UNDERSTANDING TUBERCULOSIS – ANALYZING THE ORIGIN OF MYCOBACTERIUM TUBERCULOSIS PATHOGENICITY

Edited by **Pere-Joan Cardona**

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Preface

The most intriguing property of *Mycobacterium tuberculosis* is its ability to remain for years in the host tissue, in a discrete and non-replicative way, reactivating and causing disease. This skill has stimulated multiple studies to try to discern why the host is not able to effectively eradicate it, instead of "tolerating" its persistence in the tissues. In this book, different specialists dissect the different factors and cells implied in the natural and adaptive immune response against *Mycobacterium tuberculosis* in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between *Mycobacterium tuberculosis* and the *Homo sapiens*.

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Part 1

Dissecting the Interphase Host-Pathogen

1

Ten Questions to Challenge the Natural History of Tuberculosis

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1. Is Mtb a naked emperor?

Making a parallelism with the short tale of Hans Christian Andersen "The Emperor's New Clothes", this first question wants to address a primordial question in the Mtb infection: how Mtb looks at the very initial moment when is about to be phagocytosed by the alveolar macrophage (AM).

The origin of infective Mtb is in general infected aerosols from a patient with active TB. More frequently those that carry such a high concentration that the bacilli can be observed directly in the sputum using the acid fast stain. Recently it has been discovered that a vast proportion of them are in a stationary phase, or latent phase according to their transcriptomic signature and the ability to accumulate lipid bodies [Garton 2008]. This accumulation can we a strategic activity for the bacilli in order to resume as soon as possible their growth when noticing that is embedded in a proper milieu. As one of the characteristics of Mtb is to build a thick cell wall [Torrelles 2010] the lipid accumulation appears to be a paramount activity.

Overall, what we can deduce is that stressed Mtb are the responsible of starting the infection. This speculation is supported by the fact that stressed bacteria have in general more capacity to resist further stress [Wallace 1961], and before infecting the AM, the bacilli must suffer at least the physical agents from the external milieu (i.e. the UV light action or desiccation). What is probably less taken into account is that immediately after "laying" in the alveolar surface, these bacilli are embedded in the pulmonary surfactant, which is plenty of hydrolases. Interestingly enough recently it has been discovered that surfactant reduce the cell envelop from up to the 80% [Arcos 2011] thus reducing very much one of the natural defensive mechanisms of the bacilli: its cell wall. In a way we can answer to the question affirmatively. Mtb is not presented as that pathogen with a huge indestructible armour, which together with the stressed status appears to be an irreducible enemy. On the contrary, this new input shows that AM face this pathogen as the children of the tale: naked and probably quite fragile. This process has quite annoying consequences for the bacilli, as the envelope changes correlate with a decrease in AM phagocytosis, early bacterial intracellular growth, and induction of proinflammatory responses with release of TNF-a from AMs, as well as an enhancement of phagosome–lysosome fusion.

2. Do the bacilli reside in the cytosol of the AM?

Classically, intracellular Mtb growth has been related to its growth inside the phagosome [Armstrong 1971], and this was the base for understanding the immune response based in the stimulation of CD4, and even for explaining the capacity of induce a chronic infection: as CD8 cells were not enough stimulated [vanPinxteren 2000]. ESX-1 complex became a crucial as a virulence factor, able to avoid the phagosome maduration [Xu 2007] as it was before the ATP-ase pump control to avoid the acidification of the phagosome [Sturgill 1994], or the production of ammonia by Mtb [Gordon 1980]. Then the concept of autophagy came to be essential for avoiding Mtb destruction [Deretic 2009]. Finally, it appears that Mtb is also able to disrupt the phagosome and reside into the cytosol [van der Wel 2009], in a way that has recently interpreted by Ian Orme as a natural way for Mtb tending to necrotize the macrophage to become extracellular and at the end growth extracellularly in the liquefacted tissue, which is the final target of the bacilli [Ian Orme, personal communication]. In this regard, it could be interpreted the pass to the cytosol as the beginning of the end of AM: i.e. to become necrotic.

3. Polymorphonuclear cells? If you explain me what they do, I will put them in my system!

Well, this was the answer when I asked to a systems' modelist why they didn't consider the presence of polymorphonuclear cells (PMN) when building a model to reproduce virtually the induction of the Mtb granuloma. *Why? If you explain me what they do, I will put them in my system!*. This concept comes to everybody naturally: how a cell that lives for 6 hours in the tissue can control Mtb which doubles every 24 hours? First impression is that if they play a role, they would kill Mtb immediately. Then there is the issue that Mtb is mostly intracellular thus the opportunity to be seen by PMNs is really reduced compared with all those pathogens that effectively growth in the extracellular milieu. But this is not accurate, taking into account that Mtb is able to destroy the AM becoming extracellular thus leaving a window. But for a long time it has prevailed the concept that before the onset of adaptive immunity, when there are a lot of PMNs in the granuloma, the bacilli apparently grow without resistance, in a exponential way. So far this is not accurate as recently a substantial bacillary destruction has been demonstrated in this period [Gill 2009]. But what is the role of PMNs? This bactericidal effect can be induced by Natural Killer cells, for instance. It can be said that as in any other process where a destruction of the tissue takes place, PMNs appears, so that their presence is incidental… but of course they play a role. In fact, this has been recently thought as anti-inflammatory [Zhang 2009], although bactericidal effect was effectively demonstrated when apoptotic [Tan 2006, Persson 2008]. This apparent contradictory data can be explained by the recent demonstration that immature granulocytes play a regulatory effect, and this precisely appears when there is a damage in the tissue [Gabrilovich 2009]. Likewise, PMN necrosis may also occur in the extracellular matrix, thereby curtailing bacterial dissemination [Brinkmann 2007] and contributing to the formation of a granulomatous structure that can support sudden cellular entrance [Lenzi 2006].PMNs can also carry bacilli to the lymph nodes through the lymphatic capillars thus favoring the adaptive immune response [Abadie 2005]. On the other hand, little information is on the role of microabscessification inside of the granulomas, which can be also an antiphagocytosis strategy or just increasing the local inflammatory response, thus favoring

granulomatous formation. Be what it could be, a new actor appears linking the presence of PMNs to the adaptive response. It has been described the induction in Mtb infection of Th17 cells, which also promotes the attraction of the PMNs to the granulomas [Bettelli 2007].

4. How the granuloma can ever be considered as a foe? The Citadel paradox

If a student interested in granulomatous processes had the opportunity to take a look at the city map of Barcelona around the second half of the 18th century he would appreciate a magnificent "granuloma-like" structure attached to the East wall of the city. This is the Citadel: a pentagonal wall fortified by extra triangular fortifications that result in a symmetric star-like structure (Figure 1). The first impression is to interpret this as a defensive structure, although if our student would like to extend his knowledge on it, he would realize that this is not the case. Indeed, at the beginning of the 18th century, Barcelona, the capital city of Catalonia, was fiercely besieged for a whole year. This siege resulted in such a large number of casualties among the attackers that, once they took the city, they initially decided to completely destroy it. Fortunately, an engineer proposed to build the Citadel instead in order to prevent the likely future riots of Barcelona's citizens against the new rules imposed by the victors, who had decided to abolish the Catalan State (Figure 2).

Fig. 1. **Map of Barcelona in 1719** showing a nice granuloma-like structure attached to the East wall. *Taken from Ròmul Brotons. "La ciutat captiva", Albertí Editor. Barcelona 2008.*

This historical perspective illustrates a common question about the role of the granulomas, which although built by the host to face the infection appears also to hide and to allow the persistence of the bacilli inside the body. Early data strongly support a defensive role in the case of TB, as after building the granuloma, there is enough chemokine production to attract specific lymphocytes, a fact that would not be possible in the case of isolate infected macrophages [Bru 2010]. On the other hand, the special structure of the lung parenchyma of bigger mammals requires the presence of intralobar septae to support the inflated structure

Fig. 2. **Map of the previous situation of Barcelona on 1714** before the siege settled by the Borbon Army (Picture A). Picture B shows the works of the neighbors of the East wall that were forced to fall down their houses in order to clean the space at the end of the Citadel to better bomb the city. *Taken from Ròmul Brotons. "La ciutat captiva", Albertí Editor. Barcelona 2008.*

of the lung. These septae, when teased by a disruption of the usual mechanical forces, i.e. because of the presence of a lesion, proliferate and tend to encapsulate it [Gil 2010]. We do believe this encapsulation is also responsible for avoiding the drainage of non-replicating bacilli towards the alveolar space, and thus the constant endogenous reinfection which allows the persistence of the bacilli through time [Cardona 2009; Cardona &Ivanyi 2011] (Figures 3 and 4).

5. Is the disturbance of a proper antibody response the main strategy of Mtb to survive? Why we can be constantly reinfected?

As posed in the previous question, attraction of specific lymphocytes appears to be paramount to stop the bacillary growth. Immune response against Mtb is mainly based on the induction of specific Th1 lymphocytes able to activate infected AM, but this leaves a huge window in which the bacilli can grow freely inside naïve AM before they are detected. This is clearly seen by looking at the low dose aerosol model in mice: no lesion can be seen until week 3 post infection, although meanwhile the bacillary load has increased 1000 times. The only way to avoid this phenomenon would be to induce the production of specific antibodies that would be able to directly destroy the bacilli; or at least to favor the immediate destruction of them once phagocytosed [Casadevall 2004]. But it is not the case ! Mtb infection is characterized by the lack of antibody formation [Davidow 2005]. That's why even when adaptive response is present, immune subjects can be constantly reinfected [Jung 2005] and that's why it is considered that in TB coexistence of lesions of different ages is possible [Cannetti 1955]. Interestingly, some authors have already demonstrated that production of those antibodies can exert a control on the bacillary concentration [Guirado 2006]. But apparently, this approach has not been enough fashionable, and still today the

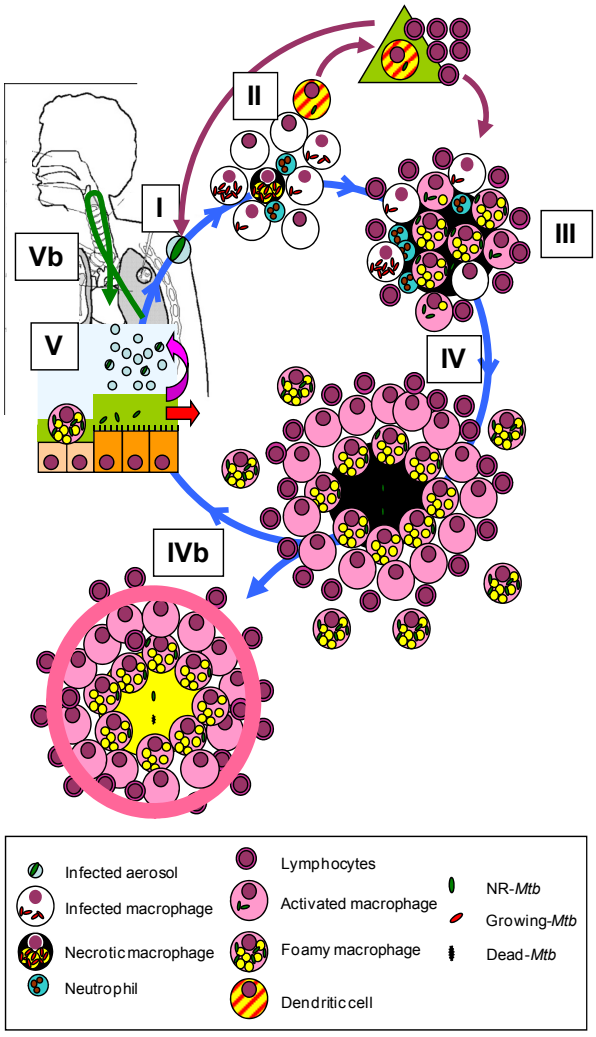

Fig. 3. **Lung pathology changing the life cycle of *Mtb* in the lungs. I.** *Mtb* transmitted by aerosol settles in the alveoli. **II.** *Mtb* growing inside macrophages, causing their necrosis. Infected monocytes become dendritic cells, that are drained to the lymph nodes (green triangle) for antigen presentation. **III**. Neutrophils, NK cells, lymphocytes and new macrophages are attracted to the granuloma**;** infected macrophages, bactericidal or bacteriostatic develop into FMs. *Mtb* changed to NR-*Mtb* in necrotic tissue are drained by FMs towards alveoli. **IVb**. Encapsulated necrotic granuloma, starting to mineralize; NR-*Mtb* cannot drain. **V**. NR-*Mtb*-infected alveolar fluid generates aerosols with the inhaled air or is swallowed and killed/drained in the gastrointestinal tract (**Vb**). Drainage of bacilli from infected lymph nodes through the thoracic ducts to the right atrium to be pumped back to the lung across the pulmonary artery also contributes to the reinfection process. Symbols: black = necrotic tissue; yellow = mineralized tissue. *Obtained from Cardona & Ivanyi 2011.*

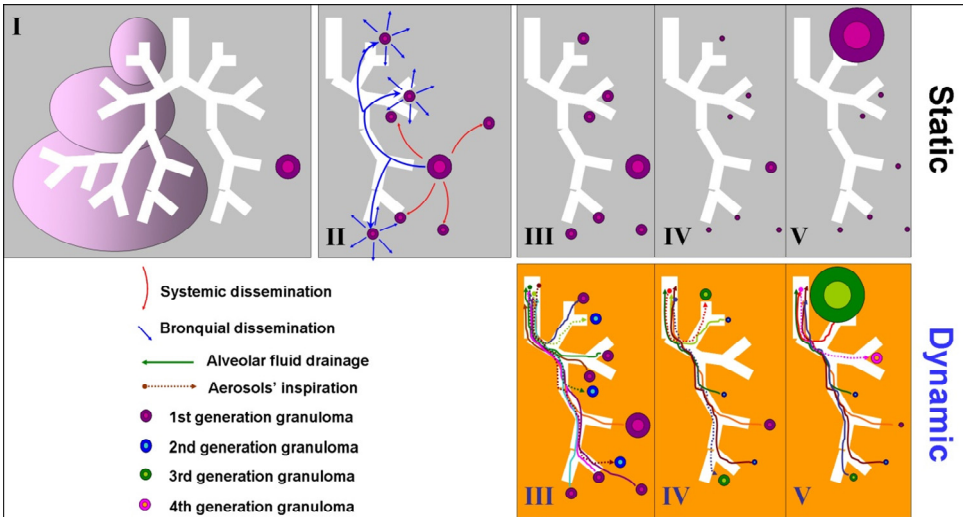

Fig. 4. **Latent TB infection (LTBI) and generation of active TB (TB).** Comparison between the traditional 'static' theory and the dynamic hypothesis. Once the initial lesion is generated (I), there is a bronchial (blue arrows) and systemic (red arrows) dissemination that generates new secondary granulomas. This process is stopped once the specific immunity is established (III). Lesions remain from then (IV) keeping dormant bacilli that have the ability to reactivate its growth after a long time (V).
In the dynamic hypothesis, there is a constant drainage of non-replicating bacilli towards the bronchial tree (solid arrows) but also the inspired aerosols (dotted arrows) can return the bacilli to generate new granulomas (III-IV). This process implies the induction of different generation of granulomas. In this process, if one of these reinfections takes place in the upper lobes, it has the opportunity to induce a cavitary lesion. *Obtained from Cardona 2009.*

majority of vaccine approaches are designed to build a strong cellular immune response giving no role to the antibody production. And what is the outcome: none of them avoid the infection by Mtb, at the most they can induce some reduction of the bacillary load [Kaufmann 2011]. That's all ! Should be resign to the fact that we will be never avoid Mtb infection?

6. What kills Mtb?

There was a time when taking into account the information coming from the experimental murine model reactive nitrogen intermediates (RNIs) appeared to be the clue to explain why after the activation of AM with interferon-gamma (IFN-γ) there was a control on the bacillary growth [Chan 1995]. This was also recreated in vitro. But the problem came when it was realized that in human AM production the role of RNIs might not be that important [Tufariello 2003]. The other mechanism could be the induction of apoptosis triggered by IFN-γ. In this case, once in an apoptotic vesicle Mtb can be effectively destroyed by any other AM regardless they activated status [Lee 2006]. This factor is also supported by the fact that Mtb tries to avoid AM apotosis [Lee 2009]. On the other hand, there is at least

another mechanism less studied but much more apparent: induction of granulomatous calcification. This is probably the oldest described bactericidal mechanism against Mtb, and very well described in human lesions [Feldman 1938]. This is a complex mechanism that our group has recently reproduced in the minipig model. Encapsulation of the lesions and turn to a fibrotic response promotes the accumulation of apoptotic vesicles in the necrotic center of the granulomas. This fact promotes the accumulation of calcium and thus the local induction of a polystress effect, based in a increased pH, hypoxia, starvation and osmotic stress [Gil 2010]. In this regard, the growing issue on the protective effect of vitamin D should be also related to this mechanism, not only devoted to the ability of trigger immunological mechanisms [Liu 2007]. Again, the obsession constantly seen by the majority of the authors to induce Th1 responses is not correct at all. This can be useful at the beginning of the infection to induce the apoptosis… but at the end, a fibrotic response is also need to induced calcification; and also to avoid the drainage of "latent" bacilli.

7. How an aircraft carrier be hidden? Does really latency exists in Mtb infection?

Ian Orme challenged years ago the TB community with a paper entitled "Latent bacilli? (I'll let you know if I ever meet one)" [Orme 2001]. The concept latency comes very much from the latent viral pathogens. Those viruses that have the ability to effectively hide and become silent and apparently non-noticed by the host, using strategies like to become part of the host's DNA [Knipe 2008]. But this is a virus… it is not the case for a bacilli, a sort of "aircraft carrier" compared with a virus, that on the contrary could be considered as a children's toy boat. It is true that Mtb has a stress response that induces a defensive metabolism including a growth disturbance, that has been called "latency"… but there is nothing special in this, as it is an universal behavior [Buchanan 1918]. In fact considering the Mtb infection as a constant reinfection process, it is clear that the bacilli are constantly noticed by the host, and that in every case it triggers very specific and efficacious responses. Looking at Figure 3 once the bacillary growth stops with the immune response, the stressed bacilli, retained mainly in the necrotic tissue, is constantly drained towards the gastrointestinal tract in a organic way that considers the degradation of AM towards foamy macrophages (FM) and thus allowing the effective drainage [Cardona 2009]. It is true that a tiny window is left by allowing the reinfection process with the production of aerosols from the alveolar fluid, but this process is only important at the very begging of the infection [Gil 2010], becoming less and less frequent with time, lowering the chance to induce active TB. All this process means that contrary to what is generally accepted, the bacilli could never become "invisible" to the host as herpes virus can do… becoming the paradigm of latency.

8. So, how active TB is induced?

The most frequent manifestation of active TB is the induction of cavitation in the lung. This happens because a liquefaction process is induced locally, favors the extracellular growth of the bacilli and makes possible the induction of a big lesion [Grosset 2003]. One of the main factors is the tropism. Again, as in other pathogens, Mtb has a special site that favors their growth. This is the upper lobe.

Cavity formation has traditionally been considered to occur from solid caseum, and a lot of controversies were raised to understand who is the responsible of inducing liquefaction: the reactivation of the bacilli trapped in the caseum of old lesions? the macrophage through the extracellular release of hydrolytic enzymes?

We understand liquefaction as one of the three possible outcomes (the other two being control and dissemination) of the constant endogenous reinfection process which would maintain LTBI [Cardona 2011]. The induction of a higher number of new lesions would increase the probability of one of them occurring in the appropriate location to induce liquefaction as upper lobes (Figure 5). These lobes favor higher bacillary load before the

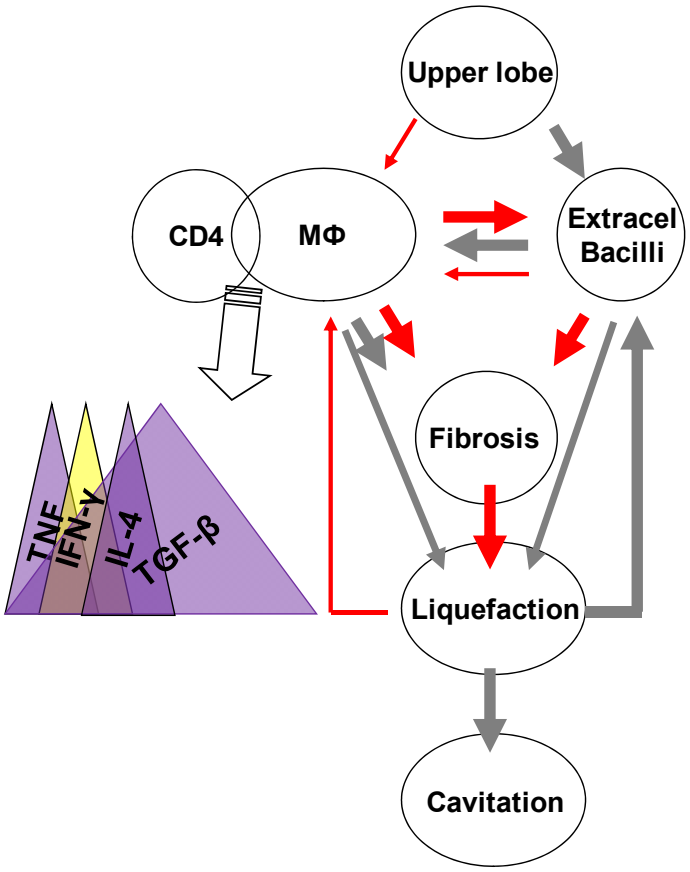

Fig. 5. **Interactions between the factors involved in the liquefaction process**. The colour of the arrows shows the ability to induce a process (in gray) or inhibit it (in red), and the thickness of the arrow is proportional to the intensity of this induction.
The upper lobe appears to be the *sine qua non* condition for the process to take place. Macrophage (MΦ) activation and the presence of CD4 is linked to the appearance of different cytokines with time: TNF initially, followed by IFN-γ and IL-4, and TGF-β from the

onset and peaking at the chronic phase. All those cytokines are profibrotic (in violet) except for IFN-γ (in yellow). This site mainly undergoes a profibrotic process, although there is also a nonspecific anti-fibrotic effect arising from the macrophages and their enzymatic activity. Extracellular bacilli also have antifibrotic activity and promote macrophage activation, although they are also thought to inhibit such activation to some extent. Fibrosis prevents liquefaction, whereas liquefaction is promoted by macrophages; the immune response, by promoting the apoptosis of infected macrophages; and extracellular bacilli. Liquefaction induces cavitation, inhibits macrophage activation (indeed, it appears to destroy them) and promotes extracellular bacillary growth.
Overall, liquefaction comes first, and then the extracellular multiplication of bacilli occurs. Fibrosis, and thus resume of the liquefaction would occur only after the number of extracellular bacilli is reduced sufficiently to allow attempts at healing to take place. Finally, a large number of extracellular bacilli results in tissue destruction, cavity formation and the death of the macrophages that attempt to inhibit such bacillary growth.
Obtained from Cardona 2011.

immune response appears by directly promoting bacillary growth and delaying the local onset of the immune response. Once this response appears, however, the synchronized induction of apoptosis/necrosis of infected macrophages together with a high IFN-γ concentration and the release of metalloproteinases by new incoming macrophages would be critical factors to promote the inhibition of localized fibrosis of the lesion and thus liquefaction. A high ability to generate a nonspecific inflammatory response, which is structurally present in males (i.e. high levels of ferritin), lower ability to produce collagen with age, or lack of proper healing of the lesions, as seen in diabetes mellitus were there is combination of local inflammation together with excessive production of metalloproteinases, could hypothetically promote this liquefaction.

Although this process can be redirected with time, with fibrosis finally taking place, another factor, the extracellular bacillary growth, even if slow, should be taken into account. Such growth might be essential to allow the irreversibility of the liquefaction process already triggered due to the so-called bacillus factor, i.e. fibrinolitic properties of proteins from the bacillary cell wall, or by infecting the macrophages surrounding the liquefaction. This would maintain the Th1 response favoring liquefaction to persist, whereby the presence of a large volume of liquefaction product leads to the destruction of new incoming macrophages (due to the high concentration of free fatty acids) and fibroblasts, thereby preventing the structuration of the site.

It could be said that liquefaction appears to be a stochastic effect due to disturbance in the organization of the intragranulomatous necrosis. The immune response and its magnitude, the bacillary load, the speed of the bacillary growth and the amount of extracellular bacilli, as well as mechanic and chemical factors (due to the distribution of the blood flow) are involved in it. Animal models have provided evidences to infer some of these factors, but more efforts on developing new models should be done in order to better mimic the human disease. Interestingly, this scenario supports the "damage framework" [Casadevall 2003] of infectious diseases that in the case of TB supports the fact that liquefaction and cavity formation is the cause of an excessive immune response against the bacilli [Cardona 2010] (Figure 6).

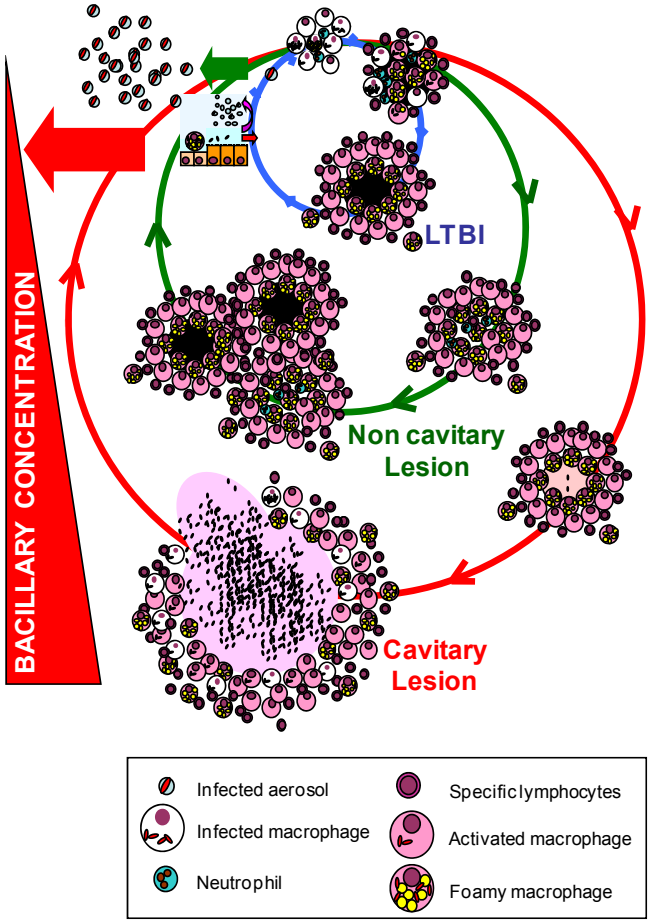

Fig. 6. **Transmission of *Mtb* infection.** LTBI (green circles) results from protracted endogenous re-infection of macrophages from drained NR-*Mtb*. Aerosol spread of drained NR-*Mtb* to susceptible hosts occurs from cavitary (i.e. in those patients that overreact against the presence of the bacilli) (red circles), and less frequently from non-cavitary (e.g. immunocompromised patients) (green circles) granuloma lesions
Symbols: black = necrotic tissue; pink = liquefacted tissue.
Obtained from Cardona & Ivanyi 2011.

9. Is Mtb fitness that important?

Considering that induction of active TB needs to be generated in that specific setting, and that tropism is that important, it appears that the most important fact comes from the chance of one person to have this site infected (Figure 6). Of course the best way is to be constantly reinfected, so the higher the prevalence of infection in the geographic region were the host resides, the higher the chance to infect the upper lobes and thus to generated active TB. In a way, also this depends on the index case. If the source of aerosols has a very intensive social

live, it has more chance to infect more people [Caminero 2001]; even more, if he or she is a good aerosol maker, the capacity to infect other people is even higher [Fennelly 2004]. So, the epidemiological factor is the most important. The host factor is also important in a way that the higher the reactivity the higher the chance to liquefact the tissue. In this regard, host polymorphism was soon detected as being a paramount factor in TB susceptibility [Dubos 1952], a fact that is nowadays clearly consolidated [Moller 2010]. Furthermore, far from the tropism issue, if the host has a depth immunosuppression and has a poor immunological capacity; or even a diminished capacity to heal lesions (i.e. to induce a correct fibrotic response) like in diabetes mellitus, the chance to develop active TB is huge.

At the end we have the third factor: the bacilli. In this case it appears that probably is the less important once taking into account the previous factors, as demonstrated by some authors [North 1999]. In this regard, the capacity to generate liquefaction by itself appears to be limited: it needs the special site and the inflammatory response generated by the host…, so it is not risky to predict that the variability of the bacilli is not really important to keep the life cycle of Mtb. That's probably why there are not really big differences among clinical strains, and that the bacilli has a very low mutation ration. It has no need so far… Figure 7.

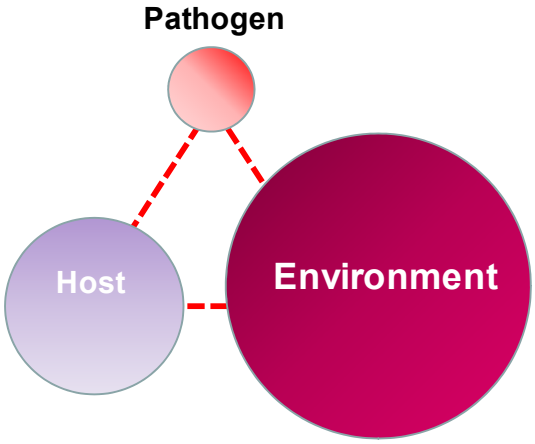

Fig. 7. **Relative importance of the different factors implied in the development of active TB.**

10. Towards new therapeutic approaches: Does host response ruin chemotherapy?

Mtb is a really slow pathogen. If *E. coli* divides every 20 minutes, Mtb needs about 24 hours, so it is 72 times slower. In this regard, if a standard antibiotic treatment of an *E. coli* infection requires 1 week, Mtb should require 72 ! Fortunately is not the case, the actual treatment needs "only" 24 weeks. This means that the actual drug combination is targeting very much very initial metabolic pathways, compared with the treatment of other bacteria. Of course the discover of a drug able to reduce even more this administration time would be desirable, but taking into account the global experience in quicker germens, it appears that we are reaching a kind of "glass roof" in this respect.

One growing issue is trying to lie Mtb by favoring artificially their growth stopping for instance the inflammatory response [Wallis 2005]. If the problem is that the stressful conditions change the bacilli metabolism in a way that make it less accessible to the drug targets, the solution should be the administration of anti-inflammatory drugs and even depress the immune response to lie the bug and "tell it" that it can finally grow !

My perception of the problem is that even in these circumstances, the reduction of the drug administration will be really neglectible. Why? Because the problem resides in that a majority of those non-replicating bacilli resides in the necrotic tissue, and to drain all of the bacilli require the elimination of all this material, and this takes time. In fact, in the case of LTBI, where the lesions are tiny, this requires up to 9 months… (Figure 8). In the case of active TB where the necrotic tissue is massive and this process would take years… So, again, the only hopeness to reduce the treatment would come from that ideal drug able to "make a hole" in the cell wall as soap, without needing any enzyme to disrupt… something very "physical" of course without hampering the much weaker host cell membranes…

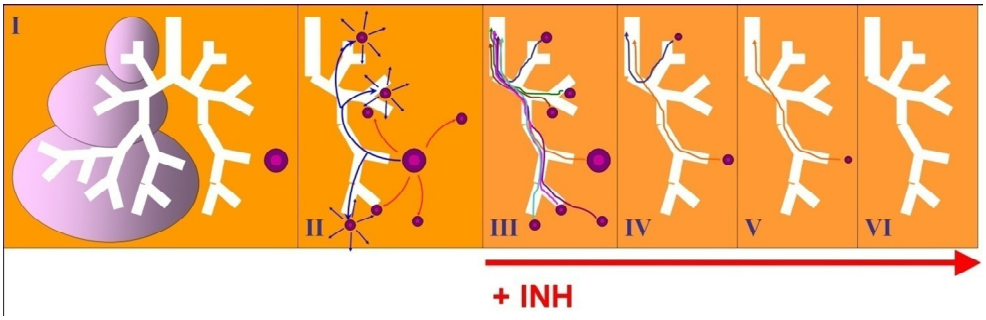

Fig. 8. **Mechanism of long-term isoniazid (INH) treatment of the latent TB infection (LTBI) according to the dynamic hypothesis.** This treatment allows the drainage of the nonreplicating bacilli, and in the case of endogenous reinfection through inspired aerosols reach the parenchyma, the bacilli have no chance to reactivate. In this case the lesions disappear with time and the opportunity to reach the upper lobes and generates the cavitary lesion is avoided. *Obtained from Cardona 2009.*

In this regard, our group promoted years ago the combination of short term chemotherapy together with a therapeutic polyantigenic vaccine (RUTI) [Cardona 2005], an approach that has already successfully finished a Phase II clinical trial [Vilaplana 2010, Archivel 2011]. The rational was to avoid precisely the sudden immunosuppression induced after chemotherapy, which is deleterious because the short time of antibiotic administration is not been able to cover all the bacilli drainage period. This attempt maybe does not induce a miraculous sterilization of the tissues but at least combines the destruction of growing bacilli, and avoids the sudden promotion of reactivation after finishing the chemotherapy. It also promotes a wider immune response, able also to help the detection of non-growing bacilli [Guirado 2008].

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2

Inflammation and Immunopathogenesis of Tuberculosis Progression

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1. Introduction

Approximately one third of the human population is infected with *Mycobacterium tuberculosis (Mtb)*. Most individuals establish latent infection. In approximately 10% of infected individuals active disease develops (Raviglione, 2003). It is accepted that the outcome of infection largely depends on the peculiarities of host immune reactivity that are controlled genetically.

A lot of efforts have been made to elucidate immune mechanisms of TB defense. The studies have identified immune cells, molecules and pathways essential for TB protection. It has been demonstrated that protection depends primarily on the activity of Th1 lymphocytes and macrophages (Schluger & Rom, 1998; Flynn & Chan, 2001; Boom et al., 2003; North & Jung, 2004; Kaufmann, 2006). Th1 cells produce immune mediators, such as IFN-γ and TNF-α that activate macrophages. Activated macrophages produce bactericidal molecules (e.g., reactive nitrogen and oxygen species) that kill mycobacteria. Both macrophages and T cells secrete a wide range of soluble factors that promote migration of other immune cells to the site of infection. At the site, immune cells settle to form granuloma that prevents mycobacteria dissemination. Overall, immune protection depends on efficient pathogen killing (i.e., antibacterial response) and efficient concentration of immune cells at the site of infection (i.e., inflammatory response). Multiple studies have demonstrated that deficiency in cells and molecules implicated in either of these responses results in extremely severe TB, supporting a concept that TB develops as a result of immune deficiency. On the other hand, since Koch's studies, TB has been considered as an immunopathological disease. In this concept, disease develops due to uncontrolled inflammatory reactivity of the host to the pathogen. Direct evidences for this concept had not been available, but are now accumulating, raising a general question on the role for immune deficiency and hyperreactivity in the pathogenesis of tuberculosis.

As noted above, the outcomes of *Mtb* infection are very diverse. The diversity consists not only in the establishment of latent infection *vs* progression to active disease, but also in a great variability in the manifestations of active disease. These manifestations differ by the type and the extent of lung tissue pathology, clinical disease characteristics, the rate of disease progression, and patient's responsiveness to treatment. Immune mechanisms operating during the onset of *Mtb* infection and during active disease differ. In particular,

inflammatory response is prerequisite for efficient control of *Mtb* at initial stages of the infection, but may become deleterious at chronic stage of disease. While mechanisms of initial TB control have been studied extensively, pathogenesis of TB progression is much less understood.

This review summarizes recent studies on TB immunopathogenesis focusing on the role for host inflammatory response in TB progression.

2. *Mtb* infection and host immune response in the lungs

2.1 Cellular immune responses to *Mtb* infection

Before considering processes ongoing during TB progression, it is important to summarize current view of the onset of pulmonary *Mtb* infection.

Mycobacteria enter the lungs trough the respiratory tract. Following the inhalation, the bacilli are phagocytosed by alveolar macrophages (AM) and airway dendritic cells (DC). Infected cells migrate to distal sites of the lung and undergo necrosis allowing *Mtb* to enter parenchyma and infect parenchymal phagocytes. Pattern recognition receptors (PRRs) expressed by macrophages, DC and epithelial cells (both on the surface and within the cell) interact with *Mtb* ligands (reviewed in detail in Kleinnijenhuis et al., 2001; van Crevel et al., 2002; Dorhoi et al., 2011; Sasindran & Torrelles, 2011). The interaction drives host cells to enhance the expression of adhesion molecules and produce inflammatory cytokines and chemokines that recruit new immune cells (i.e., neutrophils, monocytes, lymphocytes) to the infectious focus. The accumulating cells initiate formation of granuloma (reviewed in detail in van Crevel et al., 2002; Russel et al., 2009; Flynn et al., 2011).

Infected DC assisted by neutrophils (Abadie et al., 2005; Blomgran & Ernst, 2011) migrate to the lymph nodes and initiate T cell response. Due to a high production of IL-12, the response is largely polarized towards a Th1 type. Th1 cells generated in the lymph nodes migrate to the site of mycobacterial infection.

At the site, effector Th1 cells undergo functional maturation (Kapina et al., 2007) and increase their production of chemokines and effector cytokines. Chemokines attract new immune cells, amplifying local inflammatory DTH-type reaction and promoting granuloma formation. The cytokines IFN-γ and TNF-α activate adjacent macrophages (Schluger & Rom, 1998; Flynn & Chan, 2001; Pearl et al., 2001). Activated macrophages produce reactive nitrogen and oxygen intermediates (RNI, ROI), enhance surface expression of MHC class II molecules and increase secretion of inflammatory mediators, i.e. acquire ability to kill *Mtb*, enhance antigen presentation and propagate local inflammation and granuloma formation.

Besides macrophages and $CD4^+$ Th1 cells, other immune cells accumulate at the infectious focus. $CD8^+$ T cells produce IFN-γ and may exhibit cytotoxic effect against *Mtb*-infected cells (Lalvani et al., 1998; Cooper, 2009); Th17 cells promote Th1 immunity and neutrophil recruitment (Khader & Cooper, 2008); granulocytes phagocyte mycobacteria, mediate bactericidal effect, and contribute to granuloma formation (Korbel et al., 2008; Rivas-Santiago et al., 2008); B lymphocytes together with T cells form follicular structures (so-called "tertiary lymphoid tissues") that orchestrate immune response ongoing in the lungs

(Ulrichs et al., 2004). NK cells, unconventional T lymphocytes, regulatory T cells are also attracted (Cooper, 2009).

Overall, TB protection is achieved by two major mechanisms: *Mtb* killing that, ideally, stops the infection, and formation of granulomas that prevents *Mtb* dissemination. Both processes depend upon proper functioning of sets of surface receptors and soluble factors that provide immune cell activation, migration and effector activity. Factors essential for the current review are briefly discussed below.

2.2 Molecular mediators of immune response

Innate immune cells recognize *Mtb* ligands by a set of PRRs that include Toll-like receptors (TLRs), C-lectin type receptors (CLRs), scavenger receptors (SRs), immunoglobulin Fc receptors (FcRs), NOD-like receptors (NLRs) (reviewed in detail in van Crevel et al., 2002; Sasindran & Torrelles, 2011; Dorhoi et al, 2011). Ligation of PRRs induces gene expression, primarily the expression of genes for early response cytokines IL-1β, TNF-α, IL-6. All, IL-1β, TNF-α and IL-6 promote further activation of macrophages (Kishimoto, 2005). In addition, IL-1β is highly chemotactic for T lymphocytes, stimulates $CD4^+$ T cell proliferation and IFN-γ production, controls early processes of granuloma formation, and stimulates the generation and the recruitment of neutrophils (Hunninghake et al., 1987; Sugawara et al., 2001; Miller et al., 2007; Oliveira et al., 2008; Ueda et al., 2009). TNF-α is critical for the continued organization of the granulomatous lesions (Kindler et al., 1989; Flynn et al., 1995; Bean et al., 1999; Roach et al., 2002) and has immunoregulatory propereties (Orme & Cooper, 1999; Motoo et al, 2009). IL-6 modulates T- cell response, is essential for antibody formation, and stimulates hematopoiesis, in particularly, the myeloid lineage (Liu et al, 1997; Kishimoto, 2005; Walker et al., 2008). All three cytokines may cause severe pathology. They have been implicated in microvascular thrombosis, capillary leak and neutrophilic chemotaxis, produce organ dysfunction, systemic inflammation, acute-phase response, cachexia and fever (Tracey et al., 1987; Hernandez-Pando et al., 1994; Chang & Bistrian, 1998; Bekker et al., 2000; Agriles et al., 2005; Oliveira et al., 2008).

Chemokines are secreted by macrophages, neutrophils, T lymphocytes, endothelial cells and other local cells. CC chemokines CCL2, CCL3, CCL4, CCL5, and other attract monocytes, lymphocytes, macrophages, DC, NK cells to the site of infection and favor Th1 response. CXC chemokines CXCL10 (IP-10) and CXCL9 (MIG) are produced in response to IFN-γ and attract predominantly T-lymphocytes and monocytes, propagating T cell response. CXC chemokines CXCL8 (IL-8), CXCL2 (MIP-2) and CXCL1 (KC) are mainly chemotactic for hematopoietic stem cells and granulocytes and are responsible for neutrophilic inflammation at advanced stages of TB (Rhoades et al., 1995; Sasindran & Torrelles, 2011).

A separate set of molecules mediate *Mtb* killing. IFN-γ activates macrophage for *Mtb* killing. Granzymes and perforin mediate cytotoxicity of $CD8^+$ T cells and NK cells. ROI and RNI, defensins, cathelicidin, proteases and other bactericidal molecules produced by IFN-γ-activated macrophages and by neutrophils mediate *Mtb* killing (Flynn & Chan, 2001; van Crevel et al., 2002; Rivas-Santiago et al., 2008). Of note, these molecules are released not only intracellularly but also extracellularly. Thus, extracellular milieu at the focus of the infection becomes highly inflammatory containing multiple mediators that are not present in healthy lungs and that potentially are highly deleterious. During active TB, cytokines,

chemokines and other mediators released by immune cells, as well as activated immune cells themselves, are found not only at the focus of the infection, but also in the bronchoalveolar fluid (BAL) and in the circulation where they may mediate systemic inflammatory response.

2.3 Granuloma in TB protection and pathology

It is generally assumed that formation of granuloma represents a host strategy to contain the infection and limit pathogen dissemination. However, granulomatous response is observed not only in individuals with latent infection, but also in TB patients, including patients with extremely severe and rapidly progressing TB. Thus, it was suggested that a mere formation of granuloma is not enough to prevent TB, rather it is important how "proper" granuloma is functioning (Flynn et al., 2011).

In humans, granulomas observed during latent infection ("tuberculomas") are usually small, compact, solid and not numerous. They consist of macrophages and lymphocytes and a small amount of neutrophils. A central core contains epithelioid macrophages and a few neutrophils and multinucleated giant cells (Lin et al., 2009). The wall is well organized and contains follicle-like structures, in which proliferating lymphocytes reside (Ulrichs & Kaufmann, 2006). It is believed that such granulomas are able to control the infection and keep *Mtb* in a dormant state (Flynn et al., 2011).

During active disease, the granuloma cannot contain the infection. Immune cells continue to arrive, the granuloma grows and its organized structure disrupts (Ulrichs & Kaufmann, 2006; Russel et al., 2009; Cardona et al., 2011). Macrophages differentiate into epithelioid cells. The neutrophil influx increases and the centrum of the granuloma necrotizes and then caseates resulting in the formation of necrotizing and caseating granulomas. In necrotizing granulomas the central area consists mainly of degenerating neutrophils; in caseating granulomas it is presented by cell debris. The centrum is surrounded by a dense zone of epithelioid macrophages, multinucleated giant cells and lymphocytes (Lin et al., 2009). Eventually, the centrum caseous breaks into the bronchus, releasing bacteria into the respiratory tract and resulting in the formation of cavities. *Mtb* replication goes out of control. It has long been thought that the caseum represents a nutritional site for rapid *Mtb* replication. However, recent data have shown that: (i) many necrotic areas are devoid of *Mtb* (Ulrichs & Kaufmann, 2006), (ii) microbes located in the caseum resemble stationary-phase organisms, whereas replicating *Mtb* are found in sputum and BAL, and in connection with neutrophils (Eum et al., 2010). It is therefore suggested that *Mtb* replication does not occur in the liquefying cavity, but rather starts upon the exit of the bacilli from that cavity into the sputum.

Many questions regarding the functioning of granulomas remain unclear. What is an association between caseation and TB activity? Caseation is often considered as a hallmark of active disease. However, it has been reported that in non-human primates caseation occurs very early in granuloma formation, shortly after macrophages in the lungs become infected, i.e. during the very early, latent stage of the infection. In this model, caseating granuloma could successfully contain the pathogen and did not necessarily proceed to active TB (Lin et al., 2006), suggesting that it is probably the extent of caseation, but not the caseation itself that determines disease activity. Next, caseous (tuberculous) pneumonia is

an example of active TB disease that is not accompanied by the formation of classical granulomas. In this disease, extensive areas of parenchymal infiltration with multiple necrotizing and caseating foci are observed, but these foci are not structured, instead they extend contiguously in the lung parenchyma. Clinical manifestations of caseous pneumonia are extremely severe with evident signs of severe systemic inflammation; but exact pathways underlying the development of caseous pneumonia (*vs* caseating granulomas) are poorly understood. Another important question is whether caseation (either during pneumonia or in granulomas) represents host response to unlimited *Mtb* growth (i.e., is a result of inefficient *Mtb* control) or whether it is a result of "improper" host reactivity to a relatively small number of dormant *Mtb* persisting in early or mature granulomas. Several recent reviews have discussed these questions and suggested hypotheses to explain the development of active TB in non-immunosuppressed patients (Russel, 2009; Cardona, 2009; 2010; Flynn et al., 2011).

3. TB infection in hosts with immune deficiency

A role for immune deficiency in the pathogenesis of *Mtb* infection has been addressed in multiple studies.

In mice, targeted mutations of PRR genes impaired host resistance to *Mtb* (Drennan et al., 2004; Divangahi et al., 2008; Mayer-Barber et al., 2010). Deficiency on PRR adaptor molecules, e.g., MyD88, CARD9, TIR8, resulted in extremely high susceptibility (Garlanda et al., 2007; Dorhoi et al., 210; Mayer-Barber et al., 2010). Lack of "early response cytokines" (i.e., IL-1β, TNF-α, IL-6) or their receptors impaired granuloma formation, cytokine and chemokine synthesis and rendered mice extremely susceptible to *Mtb* infection. (Ladel et al., 1997; Bean et al., 1999; Juffermans et al., 2000; Yamada et al., 2000; Roach et al., 2002; Fremond et al., 2007).

Defects in acquired immunity also led to disease exacerbation. Mice deficient in T cells, (especially, in $CD4^+$ subset) and Th1 type cytokines (i.e., IL-12p40, IFN-γ) succumbed early to *Mtb* infection with high bacterial loads (Cooper et al., 1993; Flynn et al., 1993; 1995; Cooper et al., 1997; Mogues et al., 2001). Similar effects were observed in mice with defects in enzymes involved in the generation of host bactericidal molecules (e.g., iNOS, p47phox (MacMicking et al., 1997; Cooper et al., 2000; Scanga et al., 2001; Jung et al., 2002).

Observations in humans are in line with results obtained in mice. A role for TNF-α in host defense is supported by reactivation of TB in rheumatoid arthritis patients receiving anti-TNF therapy (Keane et al., 2001). An essential role for $CD4^+$ T cells in anti-TB defense is evident from a high incidence of TB and altered histopathological characteristics of TB (i.e., diffuse necrotic lesions instead of structured granulomas) in humans co-infected with immunodeficiency virus (Chaisson et al, 1987). Finally, humans with mutations in molecules involved in Th1 immunity, i.e., the IL-12p40 subunit, the IL-12 receptor β1 chain, the IFN-γ-receptor ligand binding chain, STAT1, exhibit high susceptibility to mycobacterial infections induced by *Mtb*, BCG or environmental mycobacteria (Altare et al., 1998; Dorman et al., 2000; Casanova & Abel, 2002).

Altogether, multiple studies have associated severe *Mtb* infection with immune deficiency and poor control of pathogen growth. This association explains why hosts with genetic or

acquired immune deficiencies suffer from severe mycobacterial infections. However, it cannot explain why active TB occurs in immunologically-competent hosts, or why TB exhibits so many different clinical manifestations.

4. Inflammation and TB progression

Inflammation always accompanies infection and represents a critical component of host immune defense. However, inflammatory reactions may also be deleterious and promote disease exacerbation. The first indication of a damaging role of host immune response during TB was obtained by Koch who described local and systemic reactions and disease worsening following treatment of TB patients with *Mtb* extract (Koch, 1890, as cited in Moreira et al., 2002). Thereafter, multiple observations have associated severe TB course with high inflammatory reactions. However, it is usually very difficult to dissect whether severe inflammation is a cause or a result of disease severity, i.e., whether it develops due to intrinsic host hyperreactivity to the pathogen or whether it mirrors high pathogen load (i.e. deficient *Mtb* control). Gene targeting approach is not very helpful with this respect as the majority of factors mediating inflammation are prerequisite for the development of protection and therefore their targeting or neutralization results in disease exacerbation and masks potential pathological properties. Yet, several experimental settings made this dissection possible. Detailed analysis of theses studies allows identifying immunological features critical for TB progression.

4.1 *Mtb* infection in mice with deficiency in negative regulators of inflammation

Several studies examined the course of TB in mice with deficiency in negative regulators of inflammation. TIR-8 (Toll/IL-1R), a member of the IL-1R family, is an inhibitor of inflammation. The receptor functions by trapping of TNFR-associated factor 6 and IL-1R associated kinase 1 and inhibiting activation of NF-kB induced by members of the IL-1/TLR family (Polentarutti et al., 2003; Garlanda et al., 2007). In *Mtb* infected Tir-8$^{-/-}$ mice control of mycobacteria growth and T cell responses were unimpaired. Nevertheless, the mice were rapidly killed by low doses of *Mtb*. The disease was characterized by overwhelming inflammatory response in the lungs that manifested as increased production of IL-1β and TNF-α and increased lung infiltration with neutrophils and macrophages. Blocking IL-1β and TNF-α with a mix of anti-cytokine antibodies significantly prolonged survival of Tir-8$^{-/-}$ mice supporting that their exaggerated mortality was associated with exacerbated inflammation and tissue damage (Garlanda et al., 2007).

Similarly, mice lacking D6, a decoy and scavenger receptor for inflammatory CC chemokines, had normal control of bacteria replication but responded to *Mtb* infection by uncontrolled systemic inflammation and died from a fatal infection (Di Liberto et al., 2005).

WSX-1, a component of IL-27R complex, is another molecule that plays a regulatory role during *Mtb* infection, mainly by dampening Th1 response. In the absence of WSX-1, *Mtb* infection induced elevated production of the pro-inflammatory cytokines TNF-α and IL-12p40. This led to concomitant activation of CD4 T cells, increase in IFN-γ production and macrophage effector functions. Bacterial loads were reduced, but mortality was accelerated, which was attributed to chronic hyperinflammatory response (Holscher et al., 2005).

4.2 *Mtb* infection in mice with deficiency in positive regulators of inflammation

As discussed above, deficiency in molecules mediating inflammatory signals alters host control of *Mtb* replication and exacerbates *Mtb* infection. Strikingly, even when inflammatory pathways are altered, fatal infection is accompanied by overwhelming inflammation, supporting a concept that TB progression and lethality are associated with hyperinflammatory reactions.

Dorhoi and coauthors (Dorhoi et al., 2010) examined *Mtb* infection in mice that lack CARD9, an adaptor molecule that collects signals from several PRRs. Mice developed a lethal infection accompanied by uncontrolled *Mtb* replication, by besides that – by a severe neutrophilic inflammation of the lung tissue and overproduction of factors involved in granulocyte generation and chemoattraction (i.e., G-CSF, KC). Neutralization of G-CSF or depletion of neutrophils reduced lung inflammation and prolonged host survival without affecting bacterial burdens. Thus, dampening neutrophilic inflammation at advanced stage of disease was enough to decrease disease severity.

Mice with a deficiency in IL-1R developed lethal infection characterized by extremely high numbers of *Mtb* in their lungs. Of note, a characteristic feature of lethal infection was an elevated (but not a deficient) production of major proinflammatory cytokines, e.g., IL-1β, IL-6, TNF-α (Fremond et al., 2007).

Mice deficient in TNF-α or TNF-α receptor developed extremely severe disease due to defects in granuloma formation (Bean et al.,1999). Of note, a characteristic feature of this infection was a prominent infiltration of the lung tissue with neutrophils.

In humans, S180L polymorphism in TIRAP gene implicated in the TLR2- and TLR4-mediated signaling, leads to the attenuation of inflammation and decreases the risk of TB development (Castiblanco et al., 2008).

4.3 Anti-inflammatory treatment improves TB outcome

Several groups examined the possibility to improve TB outcome by limiting immune inflammation. In patients with pulmonary TB, treatment with adjunctive corticosteroid therapy together with antibiotics accelerated sputum culture conversion in comparison with patients who received antibiotic treatment alone (Bilaceroglu et al., 1999). Adjunctive treatment with etanercept, a soluble TNF-receptor, reduced time to sputum culture conversion and improved clinical signs of TB in HIV infected patients (Wallis et al., 2004). Thalidomide, an inhibitor of TNF-α production, improved treatment outcome in patients with pulmonary TB (Tramontano et al., 1995; Coral et al., 1996). Recently, an inhibitory effect of CC-3052, an inhibitor of phosphodiesterase-4, on TNF-α production was shown. Co-treatment of *Mtb* infected rabbits and mice with isoniazid plus CC-3052 significantly reduced the level of TNF-α expression and the extent of disease (Koo et al., 2011; Subbian et al., 2011). As mentioned above, simultaneous blocking of IL-1β and TNF-α significantly prolonged survival of Tir-8-/- mice, and neutralization of G-CSF or depletion of neutrophils decreased disease severity in CARD9-/- mice (Garlanda et al., 2007; Dorhoi et al., 2010). In contrast to anti-TNF treatment, treatment of mice with TNF-α or BCG expressing TNF-α significantly increased lung tissue inflammation and resulted in accelerated mortality without affecting the bacillary load (Moreira et al., 2002). Altogether, the studies show that

dampening immune inflammation during TB may significantly ameliorate disease outcome without affecting *Mtb* replication.

4.4 *Mtb* infection in hosts with genetic differences in the extent of inflammation

Studies reviewed above are largely based on the analysis of TB infection in hosts with artificially altered or modulated immune responses. Such interventions may interfere with processes naturally operating in the infected host. To elucidate whether the extent of inflammation affects TB progression in a "normal" population, several groups have compared immune responses in mice genetically resistant and susceptible to TB. In different models, susceptible mice produced more proinflammatory cytokines and developed stronger neutrophilic inflammation than resistant mice (Cardona et al., 2003; Eruslanov et al., 2004; Eruslanov et al., 2005; Keller et al., 2006). To directly address an association between inflammatory reactions and TB progression, we have recently analyzed TB severity and immune reactivity in a panel of genetically heterogeneous (A/SnxI/St)F2 hybrid mice (Lyadova et al., 2010). The hybrids originated from TB-highly-susceptible I/St and more resistant A/Sn mice that following challenge with *Mtb* displayed different rates of TB progression (Lyadova et al., 2000; Sanchez et al., 2003; Eruslanov et al., 2004). In F2 mice, the rate of TB progression did not depend on lung *Mtb* loads or the levels of lung expression of iNOS, IFN-γ, IL-12, or CCL5, i.e. genes controlling antibacterial response. Instead, it directly correlated with high lung expression of inflammation-related factors, such as IL-1β, IL-6, IL-11, CXCL2, several metalloproteinases. Another characteristic feature of rapidly progressing TB was the accumulation in the infected lungs of Gr-1-positive cells (see below for details). Thus, similarly to gene-targeted mice, in F2 mice severe infection was characterized by: (i) overexpression of proinflammatory factors and (ii) excessive infiltration of the lung tissue with neutrophil-like cells. Further analysis suggested that these manifestations were a consequence of increased transcription of proinflammatory factors in host macrophages and were predetermined genetically (Lyadova et al., 2010).

A role for host genetic factors in the control of inflammation and TB progression was directly demonstrated in the studies by Kramnik's group (Pan et al., 2005; Yan et al., 2007). The authors identified *sst1* genetic loci on mouse chromosome 1 that controls progression of pulmonary TB. Different susceptibility of *sst1* congenic mice to *Mtb* infection was associated with neither Th1 cell activation nor with iNOS/NO responses but was due to different host capacity to mount necrotic lung inflammation and was mediated by macrophages.

4.5 T lymphocytes in TB exacerbation

T lymphocytes are responsible for efficient protection against mycobacteria. However, they may also contribute to TB exacerbation. A series of recent studies performed in programmed death-1 (PD-1) knockout mice has clearly demonstrated that (Lázár-Molnár et al., 2010; Barbar et al., 2011).

PD-1 is an inhibitory receptor expressed on exhausting T cells; its engagement inhibits T cell proliferation and cytokine secretion. PD-1-deficient mice infected with *Mtb* developed unaltered or even increased $CD4^+$ T cell and NO responses. Yet, they died because of severe infection characterized by uncontrolled bacterial proliferation, increased lung tissue pathology, neutrophilic infiltration, and high lung expression of proinflammatory cytokines

TNF-α, IL-1β, IL-6 and IL-17 (Lázár-Molnár et al., 2010; Barbar et al., 2011). Depletion of $CD4^+$ T cells ameliorated TB course, indicating that $CD4^+$ T cells themselves drove the increased bacterial loads and pathology seen in infected PD-1-deficient mice. In contrast to *Mtb* infection, resistance to viral infections was increased in PD-1 deficient mice (Velu et al., 2009). Thus, in TB imbalanced T cell responses are more deleterious than during other infections.

Our observations made in F2 model support the involvement of T cells in TB exacerbation (Lyadova et al., 2010). In this model, susceptible mice displayed first signs of TB progression (i.e., wasting) on day 16 post-challenge and died on days 26-35 post-challenge. Mice that had not succumbed to infection by the end of week 5 survived for as long as 140 days (the time of observation). It is well established that *Mtb*–specific Th1 response appears at week 2 and reaches its plateau at week 4 post-challenge. Thus, the most susceptible F2 mice succumbed to *Mtb* infection at a time when T cell response started to operate; mice that survived this period, lived for a long time. We believe that the onset (or a sudden increase, as in Koch's studies) of T cell response represents a risk factor that may provoke disease exacerbation. The underlying mechanism likely involves T-cell dependent propagation of inflammation mediated by innate immune cells.

The role for T cells in hyperinflammatory reactions and TB exacerbation is also supported by the immune restoration syndrome (IRS) observed in patients co-infected with HIV-1 and *Mtb* and initiating highly active antiretroviral therapy. The syndrome is characterized by the exacerbation of granulomatous lesions and massive inflammatory and Th1 cytokine storm. The syndrom has been associated with a sudden restoration of immune competence, i.e. an increase in the numbers of activated tuberculin-specific effector memory CD4 T cells (Autran et al., 2009).

4.6 Infection induced by *imp Mtb* mutants

Recently, several mutant *Mtb* strains bearing immunopathology (*imp*) phenotype have been generated. The mutants have unaltered capacity to grow and persist in mouse lungs, but induce poor inflammation and attenuated disease. TB-susceptible DBA/2 mice challenged with ΔSigC mutant had decreased mortality associated with lower numbers of neutrophils and reduced levels of TNF-α, IL-1β, IL-6 and IFN-γ in their lungs (Khairul-Bariah et al., 2008). Similar results were obtained when SCID mice were challenged with ΔSigC mutant. whiB3 *Mtb* mutant induced milder disease than wild type *Mtb* strain due to reduced granulomatous inflammation in the lungs (Steyn et al., 2002). ΔSigH *Mtb* mutant produced high bacterial counts in the lungs, but recruited fewer $CD4^+$ and $CD8^+$ T cells and was nonlethal in TB-susceptible C3H mice (Kaushal et al., 2002).

Thus, peculiarities of infecting *Mtb* strain represent another factor that determines TB outcome by affecting inflammatory reactions.

4.7 Inflammatory responses in patients with pulmonary TB

In TB patients, severe infection is also associated with excessive inflammatory reactions. Patients with pulmonary TB have higher levels of proinflammatory cytokines IL-1β, IL-6, TNF-α, IL-8, and their inhibitors TNFRI, IL-1Ra and TGF-β in sera and BAL fluid than

healthy controls and TB contacts (Zhang et al., 1995; Tsao et al., 1999; Tsao et al., 2000; Nemeth et al., 2011). Among TB patients, serum levels of TNF-α and TGF-β are significantly higher in patients with advanced TB compared to patients with mild-moderate TB (Fiorenza et al., 2005). In involved sites of TB, spontaneous release of IL-1β, IL-6 and TNF-α is significantly higher than in uninvolved sites and in miliary TB (Law et al., 1996). Patients with large TB cavity have much higher concentrations of TNF-α and IL-1β than patients who have small or no cavity. Importantly, the ratios of TNF-α to sTNF-RI and IL-1β to IL-1RA in the BAL fluids are also higher in patients with large cavity. Thus, a role for the relative abundance of TNF-α and IL-1β in tissue necrosis and cavity formation was suggested (Tsao et al., 2000).

Besides high levels of proinflammatory factors, a characteristic feature of progressing pulmonary TB is high numbers of granulocytic cells in the BAL fluid (Law et al., 1996; Barry et al., 2008). It was demonstrated that in sputum and BAL fluids of patients with pulmonary TB neutrophils are more abundant and contain more intracellular bacilli than macrophages (Eum et al., 2010).

In summary, hyperinflammatory reaction is a characteristic feature of progressing pulmonary TB in both humans and experimental animals. The reaction manifests as high expression of proinflammatory cytokines and prominent neutrophilic influx to the lung tissue. These manifestations develop irrespectively on exact pathways that have led to severe TB (e.g., defects in host capacity to control *Mtb* growth, host hyperreactivity to pathogen-derived signals, or peculiarities of infecting *Mtb* strain).

Mechanisms whereby proinflammatory cytokines mediate their pathological activity have been studied during different pathological conditions and reviewed in detail elsewhere (Chang & Bistrian, 1998; Thacker, 2006; Mootoo et al., 2009; Argiles et al., 2005). In contrast, data on the role for neutrophils in TB pathogenesis are contradictory and require special consideration.

5. Neutrophils

Physiological activities of neutrophils involve adhesion, migration to the site of inflammation, phagocytosis, degranulation, and release of inflammatory mediators. We will briefly review activities related to TB and discuss the controversial results of the studies that addressed the role of these cells in tuberculosis.

5.1 Functional activities

Neutrophils are among the first cells that arrive at the inflammatory focus (Appelberg & Silva, 1989). The process involves adhesion of circulating neutrophils to the endothelial cells and migration through the endothelial barrier and within the inflamed tissues. Neutrophils' migration is driven by the inflammatory cytokines IL-β and TNF-α, the chemokines IL-8, CXCL2, CXCL1, bacteria products and molecules released by dying cells, i.e. it occurs in response to inflammation and tissue injury (reviewed in Witko-Sarsat et al., 2000).

At the site of infection, neutrophils phagocytoze IgG- and complement-opsonized targets and exhibit bactericidal activity. The later is mediated by the production of ROI and release

of bactericidal molecules stored in neutrophils' granules. ROI include: (i) superoxide anion and hydrogen peroxide generated by NADPH-dependent oxidase; (ii) hypochlorus acid and chloramines, generated by neutrophil-specific enzyme metalloperoxidase. Granule-associated bactericidal molecules are numerous and include short bactericidal peptides (e.g., human neutrophil peptides (HNPs) 1–3, cathelicidin LL-37, lipocalin 2); lactoferrin; serine proteases; metalloproteinases (Witko-Sarsat et al., 2000; Fu, 2003; Martineau, et al. 2007; Rivas-Santiago et al., 2008). Macrophages utilize neutrophil bactericidal peptides to increase their antibacterial activity: they phagocyte apoptotic neutrophils and deploy neutrophils' bactericidal peptides to combat intracellular *Mtb* (Tan et al., 2006).

An additional bactericidal mechanism is formation of extracellular traps (NETs) - a web of chromatin fibers that contain serine proteases and can trap and kill extracellular microbes (Brinkmann et al., 2004).

Neutrophils are involved in the formation of granuloma (Seiler et al., 2003) and in the initiation of T cell response: they were shown to transport live mycobacteria from peripheral tissues to the lymphoid organs and to deliver mycobacterial antigens to DC in a form that makes DC more effective initiators of naïve $CD4^+$ T cell activation (Abadie et al., 2005; Blomgran et al., 2011).

An important activity of neutrophils is a secretion of inflammatory mediators and their inhibitors. The list includes proinflammatory cytokines IL-1β and TNF-α, the major neutrophil attracting chemokines IL-8 and CXCL2, growth factors GM-CSF and VEGF, several metalloproteinases, IL-1Ra, TGF-β (McColl et al., 1992; Cassatella, 1995; Riedel & Kaufamm, 1997; Petrofsky et al., 1999; Scapini et al., 2000; Matzer et al., 2001; Sawant & McMurray, 2007; Lyadova et al., 2010). The secretion is not high, but when neutrophils accumulate in high numbers, it may represent an important source of inflammatory factors. Interestingly, many neutrophils contain intracellular IFN-γ. This was shown during *Mtb* infection (our unpublished observations) and also in other models (Terri & Beaman, 2002). Neutrophils not only produce proinflammatory cytokines by themselves, but also stimulate proinflammatory activity of macrophages (Persson et al., 2008).

An important issue is that factors produced by neutrophils are the major positive regulators of their activity: TNF-α and IL-1β enhance neutrophils' migration, degranulation, oxidative and secretory activities; IL-8 and CXCL2 are the major neutrophil-attracting chemokines; IFN-γ promotes secretory activity; metalloproteinases degrade extracellular matrix facilitating cell migration within the inflamed tissue. Thus, neutrophilic inflammation is under an autocrine regulation. The major inhibitors of cytokine production by neutrophils are IL-10, IL-4, and IL-13 (Witko-Sarsat et al., 2000), but they are poorly produced during TB.

Neutrophils release bactericidal molecules and enzymes not only into the phagosome, but also into the extracellular milieu. This allows killing extracellular microbes, but on the other part is detrimental: serine proteinases degrade almost all components of extracellular matrix and a variety of plasma proteins; collagenase (MMP8) and gelatinase (MMP9) cleave different types of collagen; ROI and chlorinated oxidants inactivate inhibitors of proteinases, activate metalloproteinases and may mediate direct cytotoxic effect (Weiss, 1989; Witko-Sarsat et al., 2000)

The only way to resolve neutrophilic inflammation is to clear the infection: in this case emigration of new neutrophils stops; neutrophils that had migrated to the inflamed sites undergo apoptosis and are phagocytosed by macrophages. During chronic infections, neutrophilic inflammation becomes uncontrolled.

5.2 Neutrophils during *Mtb* infection

5.2.1 Antimycobacterial activity of neutrophils and TB prevention

Neutrophils are among the first cells that migrate to the focus of lung *Mtb* infection, and they progressively accumulate at the infectious site during the chronic stage of disease. Human and mouse neutrophils efficiently phagocyte mycobacteria (Kisich et al., 2002; Eruslanov et al., 2005), but their capacity to kill mycobacteria is disputable.

Denis and Andersen reported that human neutrophils stimulated with IFN-γ failed to kill *Mtb* (Denis & Andersen, 1991). In line with this, in our previous studies mouse neutrophils displayed low antimycobacterial activity that could not be enhanced by the addition of exogenous IFN-γ (Eruslanov et al., 2005). In a recent study by Eum and coauthors (Eum et al., 2010), neutrophils present in the sputum and BAL fluids of patients with active pulmonary TB contained *Mtb* that exhibited signs of replication. Based on these observations, it is concluded that neutrophils have poor antimycobacterial activity and during TB act by hiding *Mtb* from macrophages and permitting *Mtb* replication (Eruslanov et al., 2005; Eum et al., 2010).

In other studies, neutrophils were shown to kill *Mtb*. The effect was mediated by α-defensins, LL37 and lipocalin and promoted by TNF-α (Kisich et al., 2002). Of note, IFN-γ did not enhance killing, which may explain a failure to detect neutrophil-mediated *Mtb* killing in the studies described above (Denis & Andersen, 1991; Eruslanov et al., 2005). Recently, an association between low plasma levels of HNP1-3 and the development of multi-drug resistant TB has been demonstrated (Zhu et al., 2011), supporting the involvement of neutrophils in TB protection. In line with this, it has been demonstrated that black African participants (known to have a relatively high susceptibility to TB) have lower counts of neutrophils and lower concentrations of circulating HNP1–3 and lipocalin 2 than white participants; in TB contacts, the counts of peripheral blood neutrophils inversely correlated with risk of TB development (Martineau et al., 2007). Thus, multiple studies suggest a role for neutrophils in TB prevention.

5.2.2 Neutrophils and TB progression

In contrast to early stages of *Mtb* infection, at which neutrophils are not numerous and may contribute to *Mtb* control, during active disease neutrophils become more abundant and may cause severe pathology. In humans, high numbers of neutrophils in BAL fluids have been associated with disease activity and lung tissue cavitation (Barry et al., 2009; Sutherland et al., 2009). In mice, neutrophils (Gr-1-positive cells) accumulate abundantly in the lungs of susceptible mice (e.g., I/St, DBA/2) but are less numerous in resistant mice (Eruslanov et al., 2005; Keller et al., 2006). It is believed that neutrophils contribute to disease progression by amplifying local inflammatory reactions and mediating tissue injury.

An important question is whether the propensity to develop extensive neutrophilic inflammation is a primary cause of host susceptibility to the infection. In some studies, granulocytes from susceptible mice were shown to have intrinsically high capacity for migration in response to inflammatory stimuli (Keller et al., 2006). However, neutrophilic infiltration is a characteristic future of severe TB in hosts of different genetic backgrounds, e.g., in DBA/2, C3H, I/St, 129Sv mice. It is unlikely that initial mechanisms of TB susceptibility operating in these mice are identical. Rather, at early stages of infection the disease is driven via different pathways. Such pathways may include defects in host ability to restrict *Mtb* growth, intrinsic hyperreactivity of host macrophages to *Mtb* derived ligands resulting in overproduction of neutrophil-activating factors, or high reactivity of neutrophils to inflammatory stimuli. Ultimately, different pathways converge to induce uncontrolled inflammation, characterized by high local production of proinflammatory factors and extensive neutrophilic infiltration. These inflammatory reactions become a hallmark and an important pathogenic mechanism of TB progression. It would be interesting to know whether "inflammatory" neutrophils (i.e., neutrophils residing in highly inflammatory conditions) retain their antibacterial activity and can, at least in part, mediate *Mtb* control, or whether at this stage of disease they exhibit only inflammatory functions.

5.2.3 Neutrophil depletion experiments

To directly address a role for neutrophils in anti-TB defense, several groups examined how neutralization of granulocytes affected *Mtb* infection in mice.

Pedrosa and colleagues (Pedrosa et al., 2000) found that depletion of neutrophils from TB-resistant BALB/c mice during the first week of *Mtb* infection worsened disease and increased bacillary growth. The effect was due to a decreased production of IFN-γ and NO, i.e. was mediated indirectly. Depletion of neutrophils at late stages of the infection did not have a significant effect on the growth of *Mtb* in the lungs. Appelberg and coauthors (Appelberg et al., 1995) performed studies in B6 and beige mice. Beige mice bear mutation that affects function of leukocytes, including granulocytes, and renders mice susceptible to *M. avium*. Transfusion of beige mice with B6 granulocytes at the early stage of infection increased host resistance. In contrast, depletion of neutrophils from B6 mice increased host susceptibility. Seiler and coauthors (Seiler et al., 2003) reported that early depletion of granulocytes from B6 mice did not affect host survival or *Mtb* burden, but impaired granuloma formation.

In the study by Ehlers' group (Keller et al., 2006), depletion of granulocytes from TB resistant B6 or BALB/c mice did not affect the course of *Mtb* infection. In contrast, in TB-susceptible DBA/2 mice depletion of granulocytes had beneficial effect and prolonged mice survival. In CARD9-/- mice, depletion of neutrophils on days 8-14 post-challenge significantly prolonged mice survival. The effect was due to a reduced inflammation, and was not associated with changes in bacterial burdens (Dorhoi et al., 2010).

The results of these studies are usually interpreted as contradictory. In fact, they are compatible and can be explained by (i) the differential role for neutrophils at early and late stages of *Mtb* infection; (ii) different strength of neutrophilic inflammation in genetically different hosts. We suppose that at the early stage of the infection, when neutrophils are not

numerous and neutrophil-activating proinflammatory cytokines are not abundant, neutrophils contribute to *Mtb* control. At the late stage of the disease, the action of neutrophils depends on the strength of local inflammation: if neutrophils accumulate in high numbers and are in highly inflammatory milieu (conditions usually observed in TB-susceptible mice), they become deleterious. Thus, depletion of neutrophils reduced *Mtb* control and worsened disease in resistant mice, but dampened inflammation and ameliorated disease course in susceptible mice.

5.2.4 Not all Gr-1/Ly-6G-positive cells accumulating in the lungs at the advanced stage of *Mtb* infection are neutrophils

To address a role for granulocytes in TB progression, we have recently used our F2 model of *Mtb* infection (Lyadova et al., 2010). In this model, (A/SnxI/St)F2 mice challenged with *Mtb* display different rates of TB progression. We examined the accumulation of cells expressing Gr-1 marker (marker expressed by granulocytes and to a less extent – by monocytes) and Ly-6G molecules (molecules thought to be expressed exclusively by granulocytes) in the lungs of F2 mice at advanced stage of disease (day 24 post-infection). We found that the population of Gr-1-positive cells infiltrating *Mtb*-infected lungs, was not homogeneous, and consisted of two different subsets, Gr-1^{hi} and Gr-1^{dim}. Similarly, Ly-6G-positive cells contained Ly-6G^{hi} and Ly-6G^{low} subsets. In mice with slowly progressing TB all Gr-1/Ly-6G-positive cells were Gr-1^{hi}/Ly-6G^{hi}. In contrast, in mice with severe infection a vast majority of Gr-1/Ly-6G-positive cells were Gr-1^{dim}/Ly-6G^{dim}, whereas Gr-1^{hi}/Ly-6G^{hi} cells were almost undetectable. Further analysis showed that Gr-1^{hi}/Ly-6G^{hi} cells were granulocytes: they expressed F4-80^{neg}CD11b^{hi} phenotype and had segmented nuclei. Gr-1^{dim}/Ly-6G^{dim} cells exhibited characteristics of immature myeloid cells: they had F4-80^{low}CD11b^{hi} phenotype that could be attributed nor to mature granulocytes nor to monocytes. Analysis of nuclear morphology showed that these cells had un-segmented or low-segmented nuclei. At advanced stage of *Mtb* infection, Ly-6G^{dim} cells appeared and accumulated not only in the lungs, but also in the bone marrow (Tsiganov E.N., Lyadova I.V., manuscript in preparation), suggesting that hematopoiesis was altered in mice with progressing TB and that the accumulation of Gr-1^{dim}/Ly-6G^{dim} cells in the lungs was a result of this alteration.

In connection with these data, two points should be discussed.

First, Gr-1 and even Ly-6G expressing cells accumulating in the lungs of *Mtb*-infected mice do not necessarily represent mature neutrophils. Experimental studies in which neutrophils were identified based on their Gr-1/Ly-6G-positivity should be revised to take into account the level of Gr-1/Ly-6G expression. Similarly, several studies identified neutrophils based on the expression of myeloperoxidase. This enzyme is, indeed, synthesized be granulocytes, but also - by their myeloid precursors.

Second, our data suggest that severe TB is accompanied by hematopoietic shifts that result in a progressive accumulation of immature myeloid cells and gradual disappearance of mature neutrophils from *Mtb*-infected lungs. It will be interesting to examine, whether the substitution of neutrophils by immature cells may underlie inability of "neutrophils" to control *Mtb* infection at the advanced stages of TB disease.

6. Conclusion

Inflammation plays a dual role in host immune response to mycobacteria. On the one part, it is prerequisite for successful pathogen elimination. On the other part, it mediates tissue injury and disease progression. At the onset of the infection, inflammatory reactions are largely protective; during active disease, the deleterious effect of inflammation prevails, making inflammation a paramount pathogenic factor of TB progression.

Irrespectively on genetic factors and molecular pathways that have lead to severe TB (that are different in genetically different hosts), pathogenetic mechanisms operating during advanced stage of disease are common. They include overproduction of proinflammatory factors and excessive infiltration of the lung tissue with neutrophils (or their precursors). A positive feedback loop between these reactions exists (proinflammatory factors promote neutrophilic inflammation; neutrophils produce proinflammatory factors; both induce tissue injury, *Mtb* dissemination, and another round of inflammation) making the regulation of the ongoing inflammation difficult. An additional and a new component of TB progression is alteration of host hematopoiesis that results in the generation of immature myeloid cells, their emigration and prominent accumulation in the periphery. The role for these cells in TB progression is yet to be determined.

The fact that mechanisms mediating TB progression are common has an important practical outcome: there is no need to search for exact cause that has driven severe disease in each individual; it might be possible to slow down disease progression by interfering with any of the pathways involved in hyperinflammatory response. With this respect, co-treatment of host with anti-*Mtb* and anti-inflammatory drugs opens new perspectives for efficient TB therapy (Koo et al., 2011).

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8. References

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3

Host–Pathogen Interactions in Tuberculosis

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1. Introduction

The development of massively parallel DNA sequencing is revealing the scale of mammalian bacterial colonization and suggests that *Homo sapiens* is colonized by between 10^3 and 10^4 bacterial phylotypes. The understanding of the complexity of host-bacterial interactions could explain why only a relatively tiny number of bacteria causing human diseases (Keijser et al., 2008; McKenna et al., 2008; Henderson et al., 2011). Over thousands of years microbes and mammals have co-evolve resulting in extraordinarily sophisticated molecular mechanism permitting the organism to survive together. *Mycobacterium tuberculosis* is one of the best examples of successful coevolution, since the bacilli have infected one third of the human population, but in 90% of the cases without causing overt disease (Bhowruth et al., 2008). The factors that regulate the course and outcome of infection by *M. tuberculosis* are multifaceted and involve a complex interplay between the immune system of the host and survival strategies employed by the bacilli (Mischenko et al., 2004). During the infection process and pathological development of human tuberculosis, *M. tuberculosis* expresses many molecules and recruits others from the host that allow the microorganism to recognize and be recognized by different host receptors. In this way, the knowledge of these interactions at the molecular level is of fundamental importance to understand all the events involved in entry, dissemination and persistence of the pathogenic mycobacteria and in the design of new highly specific therapeutic agents.

In this chapter, we are describing the host and the human pathogen *M. tuberculosis* molecules that are involved in the interactions with innate immune system, Extracellular Matrix Protein (ECM) and fibrinolytic system, the proposal mechanisms of interactions and the biological/pathological consequences are discussed. Also, examples are shown of how genetic variations in host and bacteria regulatory and encoded sequences can affect conditions that influence the relationship between bacteria and their host. Finally considerations are done about how the knowledge of host-pathogen interactions, can be useful in the search of new tools to fight against the disease.

2. Interaction of *M. tuberculosis* with the innate immune system

Tuberculosis is primarily acquired through inhalation of airborne droplets containing the viable bacilli which travel to distal regions of the lung where they are recognized by the pulmonary innate immune system that plays a key role in the recognition of microbes entering via the respiratory route. These early interactions constitute an important link between innate immune response and the subsequent activation of adaptive immune response that although sufficient to contain the microorganisms is enabling to eliminate them. The molecules of bacteria and host involve in these interactions are known as pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRR) respectively (Salgame, 2005).

The PRRs include the Toll-like receptors (TLRs) which are type I transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats and a cytoplasmic tail that contains a conserved region called the Toll/IL-1 receptor (TIR) domain (Medzhitov et al., 1997). Ten human and twelve murine TLRs have been characterized, TLR1 to TLR10 in humans and TLR1 to TLR9, TLR11, TLR12 and TLR13 in mice, the orthologous of TLR10 being a pseudogene. Specific interaction of TLRs with microbial ligands triggering proinflamatory citokines such as TNF-α, IL-1 and IL-12 generated through the adaptor molecule myeloid differentiation primary response protein 88 (MyD88) (Giacomini et al., 2001; Underhill et al., 1999; Kiemer et al., 2008). Moreover, the *in vivo* importance of the TLR-mediated signal in host defense to *M. tuberculosis* was highlighted in studies using mice lacking MyD88, these animals are highly susceptible to airborne infection with *M. tuberculosis*. In contrast, mice lacking individual TLRs are not dramatically susceptible to *M. tuberculosis* infection, suggesting these observations that multiple rather than single TLRs are required for the innate defense against mycobacteria. Remarkably, adaptive immunity is not impaired in MyD88-deficient mice during the course of *M. tuberculosis* infection (Fremond et al., 2004; Scanga et al., 2004; Hölscher et al., 2008).

2.1 Interaction of *M. tuberculosis* with Toll-like receptors

Studies using gene knockout mice have shown a role for TLR2, TLR4, TLR6, and TLR9 (Reiling et al., 2002; Sugawara et al., 2003; Bafica et al., 2005) in protective immunity to mycobacterial infection.

At date, the ligands for TLR2 have been identified and it is now known that TLR2 recognizes *M. tuberculosis* lipoglycans and lipoproteins, as well as and a member of the PE_PGRS family.

2.1.1 Lipoarabinomanan (LAM) and Lipomannan (LM)

LAM and LM are integral parts of the mycobacterial cell wall. They are composed of a carbohydrate backbone made of a *D*-mannan core and a *D*-arabinan domain. Several reports have described a TLR2-dependent cell activation by mycobacterial cell wall lipoglycans (mannose-capped lipoarabinomannan (ManLAM), phosphatidyl *myo*-inositol mannosides (PIM2 and PIM6)). Typically, Man-LAM from pathogenic mycobacteria have been reported to be anti-inflammatory molecules, inhibiting the production of IL-12 and TNF-α and increasing IL-10 production by dendritic cells or monocytic cell lines, whereas

phosphoinositol-substituted LAM from nonpathogenic species are proinflammatory molecules stimulating the production of TNF-α and IL-12 (Means et al., 1999; Jones et al., 2001; Gilleron et al., 2003).

LM, but none of the corresponding LAM, induce macrophage activation dependent on the presence of TLR2 and exerted also a potent inhibitory effect on TNF-α, IL-12p40, and Nitric Oxide (NO) production by Lipopolysaccharide (LPS)-activated macrophages. These results provide evidence that mycobacterial LM bear structural motifs susceptible to interact with different pattern recognition receptors with pro or anti-inflammatory effects (Quesniaux et al., 2004).

2.1.2 LpqH (*Rv3763*) or 19 kDa protein

The 19 kDa cell wall-associated and secreted glycolipoprotein (Garbe et al., 1993), induce apoptosis in differentiated THP-1 cells and monocyte-derived macrophages being this effect TLR2-mediated. This protein also inhibits IFN-γ-regulated MHC-II expression on alveolar macrophages, dependent on TLR2. Although native LpqH is a mycobacterial glycolipoprotein, based upon the use of recombinant where the acylation signal had been removed, it was concluded that it is the polypeptide component of 19 kDa is responsible for signaling through TLR2 and that the lipid moiety is not required (López et al., 2006; Fulton et al., 2004).

2.1.3 LprG (*Rv1441c*) or P27 protein

The LprG is a 27 kDa secreted glycolipoprotein (Gonzalez-Zamorano et al., 2009). It had been shown that after prolonged exposure (>16 h) of human macrophages to this protein, a marked inhibition of MHC-II antigen processing dependent on TLR2 occur. Short-term exposure (<6 h) to LprG stimulated TLR2 dependent TNF-α production. Inhibition of MHC-II antigen processing by mycobacterial lipoproteins may allow *M. tuberculosis*, within infected macrophages, to avoid recognition by CD4 T cells that represent a negative feedback mechanism for control of inflammation that may be subverted by *M. tuberculosis* for immune evasion. As observed with LpqH nonacylated LprG retains TLR2 activity (Drage et al., 2010; Gehring et al., 2004).

2.1.4 LprA (*Rv1270c*)

The LprA a 24 kDa cell wall-associated lipoprotein with no homologs outside the slow-growing mycobacteria induced expression of TNF-α, IL-10 and IL-12. The protein had agonist activity for both human and murine TLR2. LprA also induced dendritic cell maturation as shown by increased expression of CD40, CD80, and MHC-II. In macrophages, prolonged (24 h) incubation with LprA decreased IFN-γ induced MHC-II antigen processing and presentation, consistent with an observed decrease in MHC-II expression. In contrast with LpqH and LprG, only acylated LprA showed the agonist activity (Pecora et al., 2006).

2.1.5 PstS1 (*Rv0934*) or 38 kDa antigen

The 38 kDa a phosphate binding glycoprotein has been considered as an immunodominant antigen for its capacity to evoke a prominent cellular and humoral immune responses in tuberculosis (Espitia et al., 1989a; Zhu et al., 1997). The protein acting through both TLR2

and TLR4, induces the activation of the Extracellular Signal Regulated Kinase (ERK1)/2 and p38 Mitogen-activated protein kinase 1(MAPK1) pathways, which in turn play an essential role in TNF-α and IL-6 expression (Jung et al., 2006).

2.1.6 PE_PGRS33 (*Rv1818c*)

The 45 kDa protein is a member of PE_PGRS family. These sequences were initially described by Poulet and Cole in 1995 as GC rich sequences (PGRS, polymorphic GC repetitive sequence) in the genome of *M. tuberculosis* (Poulet & Cole, 1995). They have a conserved Pro–Glu motif (PE domain) in their N-terminal, and in C-terminal an alanine and glycine rich domain (PGRS domain). Nearly 100 genes belonging to the PE family scattered throughout the *M. tuberculosis* genome (Cole et al., 1998). The PE_PGRS33 is surface exposed protein (Brennan et al., 2001), that elicits TNF-α release from macrophages in a TLR2 -dependent manner. ASK1 (apoptosis signal-regulating kinase 1) is activated downstream of TLR2. ASK1 activates the MAPKs p38 and c-Jun N-terminal kinases (JNK). PE_PGRS33-induced signaling leads to enhanced expression of TNF-α and TNF-α receptor (TNFR1) genes. Release of TNF-α plays the determining role in triggering apoptosis in macrophages challenged with PE_PGRS33 (Basu et al., 2007).

2.2 Interaction of *M. tuberculosis* with non-Toll-like receptors

Several recent findings have indicated that PRRs other than TLRs (non-TLRs) evoke innate immune responses. Among non-TLRs that interact with *M. tuberculosis* are the complement receptors 3 (CR3). The nucleotide-binding oligomerization domain (NOD)-like receptor 2 (NOD2) and members of C-type lectins receptors.

2.2.1 Complement receptor 3 (CR3)

M. tuberculosis can bind to several types of receptors on the surface of mononuclear phagocytes including complement receptors 3 (CD11b/CD18) which is a heterodimer belonging to the leukocyte 2-integrin family. This receptor binds complement fragment C3bi and also contains a carbohydrate binding site. *M. tuberculosis* can bind to the complement receptors via both complement-dependent and independent pathways (Cywes et al., 1997). The presence of human serum containing active complement components was found to enhance the binding of *M. tuberculosis* on the surface of human monocytes and monocyte-derived macrophages. CR3 was identified as the major component in human serum involved in enhancing the adherence and uptake of *M. tuberculosis* by mononuclear phagocytes (Schlesinger et al., 1990). By using affinity blot with C3 complement protein it was found that C-terminal region of *M. tuberculosis* heparin-binding hemagglutinin (HBHA (*Rv0475*) bound human C3. The presence of complement-sufficient serum increased the adherence of the HBHA-coated beads to the J774 cells, suggesting these results that the protein may enhance the adherence and phagocytosis of *M. tuberculosis* to mononuclear phagocytes through the binding of C3 and interaction with C3 receptors on mononuclear phagocytes (Mueller-Ortiz et al., 2001, 2002).

2.2.2 Nucleotide-binding oligomerization domain (NOD)-like receptor 2 (NOD2)

NOD2 also known as CARD15, this protein activates innate immunity in response to peptidoglycan-derived muramyl dipeptide (MDP). Indeed, defects in NOD2 signaling lead

to impaired *in vitro* mycobacteria recognition by human or murine-derived macrophages (Saiga et al., 2011). Studies in macrophages from NOD2-deficient mice indicate that NOD2 mediates resistance to mycobacterial infection via both innate and adaptive immunity (Divangahi et al., 2008).

2.3 C-type lectin receptors involved in the recognition of mycobacteria

C-type lectin receptors are a family of proteins of innate immune system that bind surfactants and mannose-binding lectin protein (MBL). In addition, there are cell-associated C-type lectins, including the mannose receptor (MR), dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), DC-associated C-type lectin-1 (Dectin-1) and macrophage inducible C-type lectin (Mincle) (Torreles et al., 2008). Some C-type lectin receptors are expressed on the plasma membrane or on the endosomal/phagosomal membrane, whereas NOD-like receptors are expressed within the cytoplasm. Indeed, distinct patterns of TLR and NOD like receptor-mediated gene expression profiles have been demonstrated in infection with intracellular bacteria (Saiga et al., 2011).

2.3.1 Macrophage Mannose Receptor (MR)

Macrophages primarily use the MR as well as CR3 for the phagocytosis of *M. tuberculosis*. Interaction of pathogenic *M. tuberculosis* with the human MR was first demonstrated by Schlesinger., 1973, and ManLAM was proposed as the molecule responsible for the specific MR-mediated phagocytosis of pathogenic (Schlesinger et al., 1994). It was also demonstrated that engagement of the MR by ManLAM during the phagocytic process directs *M. tuberculosis* to its initial phagosomal niche, thereby enhancing survival in human macrophages in part by limiting phagosome lysosome fusion, being ManLAM responsible for blocks phagosome maturation (Kang et al., 2005). It was also shown that the macrophage MR, may interact with mannose residues of mycobacterial lipoglycoprotein LpqH promoving phagocytosis of mycobacteria (Diaz-Silvestre et al., 2005).

2.3.2 Dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)

This Calcium-dependent carbohydrate-binding protein has specificity for mannose-containing glycoconjugates and fucose-containing Lewis antigens. In recent years, DC-SIGN has gained an exponential increase in attention because of its involvement in multiple aspects of immune function. Besides being an adhesion molecule, particularly in binding intercellular adhesion molecule 2 (ICAM-2) and intercellular adhesion molecule 3 (ICAM-3), it is also crucial in recognizing several endogenous and exogenous antigens. Additionally, the intracellular domain of DC-SIGN includes molecular motifs, which enable the activation of signal transduction pathways (Švajger et al., 2010). Mannosylated moieties of the *M. tuberculosis* cell wall, such as ManLAM or PIMs were previously shown to bind to DC-SIGN on immature dendritic cells and macrophage subpopulations. This interaction reportedly impaired dendritic cell maturation, modulated cytokine secretion by phagocytes and dendritic cells and was postulated to cause suppression of protective immunity to tuberculosis (Geijtenbeek et al., 2003). However, experimental *M. tuberculosis* infections in mice transgenic for human DC-SIGN revealed that, instead of favoring immune evasion of

mycobacteria, DC-SIGN may promote host protection by limiting tissue pathology. Furthermore, infection studies with mycobacterial strains genetically engineered to lack ManLAM or PIMs demonstrated that the ManLAM/PIM–DC-SIGN interaction was not critical for cytokine secretion *in vitro* and protective immunity *in vivo*. The dominant *M. tuberculosis*-derived ligands for DC-SIGN are presently unknown, and a major role of DC-SIGN in the immune response to *M. tuberculosis* infection may lie in its capacity to maintain a balanced inflammatory state during chronic tuberculosis (Ehlers, 2010). Recent studies suggest more varied modes of binding to multiple mycobacterial ligands. Four novel ligands of *M. bovis* BCG that bind to DC-SIGN were identified; chaperone protein DnaK, 60 kDa chaperonin-1 (Cpn60.1), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and lipoprotein LprG. Of these, only LprG appears to bind DC-SIGN via typical proteinglycan interactions (Carroll et al., 2010). Additional ligands, possibly including the mannosylated 19 kDa (LpqH) and 45 kDa (Apa) glycoproteins had been also proposed as a potential targets of DC-SIGN (Pitarque et al., 2005).

2.3.3 Dendritic cell-associated C-type lectin-1 (Dectin-1)

This is a fungal pattern recognition receptor that binds to β-glucans and triggers cytokine production by facilitating interaction with TLR2 or by directly activating spleen tyrosine kinase (Syk). Recognition of mycobacteria by Dectin-1 has been shown to induce expression of TNF-α, IL-6, and IL-12. The significance of these findings is unclear at present because mycobacteria are not known to contain β-glucans (Yadav & Schorey, 2006; Rothfuchs et al., 2007).

2.3.4 Macrophage inducible C-type lectin (Mincle)

This molecule is expressed in macrophages subjected to several types of stress. Mincle possesses carbohydrate-recognition domain (CRD) within the extracellular region. It has recently been shown that Mincle recognize the trehalose-6,6-dimycolate (TDM, also called cord factor), a mycobacterial cell wall glycolipid that is the most studied immunostimulatory component of *M. tuberculosis*, thereafter modulating macrophage activation. TDM activated macrophages to produce inflammatory cytokines and NO, which are completely suppressed in Mincle-deficient macrophages (Ishikawa et al., 2009).

2.3.5 Pulmonary surfactant protein A and D (Sp-A and Sp-D)

C-type Ca^{2+}-dependent lectins are pulmonary collagenous soluble proteins that are secreted into the alveoli by resident type II alveolar epithelial cells and distal bronchiolar Clara cells. They form a complex structure with lipids and proteins that reduces surface tension of alveoli and promotes lung expansion. These proteins bind to pathogens, mediate uptake into phagocytes, and modulate effector mechanisms such as oxidant production, lung inflammation, and bacterial killing (LeVine et al., 2000). Ferguson and Schlesinger identified ManLAM as a potential ligand of human Sp-D (Ferguson & Schlesinger, 2000). It was also found that purified human Sp-A exclusively binds to alanine and proline-rich antigenic (APA or 45/57 kDa antigen (*Rv1860*) glycoprotein. This result was supported by direct binding of Sp-A to purified APA. Moreover, EDTA addition or deglycosylation of purified

Apa samples completely abolished the interaction, demonstrating that the interaction is Calcium and Mannose-dependent, as expected (Ragas et al., 2007).

2.3.6 Mannose Binding Lectin (MBL)

This 32 kDa protein provides first-line defense against several microbes, the protein possess a collagen-like domain as well as a CRD that binds to high mannose and *N*-acetylglucosamine oligosaccharides present on a range of pathogens, including *M. tuberculosis* (Garred et al., 1997). MBL activates the complement pathway in an antibody-independent manner in conjunction with the MBL-associated serine protease and leads to phagocytosis through the complement or collectin receptors (Berrington & Hawn, 2007).

3. Interaction of *M. tuberculosis* with extracellular matrix proteins

Bacterial species gain access to the human body through different tissues and invasion is generally mediated by bacterial surface and secreted molecules (Sun, 2006). Bacterial colonization, whether benign or pathological, requires the colonizing organism to bind with some avidity to the host. Bacteria have evolved a wide range of molecules, known as adhesins to enable them to bind to selected host molecules. Most high-affinity bacterial adhesins are proteins, and the major targets for them are the host extracellular matrix proteins **(**ECM) which are also present in the cell surface of animal cells (Henderson et al., 2011). Pathogenic bacteria have evolved mechanisms to exploit molecules present in membranes for their own purposes including mediating attachment to target cells through the interaction with ECM proteins such as proteoglycans, fibronectin (Fn) and laminin among others.

Tuberculosis commonly affects the lung but, from the initial focus of infection the bacilli can spread mainly through the blood, but also via lymphatic to other areas of the lung and other organs (Leung, 1999). The dissemination process may allow the bacilli reach regions such as central nervous system, genital tract, intestine, skin, bones, digestive system tissue associated and integumentary system. The ability to spread seems to be determined more by mycobacterial factors since it has been observed that extrapulmonary strains showed a greater ability to spread and were more efficient for the invasion (Mischenko et al., 2004; Garcia de Viedma et al., 2003, 2005).

For many years, it was thought that macrophages were the cells primarily involved in the interaction of tubercles bacilli with the host. However, it is now known that *M. tuberculosis* is also able to invade non-phagocytic cells such as the respiratory epithelial cells and epithelial cells M. Direct adherence and penetration may be important to gain access to the hematogenous and lymphatic systems and therefore dissemination (Menozzi et al., 2002; Teitelbaum et al., 1999). Bacilli also may interact with pneumocytes causing necrosis and destruction of cellular barriers, which could facilitate its passage into the bloodstream and allow the invasion. Binding to ECM may be of relevance to the entry of pathogenic mycobacteria into nonprofessional phagocytic cells (Bermudez et al., 2002).

3.1 Mycobacterial fibronectin binding proteins

Fn is a large and essential multidomain glycoprotein with multiple adhesive properties, functioning as a key link between cells and their ECM. Fn is recognized to be the target

for a large number of bacterial proteins, which are generally considered to function as bacterial adhesins. The ability to bind to Fn has been reported for bacterial pathogens like *Staphylococcus aureus*, *Streptococcus pyogenes* and *Borrelia burgdorferi*, and protozoa like *Trypanosoma cruzi*. Fn plays a vital role in a variety of normal physiological processes, its targeting appears to be another example of the exploitation of a host cell process in the dissemination, establishment and maintenance of infection (Pasula et al., 2002; Henderson et al., 2011). The interaction of Fn with mycobacteria seems to be conserved within the genus. Fn is required for the anti-tumoral effect of *M. bovis* BCG on superficial bladder tumors. Fn is not exposed on normal bladder epithelia, and adhere of BCG cells only occur when uroepithelia is damaged. Blocking of BCG attachment to Fn consequently prevents immune responses and inhibits the expression of anti-tumor (Hudson et al., 1990; Sinn et al, 2008).

3.1.1 Proteins of antigen 85 complex

One of the first proteins described as a Fn binding proteins (FnBPs) were the members of the antigen 85 complex (Abou-Zeid et al., 1988) which consists of three proteins termed antigen 85A, 85B and 85C, encoded by three different genes (*Rv3804c*, *Rv1886c* and *Rv0129c* respectively). They are mycolic acid transferases present in many mycobacteria species. The complex a potent immunogen, is clearly one of the major antigen in the immune response to *M. tuberculosis* infection. For this reason is one of the stronger vaccine candidates (Giri et al., 2006; Romano et al., 2006).

The interaction of antigen 85B with Fn involves the binding of multiple regions of this protein to the collagen-binding domain of Fn (Peake et al., 1993). Peptide mapping of the 84-110 sequence defined residues 98-108 as the minimum inhibitory motif with six residues (FEWYYQ) to be the most important for Fn interaction. This motif forms a helix at the surface of the protein and has no homology to other known prokaryotic and eukaryotic FnBP features and appears to be unique to the mycobacteria (Naito et al., 1998).

3.1.2 Malate synthase G (*Rv1837c*)

This cytoplasmic protein is involved in the glyoxylate pathway. The binding site in malate synthase G (MS) for Fn, lies in a C-terminal region of the protein that is unique to *M. tuberculosis*. The protein is secreted and is anchored on the cell wall by an undefined mechanism. MS expressed in *M. smegmatis* localizes to the cell wall and enhances the adherence of bacteria to lung epithelial A549 cells. Present in the bacterial surface this protein is also able to bind to laminin. These studies show that a housekeeping enzyme of *M. tuberculosis* contributes to its armamentarium of virulence promoting factors (Kinhikar et al., 2006).

3.1.3 Alanine and proline-rich antigenic (APA) (*Rv1860*) or 45/47 kDa antigen

These 40/45 kDa glycoproteins (Espitia et al., 1989b; Dobos et al., 1996) have a very highly conserved alanine and proline-rich 300–350-residue sequence and is apparently unique to mycobacteria. Using synthetic peptides the minimal binding sequence to Fn was determined to be 12 amino acids, 269–280 and sequence necessary for Fn binding is a motif RWFV (273–276). Furthermore, the data suggest that mycobacterial Fn-attachment protein (FAP)

proteins, all of which share the RWFV binding motif, constitute a family of highly homologous proteins that bind Fn in a unique manner (Zhao et al., 1999; Schorey et al., 1996).

3.1.4 PE_PGRS proteins

Although, PE_PGRs are a large family of proteins, the capacity to bind to Fn had been only determine in a few members of the family like Wag22 (*Rv1759c*) antigen (Espitia et al., 1999). More recently, we also found that PE_PGRS33 (*Rv1818c*) and PE_PGRS1 (*Rv0109*) are also bind Fn, being the Fn binding site localized in the PGRS domain, however the motifs involved are unknown (unpublished observations).

3.1.5 Glutamina syntethase A1 (GlnA1) (*Rv2220*)

An essential protein of *M. tuberculosis* that plays a role in nitrogen metabolism. Its enzymatic activity detected in culture filtrates of pathogenic but not of nonpathogenic mycobacteria, has been associated with virulence. Interestingly, we found that GlnA1 was also able to bind to Fn, a feature so far not described in spite of evidence indicating the presence of Fn-binding molecules in the range of 57–60 kDa the GlnA1 molecular mass (Xolalpa et al., 2007).

3.2 Mycobacterial heparin binding protein

Heparin, a sulfated polysaccharide belonging to the family of glycosaminoglycans, has numerous important biological activities, associated with its interaction with diverse proteins. Heparin and the structurally related heparan sulfate are complex linear polymers comprised of a mixture of chains of different length, having variable sequences. Heparan sulfate is ubiquitously distributed on the surfaces of animal cells and in the ECM. It also mediates various physiologic and pathophysiologic processes. In *M. tuberculosis* an heparin-binding protein has been identified.

3.2.1 Heparin-binding hemagglutinin (HBHA) (*Rv0475*)

In 1996, Menozzi *et al*. identified a mycobacterial protein of 199 amino acids with a molecular weight of 28 kDa that binds heparin (Menozzi et al., 1996). This protein promotes the binding of rabbit erythrocytes and mycobacterial aggregation. Antibodies against HBHA inhibit haemaglutination, bacterial aggregation and binding the bacteria to epithelial cells. HBHA binds to sulfated carbohydrates from a region rich in lysine and proline, thus promoting its binding to host tissues (Menozzi et al., 1998). Later, it was demonstrated that this protein is involved in extrapulmonary dissemination in a mouse model of tuberculosis, bacteria mutated in HBHA decreased spread to the lung, liver and spleen and this ability was restored by complementing the mutants with wild-type gene. These results suggest that HBHA is essential for the escape of mycobacteria in the lung and the establishment of extrapulmonary (Pethe et al., 2001).

Menozzi *et al*. showed in 2006 that this protein induces a reorganization of the actin filaments in a barrier of endothelial cells, but does not affect the tight junctions. This protein mediates the binding and internalization of mycobacteria in human laryngeal epithelial cell line (HEp-2) and type II pneumocytes cell line (A549). Apparently lysine rich C-terminal

region is mediating these biological effects (Menozzi et al., 2006). In addition to be an important adhesin in tuberculosis HBHA is also an important antigen.

3.3 Mycobacterial laminin binding proteins

Laminin is a large (900 kDa), highly glycosylated multidomain protein found in all human tissues. The laminins are an important and biologically active part of the basal lamina, influencing cell differentiation, migration, adhesion as well as phenotype and survival. Laminins are trimeric proteins that contain an α-chain, a β-chain, and a γ-chain. The trimeric proteins intersect to form a cross-like structure that can bind to other cell membrane and ECM (Timpl et al., 1979; Aumailley et al., 2005). Adhesion to laminin it is a starting point of tissue invasion for many pathogenic bacteria. Mycobacterial protein capable of binds laminin had been also identified.

3.3.1 Histone-Like Protein (HLP) (*Rv2986c*) or HupB protein

A cationic surface protein of twenty amino acids was identified as laminin binding protein in *Mycobacterium lepra*e (ML-LBP21). This cell wall protein increased the binding of mycobacteria to the surface of Schwann cells through its binding to laminin-2 α chains. ML-LBP21 showed 78% of identity with the HupB/HLP protein of *M. tuberculosis* which is also able to bind to laminin of cell surface of murine sarcoma, epithelial cells and human pneumocytes (Prabhakar et al., 1998; Shimoji et al., 1999; De Melo et al., 2000). In addition, two heparan sulphate binding sites were found in Hlp (Portugal et al., 2008).

3.3.2 Early Secretory Antigenic Target (ESAT-6) (*Rv3875*) or EsxA protein

Comparative studies have identified 16 regions of difference (RD1-16) between the genomes of *M. tuberculosi* and *M. bovis* BCG, of which one deletion, termed RD1, is absent from all *M. bovis* BCG substrains currently used as vaccines. RD1 includes 15-gene locus (ESX-1), which encodes a secretion system type VII that enables the secretion of several proteins including ESAT-6 and CFP-10. Both proteins have been considering relevant for cell immune response against tuberculosis bacilli. ESAT-6 has recently been demonstrated to cause haemolysis and macrophage lysis and also causes cytolysis of type 1 and type 2 pneumocytes. Since both types of pneumocytes express membrane laminin, and ESAT-6 exhibits dose-dependent binding to purified human laminin, these observations suggest that the specific association of ESAT-6 with the bacterial surface is mediated thought laminin (Kinhikar et al., 2010).

3.3.3 Mammalian Cell Entry operon (MCE) proteins

Although, the host receptor for MCE proteins is unknown, it is important to mention that *mce* genes encode adhesins and invasins proteins located on the surface of mycobacteria. The first adhesion of this operon was by Arruda *et al*. in 1993; by using a gene library of *M. tuberculosis* they were able to confer to non-pathogenic *Escherichia coli* the ability to invade epithelial HeLa cells (Arruda et al., 1993). Subsequently, it was demonstrated the existence of other genes arranged in an additional *mce* operons (Parker et al., 1995).

The role of membrane protein MCE-1A in invasion of mycobacteria was demonstrated *in vitro*, this protein induces membrane invagination and entry into HeLa cells. The processes

were inhibited with cytochalasin D and nocodazole indicating that rearrangements in microtubules and filaments are necessary to membrane invagination MCE-1A-mediate. The activity of this protein is given by a domain located in the central region of the protein called INV-3 (Lu et al., 2006). By comparing the predicted secondary structure for MCE-1A, MCE-2A, MCE-3A and MCE-4A proteins was found that there is 70% similarity and contain domains alpha/beta. On analysis by predicting the structure of proteins is found that the beta domain is probably involved in cell binding (Mitra et al., 2005).

4. Pathogen interaction with the fibrinolityc system

The fibrinolytic system is composed by the zymogen plasminogen (Plg), that is activated to serine protease plasmin (Plm) by tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). The activity of tPA and uPA is regulated by plasminogen activation inhibibors 1 and 2 (PAI-1 and PAI-2), whereas Plm activity is regulated by α-2 antiplasmin and α-2 macroglobulin. Plm degrades fibrin deposition in blood cloths and also can activate latent matrix metalloproteinases (MMPs) and other protein molecules. During infectious diseases, the Plg-Plm system also called fibrinolytic system; suffer alteration in their balanced expression, caused mainly by inflammation, besides the binding and interaction of some pathogen molecules with components of this system.

In recent years, several studies have suggested important roles for fibrinolityc system in bacterial infections. The microorganims interact with the Plg-Plm system in different ways; by immobilizing Plg on their surfaces through Plg receptors (PlgR) an event that conducts to activation of Plg by host plasminogen activators (PAS) to generate enzymatically active Plm, or by expressing molecules that can activate Plg by themselves. PlgR have been detected in virus (Chaipan et al., 2009), fungus (Crowe et al., 2003), parasites (Avilán et al., 2011) and bacteria. Among bacteria are *Borrelia burgdorferi* (Fuchs et al., 1994), *Escherichia coli* (Kukkonen et al., 1998; Lähteenmäki et al., 1993; Parkkinen et al., 1991), *Salmonella typhimurium* (Korhonen et al., 1997; Kukkonen et al, 1998; Lähteenmäki et al., 1995), *Neisseria meningitidis* (Ullberg et al., 1992) and *Haemophilus influenzae* (Sjostrom et al., 1997) and group A, B and C of streptococci (Lähteenmäki et al., 2001b; Coleman & Benach, 1999). In contrast to the high number of PlgR described in bacteria, only a few PAs has been identified, the plasminogen activator in *Yersinia pestis* (Pla) (Perry & Fetherston, 1997; Sodeinde et al., 1992), the staphylokinase (SK) of *Staphylococcus aureus* (Esmon & Mather, 1998; Lijnen et al., 1994) and the streptokinase (SAK) of *Streptococcus pyogenes* (Li et al., 1999; Sun et al., 2004).

Pla is an aspartic protease that cleaves Plg at the same peptide bond as tPA and uPA. It is also an adhesin with affinity for ECM and laminin (Kukkonen et al., 2001; Lähteenmäki et al., 2001a, 2001b). SAK and SK are not enzymes themselves, but they form 1:1 complexes with Plg, inducing conformational changes in Plg that allows its activation to Plm. SK in bacterial virulence has been studied most thoroughly in group A streptococci (GAS), which is an invasive pathogen that causes diseases ranging from mild pharyngeal and skin infections to potentially fatal disorders, such as toxic shock syndrome. GAS infects only humans and is specific for human Plg and the role of SK and Plg in GAS infection was confirmed in transgenic mice expressing human Plg (Sun et al, 2004).

Adherence to ECM and Plg activation on bacteria surface either by host or bacteria owns PAs seems to be the mechanisms used for bacteria to colonize and invade the host tissues.

Since similar mechanisms are typical for tumor cell migration and invasion (Mignatti & Rifkin, 1993) where Plm directly degrades laminin, a major glycoprotein of basement and indirectly damages tissue barriers by activating MMPs, the term "bacterial metastasis" is now used in analogy to tumor cells metastasis (Plow et al., 1999; Läteenmäki et al., 2000, 2005).

It is worth of note that although the list of microorganism that possess PlgR is large, many of the identified receptors belong to a group of surface-localized housekeeping enzymes that enhance virulence of several, mainly Gram-positive bacterial species; even though the mechanism of their secretion into the cell surface is not known (Pancholi & Chhatwal, 2003). It is also remarkable that some of PlgR also bound to ECM proteins. Together these observations suggest that Plm-mediated fibrin degradation could be a mechanism that advances the spread of invasive bacteria within the mammalian host (Sun et al., 2004).

4.1 Mycobacteria interactions with human fibrinolytic system

In the course of mycobacterial infections the fibrinolytic activity demonstrated a remarkable increased, the first publication in this field was issued by Smokovitis and colleagues in 1976, showed that intradermicaly inoculation of *M. bovis* BCG in rabbits triggered lesions in the dermis, with increment of focal fibrinolytic activity caused by a PA detected in lesions fluids and by histology and fibrin slide technique; 2 weeks later, when hypersensitivity to the *M. bovis* BCG vaccine became pronounced and caseous centers developed, fibrinolytic activity was particularly high (Smokovitis et al., 1976). An intact coagulation mechanism, including tissue factor generation, appears to be important for the development of skin test induration in humans skin since anticoagulation with warfarin decreased skin test induration and tissue factor generation, but lymphocyte transformation remained unchanged (Edwards & Rickles, 1978).

Macrophages obtained from infected mouse with *M. bovis* BCG and challenged in culture with protein-purified derivative (PPD), had increased plasminogen activator activity that was dependent of presence of T cells, macrophages obtained from infected animals without boosting showed less fibrinolytic activity that those stimulated with PPD and the stimulation was abolished by depletion of T cells (Gordon & Cohn, 1978).

Together these observations indicate that the fibrinolytic system could play an integral role in the tuberculosis inflammatory response. The participation of Plg-Plm system in inflamation has been documented during the last decade. It has been demonstrated that, TNF-α induced the expression of PAI-1 and uPA in adipocytes and in human pulmonary artery cells respectively (Pandey et al., 2005; Wu & Aird, 2005). PAI-2 transcription is also upregulated under the influence of TNF-α in fribrosarcoma cells (Medcalf et al., 1988). Transcription of urokinase receptor (uPAR), protein located in the membrane of numerous cell types, is influenced by TGF-β (Lund et al., 1991). tPA transcription is downregulated by TNF-α in human umbilical vein endothelial cells (Schleef et al., 1988), whereas in fibroblasts from human pulp and gingival fibrolasts TNF-α and IL-1 induce a significant increase of tPA (Chang et al., 2003).

In pleural effusions of tuberculosis patients levels of TNF-α were higher than in complicated parapneumonic and cancer patients, whereas tPA concentration was lower Plg and PAI-1

levels were higher in tuberculosis patients than the other groups. (Aleman et al., 2003). Lu and collaborators, compared the concentration of components of fibrinolytic system in pleural and ascytic fluid from tuberculosis, cancer and liver cirrhosis patients, the results showed higher levels of uPA and uPAR from tuberculosis and cancer patients with respect to cirrhosis patients, whereas tPA level was higher in cancer and cirrhosis patients than tuberculosis patients. PAI-1, Plg and Plm levels in tuberculosis patients where statistically higher than those levels in cirrhosis and cancer patients (Lu et al., 2007).

Serum level of soluble uPAR (suPAR) in tuberculosis patients was analyzed in a community from Guinea Bissau, the results showed an elevated level of suPAR in active tuberculosis; decreasing levels of suPAR where associated with treatment, whereas lower levels of suPAR where related with survival (Eugen-Olsen et al., 2002).

The protease activity of Plm plays an important role in the turnover of ECM in a mice model of *Mycobacterium avium* infection, whereas early dissemination to organs was observed in Plm and tPA deficient mice. The authors also demonstrated that fibrin deposition and Fn increased in Plg deficient mice during the infection in comparison with wild type mice (Sato et al., 2003).

The first evidence of the presence of PlgR and PAs in *M. tuberculosis* was reported by Monroy and collaborators; the lysine-dependent binding of Plg to mycobacteria was demonstrated by FACScan analysis and affinity blotting assays. The specificity of binding and the participation of lysine residues in mycobacterial protein-Plg interaction were tested in the presence of 0.1, 1, and 2 M ε-aminocaproic acid (EACA), a lysine analog. Plg binding proteins of 30, 60, and 66 kDa detected in bacteria protein extracts was abolished by this lysine analog. Furthermore, both soluble protein and total protein extracts could activate Plg only in presence of fibrin matrices, suggesting these results, that Plg activation by *M. tuberculosis* requires attachment of Plg to a target molecule. Moreover, addition of α-2 antiplasmin did not significantly decrease activation of Plg by mycobacterial extracts, showing that pathogen-associated Plm activity is not blocked efficiently by host serpins (Monroy et al., 2000).

Besides, a recently study revealed the identity of several Plg-binding proteins present in *M. tuberculosis* and that Plg bound to mycobacterial receptors is converted into Plm by tPA, the mammalian Plg activator. By proteomic analysis together with ligand blotting assays the identity of several Plg-binding spots in the mycobacteria soluble extracts and culture filtrate proteins was determined. In ligand blotting assay in polyvinylidene difluouride (PVDF) membrane with mycobacterial proteins 2D-SDS-PAGE-resolved reactive spots bound to Plg were detected using an anti-Plg antibody, then by N-terminal sequencing and/or mass spectrometry (MS) fifteen different proteins were identified from reactive spots, identified proteins correspond to DnaK (*Rv0350*), GroES (*Rv3418c*), GlnA1 (*Rv2220*), antigen 85 complex (*Rv3804c*, *Rv1886c* and *Rv0129c*), Mpt51 (*Rv3803c*), Mpt64 (*Rv1980c*), PrcB (*Rv2110c*), MetK (*Rv1392*), SahH (*Rv3248c*), Lpd (*Rv0462*), Icl (*Rv0467*), Fba (*Rv0363c*), and EF-Tu (*Rv0685*). Interaction of Plg with these proteins was inhibited by the lysine analogue EACA, indicating that the binding was mediated by lysine residues. Among of identified *M. tuberculosis* PlgRs; only DnaK, PrcB, GroES, and EF-Tu have the C-terminal lysine.

In same work, binding of Plg to DnaK, GlnA1, and antigen 85B was confirmed with recombinant proteins by ELISA and ligand blotting assays. These results confirmed findings

Mycobacterial protein targets	Host receptors	Binding sites	References
LpqH	TLR2	Unknown	López et al., 2006
	MR	Ch	Diaz-Silvestre et al., 2006
	DC-SIGN?	Ch?	Pitarque et al., 2006
LprG	TLR2	Ch	Drage et al., 2010
	DC-SIGN	Ch	Carroll et al., 2010
LprA	TLR2	Unknown	Pecora et al., 2004
APA	Sp-A	Ch	Ragas et al, 2007
	Fn	^{273}RWFV^{276}	Schorey et al.,1996
	DC-SING?	Ch?	Pitarque et al., 2006
Hlp	Hs	Gly^{46} to Ala^{60}, Thr^{31} to Phe^{50}, C-terminus, Lys rich residues	Portugal et al., 2008 Shimoji et al., 1999
	Lm	C-terminus, Lys rich residues	Soares de Lima et al., 2005
HBHA	Hs	C-terminus, Lys rich domine	Menozzi et al., 1996 Pethe et al., 2000
MS	Fn	40 aa C-terminal region	Kinhikar et al., 2006
	Lm	Unknown	Kinhikar et al., 2006
ESAT-6	Lm	Unknown	Kinhikar et al., 2010
PE_PGRS33	TLR2	Unknown	Basu et al., 2007
	Fn	PGRS domain	Unpublished work
Wag22	Fn	PGRS domain	Espitia et al., 1999
PE_PGRS1	Fn	PGRS domain	Unpublished work
DnaK	DC-SING	Unknown	Carroll et al., 2010
	Plg	Unknown Lys residue	Xolalpa et al.,2007
GlnA1	Fn	Unknown	Xolalpa et al., 2007
	Plg	Unknown Lys residue	Xolalpa et al., 2007
SahH	Plg	Unknown Lys residue	Xolalpa et al., 2007
Lpd	Plg	Unknown Lys residue	Xolalpa et al., 2007
Icl	Plg	Unknown Lys residue	Xolalpa et al., 2007
EF-Tu	Plg	Unknown Lys residue	Xolalpa et al., 2007
MetK	Plg	Unknown Lys residue	Xolalpa et al., 2007
Fba	Plg	Unknown Lys residue	Xolalpa et al., 2007
Antigen 85 complex	Fn	^{98}FEWYYQ^{103}	Naito et al., 1998
	Plg	Unknown Lys residue	Xolalpa et al., 2007
Mpt51	Fn	Unknown	Naito et al., 1998;
	Plg	Unknown Lys residue	Xolalpa et al., 2007
PrcB	Plg	Unknown Lys residue	Xolalpa et al., 2007
Mpt64	Plg	Unknown Lys residue	Xolalpa et al., 2007

Mycobacterial protein targets	Host receptors	Binding sites	References
GroES	Plg	Unknown Lys residue	Xolalpa et al., 2007
Cnp60.1	DC-SIGN	Unknown	Carroll et al., 2010
GAPDH	DC-SIGN	Unknown	Carroll et al., 2010

Abreviations:
Ch Carbohydrates
Hs Heparan sulphate
Lm Laminin
Plg Plasminogen
MR Mannose Receptor

Table 1. Host-Mycobacteria proteins involved in interactions.

with native proteins reported before, that the interaction involved lysine residues. In addition Plg bound to recombinant mycobacterial proteins was activated to Plm tPA activator (Xolalpa et al., 2007).

Until now, our results show that *M. tuberculosis* possesses Plg binding and activating molecules present in the soluble protein extracts; based in these evidences works are in progress to identify potential PA in mycobacteria. Considering that interaction with the Plg system promotes damage of extracellular matrices as well as bacterial spread and organ invasion during infection, this suggests a common mechanism in migration of eukaryotic and prokaryotic cells that could be used by *M. tuberculosis* in disease process. *M. tuberculosis* possesses several Plg receptors suggesting that bound Plg to bacteria surface can be activated to Plg, endowing bacteria with the ability to degrade ECM and basal membranes proteins contributing to tissue injury in tuberculosis.

5. Influence of genetic variations in host-mycobacteria relationship

Human tuberculosis results from the interactions between host and bacteria, the degree to which genetic variations of both human and mycobacteria influence this relationship has become elucidated.

5.1 Mycobacterial genetic polymorphisms

Massively DNA sequencing and comparative genomic, together with in *vitro* and *in vivo* models of *M. tuberculosis* infection are contributing to define how different clinical genotypes of *M. tuberculosis* affect the innate immune response. There is now evidence that strain variation can lead to variable virulence phenotypes and can evoke or suppress host immune response (López et al., 2003).

Recently direct role for strain-variation-associated virulence in suppressing host immune response and inducing hyperlethality in mice documented in a subset of clinical isolates belonging to the W-Beijing family where the presence of a polyketide synthase-derived phenolic glycolipid endowed the strains with hypervirulent phenotype (Reed et al., 2004). A recent report also showed how the cyclopropane modification of trehalose dimycolate was critical to induce a pro-inflammatory response during the first week of infection in mice (Rao et al., 2005).

On the other wise, genetic polymorphism in *M. tuberculosis* genome is given by the presence of insertion sequence 6110 (IS*6110*). About 23 copies of this sequence are distributed along the genome. Molecular tipification based on number and localization of the IS*6110* in *M. tuberculosis* clinical isolates have been used for several molecular epidemiology studies.

However, the sequence can be inserted in promoter region of regulatory genes, modifying the expression the genes they regulate, an example is the presence of IS*6110* in the promoter region of *phoP* of *M. bovis* strain B, a multidrug resistant and hypervirulent strain that was responsible for a nosocomial outbreak of tuberculosis in Spain (Soto et al., 2004). Many copies of IS*6110* has been found in or near of *pe/ppe* genes of *M. tuberculosis* strain 210, responsible of several cases of tuberculosis in Los Angeles, California USA suggest the possibility that the insertions could be mediating recombination of these sequences that result in changes that endowed the bacteria with high capacity for transmission and/or replication (Barnes et al., 1997; Beggs et al., 2000).

PE/PPE family also showed important variation in their coded sequences. Analysis of mutations in PPE38 gene of *M. tuberculosis* clinical isolates representing all major evolutionary lineages, show hypervariability of the PPE38 region consequence of the combination of a high frequency of IS*6110* insertion events, IS*6110*-associated recombination/deletion events, homologous recombination and gene conversion events (McEvoy et al., 2009).

It is known that one of the most important sources of genetic variability in *M. tuberculosis* complex is given by PE/PPE gene family (Cole et al., 1998; Fleischmann et al., 2002). Sequence of *pe_pgrs33* gene from 123 *M. tuberculosis* clinical isolates showed variations relative to PE_PGRS33 sequence from H37Rv in 84 (68.3%) of the 123 isolates. Sequence variations included insertions, deletions and both as well as single nucleotide polymorphisms (SNPs). Variations were more frequently found in the C-terminal PGRS domain of PE_PGRS protein and affecting one or more of the glycine-alanine repeats. These variations could potentially account for some of the differences in their ability to evade the host immune system, and this idea contribute to support the role of the PE_PGRS family in antigenic variation (Talarico et al., 2005).

Furthermore, the study of variations in PE_PGRS33 gene from 649 *M. tuberculosis* clinical isolates showed a possible association of major changes (large insertions/deletions or frameshift mutations) in the PE_PGRS33 protein with clustering of tuberculosis cases and the absence of cavitations in the lungs, in contrast with patients infected with *M. tuberculosis* isolates having any or minimal changes in the protein (Talarico et al., 2007). Basu et al., 2007 also observed that deletions within the PGRS domain attenuate the induction of TNF-α-mediated by PE_PGRS33 through its TLR2. Also in this work was observed that the induction of TNF-α was specific of PE_PGRS33 and not a common feature of the PE_PGRS family, since PE_PGRS48 and PE_PGRS62 did not induced production of TNF-α at the same level of the PE_PGRS33. These results provide evidences that variations in the polymorphic repeats of the PGRS domain modulate the innate immune response (Basu et al., 2007).

It has been observed that the PE_PGRS genes are differentially expressed in different strains of *M. tuberculosis* during growth *in vitro* (Flores et al., 2003; Dheenadhayalan et al., 2006) and *in vivo* (Delogu et al., 2006). Interestingly, expression of many of these proteins seems to be widely regulated by several conditions (Voskuil et al., 2004; Vallecillo & Espitia, 2010). In addition to that, the extreme polymorphism found among the clinical isolates, suggests that they could be a major source of antigenic variation in *M. tuberculosis* (Talarico et al., 2005; Santillan et al., 2006). PE_PGRS16 and PE_PGRS26, genes are inversely regulated during persistent of *M. tuberculosis* infection, suggesting that differential expression of these two PE_PGRS genes may have a role in latency. The variations in these sequences were also studied in 200 strains, 102 (51%) and 100 (50%) showed variations within the PE_PGRS16 gene and the PE_PGRS26 gene, respectively. Variations consist in insertions and deletions, frameshifts, and SNPs. Frameshifts are predominant in PE_PGRS16 gene and in-frame deletions in PE_PGRS26 gene. The observed sequence variations could impact the function of these protein and could be asociated with different clinical manifestations of tuberculosis, but remains to be understood the specific role that these genes play in *M. tuberculosis*-host interaction (Talarico, et al., 2008).

Sequence analysis of the coded sequence of PPE18 a vaccine candidate in 225 clinical isolated of *M. tuberculosis* showed an important variability that must be considered when potential vaccine candidates are selected and evaluated. Evaluation of genetic variability could provide important information regarding the ability of the immune response induced by a vaccine candidate to recognize different field strains of *M. tuberculosis* (Hebert et al., 2007).

Differential gene expression also can contribute to modify the host-pathogen interactions. It has been demonstrated that HBHA, the heparin binding protein is differentially expressed during the infection with *M. tuberculosis*, showing a higher expression early infection when the bacteria spread from the primary site of infection. In addition, it was also found that *hbha* gene is up regulated in epithelial cells but not in macrophages (Delogu et al., 2006).

Mutation in *Rv0444* gene that codified for the anti-sigma factor K increased the expression of MB83 antigen in *M. bovis* BCG and *M. bovis* clinical isolates compare with the very low expression of this protein in *M. tuberculosis* (Charlet et al., 2005; Saïd-Salim et al., 2006).

In a recent work, genome sequences of 21 phylogeographically diverse strains of *M. tuberculosis* complex (16 *M. tuberculosis* and 4 *M. africanum* strains representative of the six human *M. tuberculosis* lineages, and one strain of *M. canettii*) were carried out. The presence of 491 human T cell epitopes experimentally determined was evaluated from the genome database. The result of the analysis showed that known human T cell epitopes are highly conserved suggesting these observations that the hyperconservation of T cell epitopes in *M. tuberculosis* is consequence of a strong selection pressure, perhaps because the immune response they elicit in humans, is essential for the survival of an infected individual, and might be partially beneficial to the pathogen. One potential mechanism by which the mycobacterium could benefit from human T cell recognition is that human T cell responses are essential for *M. tuberculosis* to establish latent infection and one effective transmission to new susceptible host (Comas et al., 2010). One important limitation of this study was the exclusion of PE/PPE genes, because as it has been commented that the sequence variations into some of these genes contributed in significant form to the dynamical parasite-host relation.

According to the observations of Comas *et al*. sequence analysis of genes encoding a major *M. tuberculosis* antigens ESAT-6, TB10.4, and antigen 85B from 88 clinical isolates of *M. tuberculosis* revealed no variability in the genes *esx*A and *esx*H in all isolates. In the case of *fbp*B (antigen 85B) only one synonymous SNP located at position 714 bp of the gene sequence, among 39 (44.3%) of the 88 strains sequenced (Davila et al., 2010).

5.2 Host genetic variability

The severity of tuberculosis disease is controlled not only by genetics variations at the level of the bacteria but also by host disparities. The presence of SNP (T597C) in TLR2 among 2 groups of Vietnamese adults with pulmonary or meningeal tuberculosis suggests a strong association of TLR2 variation with meningeal tuberculosis. The authors hypothesize that polymorphisms in genes of the innate immune response may influence the host response resulting in increased susceptibility to disease causing by some bacterial lineages but not others (Caws et al., 2008).

Crohn's disease itself resembles a chronic intestinal granulomatous infection seen in animals called Johne's disease, which appears to be caused by *Mycobacterium avium* spp. *paratuberculosis* trigger (Berrington & Hawn, 2007) a controversy exists as to whether Crohn's disease has an environmental or infectious trigger. Frameshift mutations in the N-terminal leucine-rich region of NOD2 have been associated with Crohn's disease in adults. More recently, NOD2 variants were found to be associated with susceptibility to tuberculosis in an African American population. In light of these findings, the importance of NOD2-mediated immunity during a chronic mycobacterial infection requires further study (Austin et al., 2008).

In a study from Mexico, several variants of Sp-A and Sp-D were associated with susceptibility to tuberculosis (Floros et al., 2000). These results have not been verified in an independent cohort, and the functional significance of the variants has not been reported. The continued identification of *M. tuberculosis* genes or host-encoded will ultimately enhance our knowledge of the complex and highly dynamic interaction between the pathogen and host (Zahrt et al., 2003).

6. Adhesins as therapeutic targets

The emergence of *M. tuberculosis* Multidrug-resistant strains is now a major public health problem all over the world. In this context, it is highly critical to develop a new strategy for the treatment of infected patients that supplements the conventional antimycobacterial chemotherapeutic drugs. More precise understanding of the host-bacteria interactions will pave the way for the development of an effective drug. In this way, targeting bacterial colonization through blockade of selective adhesins could be therapeutically useful (Ofek et al., 2003; Klemm et al., 2010). There are a number of examples where inactivation of FnBP genes or antibodies to FnBPs has resulted in decreased bacterial colonization/virulence (Rennermalm et al., 2001; Rivas et al., 2004). Clinically, most work has been carried out on the humanized monoclonal antibody tefibazumab, which selectively binds *S. aureus* ClfA (Hetherington et al., 2006). In tuberculosis, patient sera containing anti-HBHA antibodies neutralize the entry of *M. tuberculosis* to epithelial cells, suggest that antibody to HBHA may

play a role in protection against mycobacterial extrapulmonary dissemination (Shin et al., 2006).

Some important advances of the interaction of mycobacteria and host has been elucidated but there is more questions to answer. *M. tuberculosis* as has been shown in this chapter, posseses multiple and diverse molecules that targeting innate immune system, ECM and fibrinolytic system. The study of the molecular interactions to define, binding motifs, and specificity of these interactions is crucial in the search of new molecular targets. Bacteria receptors that result in colonization and invasion are likely to be targeted.

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4

Broadening Our View About the Role of *Mycobacterium tuberculosis* Cell Envelope Components During Infection: A Battle for Survival

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1. Introduction

TB is an ancient disease, however, it is still a major health problem in the world (Centers for Disease Control and Prevention, 2006). The number of new cases of TB worldwide roughly correlates with economic conditions: the highest incidences are seen in those countries with the lowest gross national products (countries mainly in Africa, Asia, and Latin America). WHO numbers indicate that 8 million people are newly infected and nearly 2 million people die of TB every year; translating to one person infected every 4 seconds and one person dying every 18 seconds (WHO, 2007). WHO estimates that by year 2020 up to 36 million people will die of TB every year (WHO, 2010). The current TB burden in the world is strictly associated to *Mycobacterium tuberculosis* (*M.tb*) co-infection with HIV, and the recent emergence of practically untreatable extensive-, extremely-, and totally-drug resistant *M.tb* strains (XDR-, XXDR-, and TDR-) in endemic areas. In this context, a person infected with a XDR/XXDR *M.tb* strain currently has a survival rate between 36-50%, however, for people co-infected with HIV, their survival rate drops to ~15% with a life span of 16 days upon XDR/XXDR *M.tb* infection (Gandhi *et al.*, 2006). XXDR-*M.tb* strains are resistant to all first and second line of drugs currently available, and TDR-*M.tb* strains are even resistant to the new developed drugs currently in clinical trials (Andrews *et al.*, 2010;Basu *et al.*, 2009; Gandhi *et al.*, 2006, 2010). Very little is known about the cell wall composition of XDR/XXDR/TDR *M.tb* strains. The presence of phenolic glycolipids and triglycerides in the *M.tb* cell wall has been directly related to the hypervirulence observed in some strains (Reed *et al.*, 2004, 2007). However, in the case of XDR/XXDR/TDR *M.tb* strains, it still is unknown which bacterial and host factors are involved in the induction of the overwhelming host immune response generated by these strains.

Initial interactions between *M.tb* and the host mark the pathway of infection and the subsequent host inflammatory response that defines disease outcome. Many studies have been performed analyzing the constitution of the cell wall of *M.tb*, where structural-biological function relationships for the majority of the cell wall constituents are still being elucidated. The majority of the cell wall of *M.tb* is comprised of carbohydrates and lipids,

and there is increasing evidence that microbial determinants readily exposed to the host immune system play critical roles in disease pathogenesis. Recent studies have been focused on depicting how *M.tb* adapts to the host by mimicking its cell envelope to mammalian glycoforms. Of particular interest is the fact that some *M.tb* strains are characterized by the presence of mannose-containing biomolecules, whose terminal epitopes closely resemble those on host mannoproteins. In this scenario, it is thought that *M.tb* may use this resemblance to the host to its advantage gaining entrance and establishing its particular intracellular niche within the host; thus, the initial *M.tb*-host interface may dictate the pathway of infection and the successful outcome of the disease. Many factors are involved in this interface. First the constitution of the *M.tb* cell wall, which is strain dependent. Here we will discuss the differences in the cell wall among the widely used laboratory strains (*M.tb* $H_{37}R_v$ and *M.tb* Erdman) and several relevant *M.tb* clinical isolates in endemic TB areas, including hypervirulent, MDR-, XDR, XXDR- and TDR- *M.tb* strains. We will discuss their cell wall constitution in relation to their infection outcome. Second, we will focus on the host cell that acts as a niche, the alveolar macrophage, and we will discuss the innate immunofactors present on the host cell that contribute to control or alternatively can favor the infection. Importantly, we will introduce our new results in an area frequently bypassed in many forums, the host environment and how this may challenge the old dogma of the real constitution of the *M.tb* cell wall during infection. We will discuss the alveolar microenvironment that *M.tb* encounters during infection, and how these may determine/contribute to the pathway of infection and disease outcome.

2. The cell wall of *M. tuberculosis* and its biological functions

A great effort has been made by many research groups to depict the *M.tb* cell wall structure and biosynthesis. The main distinctive feature of the *M.tb* cellular envelope cited in all books is the thick and waxy cell wall. This complex structure contributes to the main characteristics that distinguish mycobacteria, such as the acid fast staining properties, the low permeability of the cell wall, the resistance to harsh environments, and the intrinsic resistance to many hydrophobic antibiotics (Brennan & Nikaido, 1995;Jarlier & Nikaido, 1994). The properties of the cell wall barrier also contribute to the intracellular survival of the organism by acting as a direct modulator in the immunological reaction between the host and mycobacteria (Barry & Mdluli, 1996;Lederer *et al.*, 1975). *M.tb* is one of a small group of species able to survive inside the phagocytic cells of a host, so it is likely that its cell wall has special properties defending the bacterium against host microbicidal processes. Within the cell wall of *M.tb* may lie all of the elements associated with TB, including the factors responsible for caseation and other features of hypersensitization, the antigens responsible for humoral immunity, the agents of toxicity, and thus, the very antigens implicated in protective immunity (Brennan, 1988). A detailed electron microscopy study has not yet, however, identified any special features in *M.tb* compared to other non-pathogenic mycobacteria. The envelope consists of three distinct parts, the plasma membrane, the wall, and around it, the outer material. These parts are involved in providing mechanical support and osmotic protection plus transport exchange of ions and molecules with the micro-environment(s) surrounding the bacillus during the different stages of infection. Here we will discuss *M.tb* cell wall components in terms of their role in *M.tb* pathogenesis, focusing on the *M.tb* peripheral lipid layer constituents that are involved in *M.tb*-host cell recognition and pathogenesis.

2.1 The plasma membrane

Defined as a classical bilayer (Silva & Macedo, 1983), the *M.tb* membrane does, however, have some distinctive components, notably the lipoglycoconjugates mannose-capped lipoarabinomannan (ManLAM), lipomannan (LM), and phosphatidyl-*myo*-inositol mannosides. Integral membrane proteins embedded in the layers of the cell wall have also been described (Brennan & Draper, 1994). Analyses of the proteome of the plasma membrane of *M.tb* suggests that the plasma membrane of *M.tb* is likely to be rich in proteins comprising several essential enzymes, receptors and transporters (Sinha *et al.*, 2002), like other prokaryotic cell membranes (Sigler & Hofer, 1997). Bioinformatic analysis of the *M.tb* genome predicts more than 600 'putative' membrane-associated proteins with different numbers of transmembrane hydrophobic segments. These proteins undoubtedly play a role in the uptake and effects of various metabolites, peptides, drugs and antibiotics. Nonetheless, the real location, expression patterns, and function for the majority of these transmembrane proteins remain relatively unexplored (Lee *et al.*, 1992;Yokoyama & Shimizu, 2002; D.B. Young & Garbe, 1991).

2.2 The cell wall

The shape-forming properties of the wall are attributable to the peptidoglycan, whose chemical structure in *M.tb* closely resembles that found in other bacteria. Mainly, the cell wall is defined as a skeleton formed by a covalently linked structure of peptidoglycan, with a branched-chain polysaccharide, the arabinogalactan, attached by phosphodiester bonds. The arabinogalactan distal ends are esterified with high-molecular weight fatty acids, the mycolic acids, of sizes and structures unique to mycobacteria. This cell wall skeleton receives the name of the mycolyl-arabinogalactan-peptidoglycan complex (mAGP) (Besra *et al.*, 1995;Daffe *et al.*, 1990). The wall is constructed of three layers. With conventional staining using electron microscopy, their appearance is defined with an inner layer of moderate electron density, a wider electron-transparent layer, and an outer electron-opaque layer of extremely variable appearance and thickness. The outer opaque layer probably contains the outer material. The electron-transparent layer appears to be mycolated arabinogalactan, which forms a large part of the wall. Finally, the inner layer is speculated to contain both peptidoglycan and arabinogalactan (Draper, 1971).

There are many models of the mycobacterial cell envelope (Bhamidi *et al.*, 2011;Brennan and Besra, 1997;Crick *et al.*, 2003;Dmitriev *et al.*, 2000;Domenech *et al.*, 2001). Interactions of the asymmetric plasma membrane, peptidoglycan, and covalent attached arabinogalactan together with LAM and PIMs have been speculated, at least some of which are known to be associated with the plasma membrane. Mycolic acids are known to be attached to the majority of the terminal and penultimate arabinose residues of the arabinogalactan (Barry *et al.*, 1998). Since the mycolates possess two hydrocarbon chains of unequal lengths, which form an irregular monolayer, it is proposed that these are complemented by two different classes of polar lipids with medium (*e.g.* mycocerosates) and short (*e.g.* acylglycerols) fatty acyl chains, respectively (Barry *et al.*, 1998). There is also evidence for a small number of porins in the envelope, presumably within the outer hydrophobic bilayer (Senaratne *et al.*, 1998; Trias *et al.*, 1992). A recent study had committed efforts to solve the enigma involving the spatial organization of the mycobacterial cell envelope. This study compared bacteria grown *in vivo* (*i.e. Mycobacterium leprae*) *vs.* grown *in vitro* (*i.e. M.tb*) showing that bacilli

grown *in vivo* had a more compact cell envelope with more mycolic acids and more but shorter arabinogalactan molecules per peptidoglycan (Bhamidi *et al.*, 2011). This differential cell envelope spatial conformation may differentially impact the rearrangement of the outer surface exposed cell envelope components that have a critical role in *M.tb*-host recognition.

The barrier for the influx of solutes such as nutrients or drugs is associated with the parallel alignment of mycolic acids (Liu *et al.*, 1995;Liu *et al.*, 1996). As a consequence, mycobacteria develop aqueous channels formed by porin molecules in the cell wall structure. Other distinguishing cell wall components of *M.tb* include ManLAM, LM, PIMs, and a peripheral layer of lipids such as trehalose mycolates (trehalose dimycolate or TDM, trehalose monomycolate or TMM), lipooligosaccharides (LOSs), phenolic glycolipids [PGLs, described in some *M.tb* clinical isolates (Reed *et al.*, 2004;Torrelles *et al.*, 2008b)], acyl trehaloses (diacyl- or DAT and triacyl- or TAT), triglycerides and sulfolipids (SLs) (Brennan & Nikaido, 1995; Muñoz *et al.*, 1997a, 1997b). Recently, the role for individual components of the cell wall has been elucidated, and much emphasis has been placed on the identification and characterization of various genes that encode enzymes involved in the synthesis of the cell wall constituents. A better understanding of the cell wall components will lead to a better understanding of the relationship/symbiosis between *M.tb* and the infected host, which will lead to the identification of new drug targets and permit the development of new antituberculosis drugs targeting enzymes involved in the biosynthesis/maintenance of the cell wall within the host during infection.

2.2.1 Cell wall core

One distinguishing characteristic of the mycobacterial cell wall is the absence of lipoteichoic acids and lipopolysaccharides, typical structures of gram-positive and -negative bacteria, respectively. Instead, the *M.tb* cell wall has a cell wall core which is a covalently linked skeleton of the mAGP (Daffe *et al.*, 1990). This structure is composed of the peptidoglycan, which is covalently linked to AG chains via phosphoryl-*N*-acetyl-glucosaminosyl-rhamnosyl linkage units (-α-L-Rha*p*(1→3)-D-GlcNAc-P-) (Daffe *et al.*, 1990; Mikusova *et al.*, 1996). AG non-reducing ends are esterified to a variety of α-alkyl, β-hydroxy mycolic acids. Lipoglycans, lipoproteins and especially free lipids are found to be associated with the mAGP complex (Andersen & Brennan P.J., 1994;Brennan & Nikaido, 1995).

2.2.1.1 The peptidoglycan

Peptidoglycan (PG) classes are identified by the type of peptide cross-linking that they display, the PG structure in *M.tb* is of the common A1γ type, although it does have some distinguishing features (Schleifer & Kandler, 1972). The insoluble PG consists of alternating units of *N*-acetylglucosamine (GLcNAc) and modified muramic acid residues [*N*-acetyl-β-D-glucosaminyl-(1→4)-*N*-acetylmuramic acid]. The typical *N*-acetyl groups in the muramic acid of bacterial PG are further oxidized to *N*-glycolyl groups in *M.tb* [*N*-glycolylmuramic acid]. The muramic acid residues are also modified by tetrapeptide [L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine] side chains (Schleifer & Kandler, 1972). Cross-linking can occur between two meso-diaminopimelic acid (DAP) residues as well as between DAP and D-alanine residues (Crick *et al.*, 2001). The free carboxyl groups of the glutamate and DAP in the murein peptides from *M.tb* can be amidated in essentially any combination and a small percentage of the D-glutamate residues are substituted with a

glycine. PG serves as a foundation structure forming the backbone of mAGP and provides shape, strength, and rigidity to the *M.tb* cell wall. Although the role of PG in pathogenesis has been shown for other bacterial species (Boneca, 2005), in the case of *M.tb* is still unclear (Table 1). However, recent studies have been focused on the role of nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) (Franchi *et al.*, 2008;Sirard *et al.*, 2007) in the recognition of *M.tb* PG fragments. In particular the NLR Nod2, which resides within the phagocyte cytosolic compartment, is shown to recognize the *M.tb* PG fragment muramyl dipeptide (MDP), where the replacement of the *N*-acetyl group of the muramic acid of MDP with a *N*-glycolyl moiety seem to significantly increase the potency of this compound as a Nod2 agonist (Coulombe *et al.*, 2009). Other studies confirmed that during infection, intra-phagosomal *M.tb* is capable of stimulating the cytosolic Nod2 pathway, and this event requires membrane damage that is actively inflicted by the bacillus (Pandey *et al.*, 2009). Moreover, *M.tb* bacilli recognized by Nod2 trigger NF-kB activation (Ferwerda *et al.*, 2005) and production of IFN-α/β with the subsequent transcription of CCL-5 (or RANTES) via Nod2, Rip2, Tbk-1, Irf3 and Irf5 cascade (Pandey *et al.*, 2009). Recent studies by Brooks *et al.* using human macrophages showed that Nod2 plays a role in controlling pro-inflammation and *M.tb* intracellular growth (Brooks *et al.*, 2011). This is in accordance with recent human polymorphisms studies linking Nod2 mutations to susceptibility to *M.tb* infection.

2.2.1.2 The arabinogalactan

The arabinogalactan (AG) is a heteropolysaccharide chain of furanoid arabinose (Ara*f*) and galactose (Gal*f*) (Daffe *et al.*, 1990;McNeil *et al.*, 1987). The furanosyl residues are arranged into three differential regions; the galactan core, the arabinan, and the non-reducing terminal segments of arabinan. Structural analysis of the AG shows that the galactan core of AG is composed by 5- and 6-linked β-D-Gal*f* residues (Daffe *et al.*, 1990). The arabinan chains consist of linear 5-linked α-Ara*f* residues with branching introduced by 3,5-Ara*f* residues (Daffe *et al.*, 1990). The linkage of arabinan chains to the galactan core occurs at the C-5 of some of the 6-linked β-D-Gal*f* residues. Clusters of four mycolic acids are then attached to the terminal arabinofuranosyl motifs of non-reducing ends of the arabinan chain via ester linkage. Approximately two-thirds of the non-reducing ends of arabinan are mycolated at the 5 position of Ara*f* residues (Daffe *et al.*, 1993), one-third with succinyl and one-third with glucosaminosyl residues (Bhamidi *et al.*, 2008). There are approximately 2-3 arabinan chains attached to the galactan core (Baulard *et al.*, 1998;Brennan and Nikaido, 1995). Finally, the galactan core of AG, in turn, attaches to the C-6-position of muramic acids of the PG via a phosphodiester linkage of α-L-rhamnopyranose (Rha*p*)-(1→3)-D-N-acetylglucosamine (GlcNAc)-(1→phosphate) (McNeil *et al.*, 1990;Mikusova *et al.*, 1996). Although *M.tb* AG has serological activity (Kotani *et al.*, 1971;Misaki *et al.*, 1974), its capacity to generate the innate immune response is unknown (Table 1).

2.2.1.3 Mycolic acids

Mycolic acids are complex hydroxylated branched-chain fatty acids with characteristic carbon numbers (60-90 carbon atoms)(Barry *et al.*, 1998). They may also contain diverse functional groups such as methoxy, keto, epoxy ester groups and cyclopropane rings (Asselineau & Lederer, 1950). Mycolic acids found in *M.tb* are composed of an α-branch and a meromycolate branch, where the latter defines the heterogeneity in the mycolic acids, although the α-branch length frequently also generates variation (Barry *et al.*, 1998). The

possible functional groups (unsaturations, methyl branches and cyclopropanes) and polar moieties (ketones, methoxy groups) are localized only in the meromycolate branch (Barry *et al*., 1998). In particular for *M.tb*, unsaturated mycolates containing cyclopropanes (either *cis*/*trans* with sometimes an adjacent methyl branch), are known as α-mycolic acids. Mycolic acids containing a methoxy group with double bond or cyclopropane ring are known as methoxymycolic acids; similarly, mycolic acids containing an α-methyl-branched ketone are known as ketomycolic acids (Takayama *et al*., 2005). The α-mycolate is the most abundant form found in the *M.tb* cell wall [65-70%] followed by methoxy- and keto-mycolates [8-15%] (Qureshi *et al*., 1978). The majority of the mycolic acids are localized in the inner leaflet of the *M.tb* cell wall covalently bound via carboxylate ester to form the non-extractable tetramycolyl-pentarabinosyl unit (McNeil & Brennan, 1991). However, mycolic acids can also be found loosely forming extractible lipids mainly in the form of TDM and TMM (Minnikin, 1982). In this case, it is thought that TDM and TMM stabilize their position within the cell wall by associating their mycolic acid lipid tails with the AG-covalent linked mycolates. In general, mycolic acids are involved in maintaining a rigid cell shape but they also contribute to the resistance to chemical injury and to the protection of the *M.tb* bacillus against hydrophobic antibiotics (Barry *et al*., 1998). The importance of the mycolic acids in the *M.tb* cell wall is defined by the action of isoniazid, which inhibits their biosynthesis and is an efficient antimycobacterial agent (Winder & Collins, 1970). Furthermore, looking at the biological functions described for mycolic acids (Table 1), it is noticable to point out that these were the first known CD1-presented antigens capable of stimulating and activating CD1b-restricted T cells (Beckman *et al*., 1994;Montamat-Sicotte *et al*., 2011;Moody *et al*., 1999). Moreover, mycolic acids *per se* are shown to be immunomodulatory, where their structural nature (*i.e.* presence of determined functional groups) may determine the degree of virulence of a *M.tb* strain (Barry, *et al*., 1998).

2.2.2 The peripheral lipid layer in the *M. tuberculosis* cell wall

The study of mycobacterial lipids was initiated more than 70 years ago under the direction of Anderson in 1939 (Anderson, 1938). This field is still an active source of research due to the fascinating diversity of their structures and biological activities. The glycolipids are major *M.tb* cell wall constituents, known for their toxic or immunological properties (Brennan and Nikaido, 1995). They comprise the acyl trehaloses [mono- and dimycolyl trehalose (TMM, TDM), di- and triacyltrehalose (DAT, TAT) and sulfolipid (SL)], oligosaccharides containing lipids (lipooligosaccharides (LOSs), phenolglycolipid (PGL), apolar lipids such as the phthiocerol dimycocerosate (DIM), and the glycosyl derivatives of phosphatidyl-*myo*-inositol.

Trehalose-containing glycolipids share a common α-D-Glc*p*(1→1′) α-D-Glc*p* unit and are the class of *M.tb* lipids that have been most extensively studied and still fascinate the majority of lipidologists and mycobacteriologists. TDM (or cord factor) was first obtained by Bloch (Bloch, 1950) after a petroleum ether extraction from cells of a virulent strain of *M.tb.* The resulting extract was toxic when injected into mice and a drastic disorganization of the cords that *M.tb* formed at the culture medium surface was also observed. The toxic compound present in the extract was shown to be 6,6'-dimycoloyl-α-D-trehalose (Noll, 1956). TDM toxicity is due to an increase of the tissue specific nicotinamide adenine dinuclease activity decreasing the levels of NAD in several tissues by blocking the electron flow along the

mitochondrial respiratory chain and thus oxidative phosphorylation (Artman *et al.*, 1964;Barry *et al.*, 1998;Brennan, 2003). During infection and when inside of the phagosome, *M.tb* is shown to produce large quantities of TDM (Fischer *et al.*, 2001). From many studies done on TDM, it is remarkable that TDM induces lung granulomas and has immunostimulating properties (Bekierkunst, 1968) that are probably at the origin of its antitumoral activity (Bekierkunst *et al.*, 1971a) (Table 1). TDM has also been shown to have adjuvant properties generating an optimal antibody response (Bekierkunst *et al.*, 1971b) and a non-specific immune response against bacterial infections and parasitic infections (Bekierkunst, 1968;Parant *et al.*, 1977, 1978;Ribi *et al.*, 1976;Yarkoni & Bekierkunst, 1976). Recent studies uncover that TDM is actively participating in blocking the mycobacterial phagosome maturation (Indrigo *et al.*, 2003). Inhibition of the phagosome maturation is observed after phagocytosis of virulent strains of *M.tb*, allowing the bacillus to survive within the phagocyte (Schlesinger LS *et al.*, 2008). Recently, Mincle (macrophage-inducible C-type lectin) (Yamasaki *et al.*, 2008) on the macrophage surface, has been shown to recognize *M.tb* TDM, and working together with the Fcγ receptor transmembrane segment induces pro-inflammation (Ishikawa *et al.*, 2009; Schoenen *et al.*, 2010). In the case of *M.tb* TMM, this is shown to be used by the bacillus to transfer mycolic acids towards molecules like the wall-linked AG. In agreement with this fact, the known secreted immunogenic Ag 85 complex has been identified as a trehalose mycolyltransferase in *M. smegmatis* (Sathyamoorthy & Takayama, 1987) and later in *M.tb* (Belisle *et al.*, 1997). TMM is also shown to have lethal toxicity, adjuvant activity, and capable of stimulating tumour necrosis factor via activation of the protein kinase C pathway (Numata *et al.*, 1985)(Table 1).

The sulfated trehaloses (sulfolipids, designated by SL) (Middlebrook *et al.*, 1959) are also present in virulent strains of *M.tb*, specifically SL-1 (Goren, 1970a,1970b). SL-1 can be acylated by 2 to 4 very long (up to C_{64}) saturated and unsaturated, highly branched fatty acids. Sulfate derivatives are rare in natural substances, and some of the acyl chains of SL-1 are also uncommon, since they are mainly highly branched in their carboxyl end (Goren & Mor, 1990;Leigh & Bertozzi, 2008). The SLs have attracted much interest since it was shown that, like TDM, they seem to inhibit phagosome-lysosome fusion in macrophages (Goren *et al.*, 1976), and are cytotoxic (Kato & Goren, 1974a, 1974b). However, the role of SL-1 in *M.tb* pathogenesis seems to be dependent of the model system studied. In this context, results obtained from *in vitro* and *in vivo* studies (the latter using different animal models and *M.tb* strains) dispute the role of SL-1 in *M.tb* pathogenesis (Brozna *et al.*, 1991;Gangadharam *et al.*, 1963;Goren *et al.*, 1974, 1982;Pabst *et al.*, 1988;Rousseau *et al.*, 2003;L. Zhang *et al.*, 1988, 1991). While SL-1 has been shown to induce specific host cell responses, such as inhibition of phagocyte priming/activation (Brozna *et al.*, 1991;Pabst *et al.*, 1988), its mechanism of action is still unclear; although a role of a guanine nucleotide binding protein in both priming and direct activation of neutrophils by SL-1 has been suggested (L. Zhang *et al.*, 1991) (Table 1).

Diacyltrehalose (DAT, a 2, 3-diacyltrehalose) and triacyltrehalose (TAT, a 2, 3, 6-triacyltrehalose) (Gautier *et al.*, 1992), who's acyl groups are mainly branched polymethyls, are also present in the *M.tb* cell wall. The main use of these glycolipids is in *M.tb* sero-diagnosis (Muñoz *et al.*, 1997a). However, a study recently showed that *M.tb* mutants lacking DATs and sulphoglycolipids cannot block phagosome maturation and thus, revealing the importance of these molecules in the *M.tb* pathogenesis (Table 1)(Brodin *et al.*, 2010). In *M.tb*, other trehalose-based lipids are lipooligosaccharides (LOSs), which contain a polyacylated trehalose with long chain fatty acids and an oligosaccharide. It contains 2 or 3

straight or methyl-branched chains. In some LOSs, acyl residues can be distributed between the two glucose residues of the trehalose end of the polymer. Depending on the species, an oligosaccharide (2 to 6 sugar residues) is linked either on carbon 3, 4 or 6 of the trehalose end (Gilleron *et al.*, 1994;Hunter *et al.*, 1985). Pyruvic acid residues (carboxyethylidene) can also be present, giving an anionic character to the molecule. LOSs have been considered to be immunogenic and also phage receptors (Besra & Chatterjee, 1994), suggesting that they are located in the *M.tb* cell wall surface. Other *M.tb* lipids containing oligosaccharides are the phenolic glycolipids (PGLs). These have been extensively studied in *Mycobacterium leprae* (PGL-I) (Hunter *et al.*, 1983;Hunter & Brennan, 1981). Leprosy patients have antibodies against this molecule, and therefore it is a useful diagnostic tool (Cho *et al.*, 1983). PGLs have been found in the Canetti strain of *M.tb* (Daffe *et al.*, 1987) (PGL-Tb), however, serodiagnostic studies have shown that there were large variations among tuberculous patients in the response to this antigen (Daffe *et al.*, 1991). This is likely due to large differences in phenolglycolipid amounts produced by different *M.tb* strains (Cho *et al.*, 1992; Torrelles *et al.*, 2008b). Recently, the hypervirulent phenotype observed in several strains of *M.tb* (*i.e.* strain HN878) has been associated with the presence of PGL in their cell wall (Reed *et al.*, 2004).

There are many apolar lipids described forming part of the cell wall of *M.tb*, however, of particular importance is the phthiocerol dimycocerosate (or PDIM/DIM). PDIM is a major apolar lipid present in the cell wall of *M.tb*. PDIM is considered a wax containing multiple methyl and/or methylene groups (Brennan, 2003). Several studies have tied, as in the case of TDM and SL-1, the presence of PDIM to *M.tb* virulence (Goren & Brennan, 1980). *In vivo* studies using *M.tb* strains depleted of PDIM show attenuation in the growth of these strains in mice (Cox *et al.*, 1999;Ferwerda *et al.*, 2007), in accordance with previous studies in Guinea pigs using a *M.tb* clinical isolate lacking PDIM in its cell wall (Goren & Brennan, 1980) (Table 1). Recent studies using a genetically engineered *M.tb* PDIM mutant concluded that PDIM inserts into the host membrane and participates both in the receptor-dependent phagocytosis of *M.tb* and the prevention of phagosomal acidification (Astarie-Dequeker *et al.*, 2009). Using PDMI mutants also is shown that these are required for *M.tb* resistance to an IFN-γ-mediated immune response that is independent of NOS2 (Kirksey et al., 2011).

Glycosyl derivatives of phosphatidyl-*myo*-inositol in the *M.tb* cell wall are the phosphatidyl-*myo*-inositol (PI) and its mannosylated derivatives known as phosphatidyl-*myo*-inositol mannosides (PIMs), phosphatidylethanolamine, phosphatidylglycerol, diphosphatidyl-glycerol, cardiolipin, and glycosylphosphopolyisoprenols. PI and its mannosylated derivatives are important lipids in the cell wall of *M.tb*, both as key membrane constituents and as participant in essential *M.tb*-host interactions and metabolic processes. PI is an acidic (anionic) phospholipid that in essence consists of a phosphatidic acid backbone, linked via the phosphate group to inositol (hexahydroxycyclohexane). In mycobacteria, the stereochemical form is *myo*-D-inositol. As early as 1930's, it was recognized by Anderson that the phospholipidic fraction extracted from *M.tb* and related mycobacteria contained inositol and mannose (Anderson, 1938). Besides phosphatidylethanolamide, PIMs are the major phospholipid components of the *M.tb* cell wall (Brennan, 2003). PIMs are found as a mixture of compounds differing one from the other by the number of mannosyl residues and fatty acids. Their structures consist of a mannosyl unit attached to position C-2 of the *myo*-inositol of a PI anchor. Position C-6 of *myo*-inositol is further substituted by an α-D-

mannosyl or a linked trimannosyl unit, giving PIM_2 and PIM_4, respectively. PIM_4 may be further substituted at position C-2 by a α-D-mannosyl leading PIM_5, which can also be further substituted at the same position leading PIM_6 [α-D-Manp(1→2)-α-D-Manp(1→2)-α-D-Manp(1→6)-α-D-Manp(1→6)-α-D-Manp(1→6)-*myo*-inositol], the higher PIM encountered in mycobacteria (Torrelles & Schlesinger, 2010). Studies have shown that PIMs, which are known to interact with the plasma membrane, are also present on the *M.tb* cell wall surface (Ortalo-Magné *et al*., 1996). The intrinsic heterogeneity of PIMs is evident looking at their carbohydrate constitution (PIM to PIM_6). A difficulty is added, however, when we look at the acylation sites of the PIMs. Many studies have shown evidence for multiacylated forms of PIMs in *M.tb* [reviewed in (Torrelles & Schlesinger, 2010)]. Differences in the degree of acylation and the kind of fatty acid linked was studied in detail by Khoo *et al*. (Khoo *et al*., 1995), who confirm the existence of triacylated PIMs (Ac_1PIM_x) esterified by palmitic (16:0) and tuberculostearic (TBST or 10-methyl-octadecanoic) acids, and discussed the presence of tetracylated PIMs (Ac_2PIM_x), where an additional fatty acyl could be carried in the *myo*-inositol ring. This fact was later corroborated by Gilleron *et al.* (Gilleron *et al*., 1999) showing an unambiguous localization of a fourth fatty acid on the C-3 of the *myo*-inositol beside the fatty acids on C-1 and C-2 position of the glycerol and on the C-6 position of the (1→2) linked mannose. PIMs can be grouped in lower- and higher-order depending of the number of mannoses, where lower-order PIMs contain 1 to 4 mannoses and higher-order PIMs contain 5 to 6 mannoses (Torrelles *et al*., 2006). The most common PIMs found are $AcPIM_2$ and Ac_1PIM_2 (di- and triacylated PIM_2) and $AcPIM_6$ and Ac_1PIM_6 (di-and triacylated PIM_6) (Khoo *et al*., 1995). Lower-order PIMs have a terminal α(1→6)-mannose and are shown to participate in the phagocytosis process through association with the non-opsonic domain of complement receptor-3 (Villeneuve *et al*., 2005), and also participate in trafficking processes within the phagocyte by facilitating early endomal fusion with phagosomes (Vergne *et al*., 2004). Higher-order PIMs have a terminal α(1→2)-mono- or di- mannoside (Ac_xPIM_5 or Ac_xPIM_6, respectively) similar to the mannose caps of mannose-capped lipoarabinomannan [see section 2.2.2.1 for details]. Only triacylated forms of higher-order PIMs are shown to interact with the mannose receptor (MR) and interfering with trafficking pathways by limiting phagosome-lysosome fusion (Torrelles *et al*., 2006). Moreover, several studies have also shown that all *M.tb* PIMs interact with dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) (Torrelles *et al*., 2006), although differences in DC-SIGN PIM recognition specificity may be species dependent (Driessen *et al*., 2009). Additional studies have also shown that cytosolic soluble CD1e is involved in PIM_6 processing and presentation via CD1 with subsequent T cell activation (de la Salle *et al*., 2005) (Table 1).

Apart from the mannosides of phosphatidylinositol, there are other mycobacterial phosphodiacylglycerol, whose origins are based on phosphatidic acid. These are phosphatidylglycerol, diphosphatidylglycerol (DPG) and phosphatidylethanolamine. Although the role of these *M.tb* cell wall phospholipids during infection is uncertain, *M.tb* cardiolipin (a DPG molecule) is shown to be processed into lysocardiolipin by the lysosomal phospholipase A_2 during *M.tb* infection (Fischer *et al*., 2001). Antibodies against *M.tb* cardiolipin are also found in sera from TB patients (Santiago *et al*., 1989), and their production is shown to be strictly related to IL-4 and T cells (Fischer *et al*., 2002). Anti-

cardiolipin antibodies are also capable of activating complement (Santiago *et al*., 1991). Small quantities of glycosylphosphopolyisoprenols, which are involved in *M.tb* cell wall biosynthesis, have also been isolated from the cytoplasmic membranes of *M.tb*, where ribosyl-, mannosyl- and arabinosyl-phosphopolyprenols have been characterized (Takayama *et al*., 1973; Takayama & Goldman, 1970; Wolucka & De Hoffmann, 1995).

In summary, *M.tb* elaborates a great variety of glycolipids of rather unusual structure. Some of these lipids are abundant in the inner cell wall and others are exposed on the bacillus surface. These include acylglucosides, sulfatides, lipooligosaccharides, phenolic glycolipids, dimycocerosates, and the ubiquitous phosphatidyl-*myo*-inositol-mannosides. Some of these glycolipids are described as virulence factors helping *M.tb* to survive as intracellular 'parasites' that infect and reside in the host cell. The biological activities attributed to these surface-exposed glycolipids may derive, at least in part, from the modulation of cell functions through the interactions between host membranes and them, whose structures are different from those of mammalian cell membrane components. Biologically active glycolipids have been shown to profoundly affect the physical and functional properties of biologic membranes (Brandley & Schnaar, 1986) as well as inhibit both macrophage antimicrobial activities and lymphocyte proliferation (Vergne & Daffe, 1998). Therefore, the enzymes involved in their biosynthesis may represent potential drug targets (Kaur *et al.*, 2009). Nevertheless, for some of these lipids confirmation of their role in *M.tb* pathogenicity is still lacking, opening the necessity to genetically manipulate *M.tb* to obtain glycolipid deficient mutants. These mutants may be unable to elaborate determined glycolipid thought to be involved in *M.tb* pathogenesis. Thus, the lack of specific glycolipids in virulent strains of *M.tb* may help us to understand their real implication in pathogenesis.

2.2.3 Lipoglycoconjugates of the *M. tuberculosis* cell wall

An extensive study of the mannose-capped lipoarabinomannan (ManLAM) has been performed in *M.tb* [reviewed in (Torrelles & Schlesinger, 2010)]. *M.tb* ManLAM is an extremely heterogeneous lipoglycan with a defined tripartite structure that possesses a carbohydrate core, a mannosyl-phophatidyl-*myo*-inositol anchor (MPI) and various capping motifs. Following the earlier work performed by Chatterjee and co-workers (Chatterjee & Khoo, 1998), a series of detailed structural analyses have produced evidence of this tripartite structure, in which ManLAM was distinguished from the related lipomannan (LM) by virtue of having an additional immunodominant arabinan domain that extends from a common phosphatidyl-*myo*-inositol mannan core in an as yet undefined manner. The polysaccharide core of *M.tb* ManLAM consists of two very well differentiated polymers, a D-mannan and a D-arabinan. The D-mannan structure consists of a linear α(1→6) linked mannopyranosyl backbone that is linked to a phosphatidyl-*myo*-inositol anchor, and presents substitutions/ branches on their C-2 with another single mannose (α-D-Man*p*(2→1)t-α-D-Man*p*). The D-mannan size and the degree of branching can vary among *M.tb* strains. This mannosyl backbone carries an unknown number of branched arabinosyl side chains, which form the D-arabinan. To date, the linkage between the arabinan polymer and the D-mannan core is still not determined. The arabinan is based on the rare α-D-arabinofuranose (Ara*f*), and consists of a branched linear α(1→5) linked Ara*f* backbone. Branching residues carry an additional α(1→3) linked Ara*f*. At its non-reducing end we can

find two types of arrangements or motifs, a linear tetraarabinofuranoside (Ara_4) defined as β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→5)-α-D-Ara*f*-(1→5)-α-D-Ara*f* and a branched hexaarabinofuranoside (Ara_6) defined as [β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→]$_2$-3 and 5-α-D-Ara*f*-(1→5)-α-D-Ara*f*. In the case of slow-growing mycobacteria like *M.tb*, *M. leprae* and *M. bovis* BCG, some of the terminal arabinan motifs are extensively capped at C-5 with one or more α-mannoses attached to these β-Ara*f* termini. The mannooligosaccharides linked to the terminal β-Ara*f* thus define ManLAM. The mannose caps are defined as a single Man*p*, a dimannoside (α-D-Man*p*-(1→2)-α-D-Man*p*) or a trimannoside (α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*). These units are located in both tetra- and hexaarafuranosyl motifs of the arabinan non-reducing terminal (Chatterjee *et al*., 1993). Data reported from different studies shown that the disaccharide unit is the cap most frequently found in both linear (Ara_4) and branched (Ara_6) termini. Man_2Ara_4 and Man_4Ara_6 are then the most frequent motifs in all ManLAM studied (Chatterjee *et al*., 1993), however, trimannoside caps in the linear Ara_4 have also been found (*i.e.* Man_3Ara_4), and in the branched Ara_6, all three combinations of mannose caps have been found, *i.e.* ($Man_{[(1\ to\ 3)x2]}$ Ara_6). The degree of ManLAM capping varies according to the *M.tb* strain studied, where *M.tb* Erdman is the most capped when compared to *M.tb* $H_{37}R_v$ and $H_{37}R_a$ strains [reviewed in (Torrelles & Schlesinger, 2010)]. The anchor structure in *M.tb* ManLAM is similar to the one in PIMs, and consists in an *ns*-glycerol 3-phospho-(1-D-myo-inositol) unit with a α-D-mannopyranosyl residue at C-2 of the *myo*-inositol (MPI). In the C-6 position of this *myo*-inositol there is *O*-linked the mannan polymer described previously (Chatterjee & Khoo, 1998). Some of the heterogeneity that characterizes ManLAM occurs through the number, the location, and the nature of the fatty acids esterifying the PI anchor. The characteristic fatty acids described in the ManLAM anchor are 16:0 and TBST (Hunter *et al*., 1986). However, traces of stearic (18:1), myristic (14:0), heptadecanoic (17:0), 10-methyl-heptadecanoic, 12-O-(methoxypropioryl)-12-hydroxy-stearic and 12-hydroxy-tuberculostearic acids have also been described (Leopold & Fischer, 1993;Nigou *et al*., 1997). The average number of fatty acids per ManLAM molecule cannot be generalized. Some studies confirmed an average of 3 fatty acids per molecule of ManLAM in positions 1 and 2 of the *ns*-glycerol and position 6 of the Man*p* unit linked to C-2 of the *myo*-inositol (Khoo *et al*., 1995). Studies by Chatterjee and colleagues performed in an ethambutol resistant strain of *M.tb* supported the existence of tetraacylated ManLAM as the most common molecular form (Torrelles *et al*., 2004). Thus, the only fact that can be generalized is that, with the exception of the lyso-forms of ManLAM (only one fatty acid in the ManLAM anchor), ManLAM at least has two fatty acids, with both fatty acids in the *ns*-glycerol unit, where 16:0 and TBST are at position 1 and at position 2, respectively.

The presence of additional acyl groups on ManLAM has been reported by several authors. Hunter *et al.* reported the existence of succinates and lactates (Hunter *et al*., 1986). Delmas *et al.* used nuclear magnetic resonance spectroscopy to locate the succinic groups (1 to 4 per molecule) in the C-2 of the 3,5-α-D-Ara*f* and/or 5-α-D-Ara*f* residues in ManLAM from different *Mycobacterium bovis* BCG strains (Delmas *et al*., 1997). Later, studies performed by Chatterjee and colleagues analyzing the content of succinates in ManLAMs from different mycobacterial species and strains showed that *Mycobacterium leprae*, the *M.tb* laboratory strain $H_{37}R_v$, and a *M.tb* clinical isolate (CSU 20) had also succinates (Torrelles *et al*., 2004); where ManLAM from *M. leprae*, the laboratory strain $H_{37}R_v$, and CSU20 had an average

number of 7, 2 and 4 succinates, respectively. The succinates biological function in ManLAM is still a question to be resolve. Our recent studies show that a biosynthetically related lipoglycan to ManLAM, the lipomannan, also contains succinates, where succinates seem to influence CD1-Ag presentation to T cells and subsequent T cell activation (Torrelles *et al*., 2011). Recently, Treumann *et al*. using nuclear magnetic resonance spectrometry defined a new terminal sugar located in the caps of *M.tb* ManLAM (Treumann *et al*., 2002). This sugar consisted in a 5-deoxy-5-methylthio-α-xylofuranosyl (MTX), and may be involved in *M.tb*-host interactions battling the effects of reactive oxygen species by adding to the antioxidant properties of ManLAM (Turnbull *et al*., 2004). The orientation of LAM in the *M.tb* cell wall is still unresolved. There are many hypotheses, but the most accepted is that ManLAM is anchored by its lipidic anchor into the plasma membrane, and projects through the thickness of the wall so that its terminal arabinose or mannose-capped arabinose units are accessible to the outside (McNeil & Brennan, 1991). Other possibilities are that ManLAM is interacting by its lipid anchor with the mycolic acid layer and with other polar wall associated lipids (Rastogi, 1991), or that ManLAM has a non-permanent location in the cell wall, and is essentially a secreted molecule in transit through the envelope. The many studies carried out on ManLAM have led to data that supports each of these hypotheses. For example, Lemassu and Daffe demonstrate the existence of non-PI containing mannose-capped arabinomannan in the so called capsular/outer material polysaccharide associated with *M.tb* (Lemassu & Daffe, 1994). Other studies, subdivided ManLAM into two different kinds, the parietal ManLAM and the cellular ManLAM. Both had similar core structure presenting remarkable differences in the degree of mannose-capping and the acylation of the PI-anchor (Gilleron *et al*., 2000). The fact that the parietal LAM is obtained without cell disruption reinforces the hypothesis of two different locations for ManLAM. Thus, ManLAM may be firmly, but not covalently, attached to the *M.tb* cell wall and it may also be anchored to the plasma membrane. The biological function of *M.tb* ManLAM is discussed later in this chapter (see also Table 1), and information about its biosynthesis pathway(s) can be found elsewhere (Kaur *et al*., 2009).

Another remarkable lipoglycan in the *M.tb* cell wall is lipomannan (LM). The α (1→6) mannose polymer of LM presents identical characteristics to the mannan backbone of ManLAM. The mannan of LM is directly attached to position C-6 of the *myo*-inositol of its MPI anchor. The *M.tb* ManLAM and LM MPI anchor is indistinguishable from the *M.tb* dimannosylated phophatidyl-*myo*-inositol (Ac_xPIM_2), the structure of which was established by Lee and Ballou (Lee and Ballou, 1965). LMs are considered multimannosylated forms of PIMs by the fact that both types of molecules have an elaborated anchor in common (Gilleron *et al*., 1999). Their common structure with ManLAM also enforces the hypothesis that LM is a precursor of ManLAM (Besra *et al*., 1997). However, it seems that LM could also be a co-lateral final product in the biosynthetic pathway of ManLAM (Besra *et al*., 1997). Few biological properties of *M.tb* LM have been described, mainly because this molecule is still understudied (Table 1).

Mycobacterial LM is shown to regulate cytokine, oxidant and T cell responses (Barnes *et al*., 1992; Chan *et al*., 2001;Gilleron *et al*., 2001). *M.tb* LM is shown to associate with DC-SIGN and not with the MR (Torrelles *et al*., 2006), and to induce apoptosis and a pro-inflammatory response through TLR2 (Dao *et al*., 2004;Nigou *et al*., 2008). However, recent studies showed that although *M.tb* LM is capable of activating macrophages via TLR2 inducing

signaling cascades required for TNF mRNA expression, the TNF mRNA produced is poor translated and faster degraded (Rajaram *et al*., 2011).

***M.tb* cell wall location**	***M.tb* cell wall component**	**Host Cell Receptor(s)**	**Phagosome maturation blockade**	**Host Immune Response**	**Sero-activity**	**Cyto-toxicity**
Outer Material	α-Glucan	DC-SIGN, CR3?	No	Anti-inflammatory	Unknown	ND
Cell wall Core	Peptidoglycan (PG)-MDP	Nod2	ND	Pro-inflammatory	Unknown	ND
	Arabinogalactan (AG)	ND	ND	ND	Yes	ND
	Mycolic Acids	CD1 (in Ag-presentation	ND	Pro-inflammatory	Yes	Yes
Peripheral lipid layer	Trehalose dimycolate (TDM)	Mincle-FcγR TLRs	Yes	Pro-inflammatory	Yes	Yes
	Trehalose monomycolate (TMM)	ND	ND	Pro-inflammatory	Yes	Yes
	Sulfolipid-1 (SL-1)	ND	Yes	Pro-inflammatory	Yes	Yes
	Diacyl- and Triacyl-trehalose (DAT & TAT)	ND	Yes	Pro-inflammatory	Yes	ND
	Lipooligosaccharides (LOSs)	ND	ND	Pro-inflammatory	Yes	ND
	Phenolic glycolipid (PGL-TB)	CR3?	ND	Pro-inflammatory	Yes	Yes
	Triglycerides	TLRs	ND	Pro-inflammatory	Unknown	Yes
	Phthiocerol dimycocerosate (PDIM)	Direct insertion into host Mbrs	Yes	Pro-inflammatory	Unknown	Yes
	Lower-order phosphatidyl-*myo*-inositol mannosides (PIMs)	CR3, TLRs, DC-SIGN	No	Pro-inflammatory	Yes	ND
	Higher-order phosphatidyl-*myo*-inositol mannosides (PIMs)	MR, DC-SIGN	Yes (through the MR only)	Anti-inflammatory	Yes	ND
	Lipomannan (LM)	TLRs, DC-SIGN	No	Pro-inflammatory	Yes	ND
	Mannose-capped lipoarabinomannan (ManLAM)	MR, DC-SIGN	Yes (through the MR only)	Anti-inflammatory	Yes	ND

Mbrs: Membranes.

Table 1. *M.tb* cell wall components and their interaction with the host outcome.

Due to the complexity of the cell wall of *M.tb*, we may need to be careful when assessing the essentiality of a specific cell wall component thought to be a virulence factor. Current strategies are directed in creating isogenic strains of *M.tb* deficient in the production of a specific virulent factor. We may not obtain the real answer by just depleting the presence of a potential virulent factor in the cell wall of *M.tb*. In this context, efforts to discern the enzymes involved in the biosynthetic pathways of the *M.tb* lipids are critical to address their essentiality in *M.tb* survival. These will allow us to uncover novel drug targets. However, when evaluating the role of the omitted/mutated lipid in *M.tb* pathogenesis, we need to be careful in considering the rearrangement that the *M.tb* cell wall may suffer upon the lack of a specific lipid. Depending of the structural nature of the lipid depleted from the *M.tb* cell wall (*i.e.* size, charge, hydrophobicity, etc.), we may find that the bacterial cell wall is altered in a way that the absence of the lipid is unexpectedly compensated. This is the case for SL-1, where the lack of this lipid in *M.tb* isolates is being linked to TB pathogenesis, however, studies performed by Jackson and colleagues clearly showed that when using an isogenic SL-1 mutant, SL-1 deficiency did not affect *M.tb* virulence (Rousseau *et al.*, 2003). As Jackson and collaborators stated in this study, there are several explanations behind this observed discrepancy, but one of them is related to the presence of a potent attenuator lipid in the clinical isolates lacking SL-1 that compensated the SL-1 phenotype (Goren *et al.*, 1982). Other factor to account for is the synergy between *M.tb* lipids; this is also observed for SL-1 and TDM, where purified SL-1 alone at high doses was innocuous, but when administered simultaneously with TDM, a synergistic increase in the TDM cytotoxicity was observed (Kato & Goren, 1974a, 1974b). Thus, indicating that the lack of a specific lipid may also significantly alter the cytotoxic properties of other *M.tb* cell wall components. Finally, we will need to consider evaluating the constitution of the *M.tb* cell wall during infection. Are the properties of the *M.tb* cell wall altered during infection? Some studies indicate that this may happen in the case for TDM, which is overproduced during *M.tb* infection (Backus *et al.*, 2011;Fischer *et al.*, 2001). Why do clinical isolates of *M.tb* present different cell wall rearrangements than the widely studied *M.tb* laboratory strains Erdman, $H_{37}R_v$, and $H_{37}R_a$? In this context, hypervirulent *M.tb* strains of the Beijing family (Tsenova *et al.*, 2005) are shown to contain large amounts of triglycerides (Reed *et al.*, 2007), and some of them also contain the PGL-TB (Reed *et al.*, 2004). *M.tb* clinical isolates deficient in ManLAM and PIMs surface exposure, but presenting in their cell wall large quantities of triglycerides, PGL-TB, and dimycocerosates, are also shown to have reduced phagocytosis but faster intracellular growth rate in human macrophages (Torrelles *et al.*, 2008b). These studies performed by Schlesinger and colleagues concluded that the clinical spectrum of TB is not only dictated by the host but also it may be related to the amounts and ratios of specific surface-exposed *M.tb* adherence factors defined by *M.tb* strain genotype (Torrelles *et al.*, 2008b;Torrelles & Schlesinger, 2010). Is this *M.tb* genotypic/phenotypic adaptation due to their multiple passages through the host? Triglycerides and DIMs are a major part of the peripheral lipid layer in the *M.tb* cell wall; however, their role in pathogenesis has been until recently overlooked due the presence of other hydrophilic and hydrophobic cell wall components more attractive to TB researchers due to their potential (or already established) role in dictating *M.tb*-host cell interactions. Other questions demanding answers refer to the structural properties of the cell wall of MDR-, XDR-, XXDR, and TDR-*M.tb* strains. What is the cell wall constitution of these strains? Studies performed using transmission electron

and atomic force microscopy techniques started to dig into this question showing that MDR-, XDR-, XXDR- and TDR- strains have thicker cell wall and rougher cell surface (supposedly produced by the progressive erosion of their cell wall by the action of the drugs) with tubular extensions than susceptible strains (Velayati *et al.*, 2009a, 2010). Because some XDR- and TDR-*M.tb* strains are related to the Beijing family (Velayati *et al.*, 2009b), which are shown to have their cell wall overpopulated with triglycerides, it is plausible to question any relationship between the abundance of a specific hydrophobic lipid on the *M.tb* cell wall and drug resistance. Many of these questions remain unanswered.

3. *M.tb*-Host interface

The initial recognition of *M.tb* by the host is quite complex and involves alveolar resident cells and many of their surface receptors. The concept of studying the contribution of a specific receptor(s) in the *M.tb* recognition and/or uptake is critical to our understanding of the pathway(s) that the bacillus exploits to gain entrance into the host cell minimizing or triggering the immune response. However, it is important to link the results obtained studying a specific receptor to the existence of other receptors that may also participate at the same time in recognizing *M.tb* generating a completely different outcome. Normally the outcome of *M. tuberculosis*-host recognition is beneficial for the host, triggering the innate immune response; however, engagement of *M.tb* with specific phagocytic receptors is shown to be beneficial for *M.tb* leading to a pathway of survival and subversion of the immune response. Here we will describe the host phagocytic and signaling receptors [some of them known as a pattern recognition receptors, PRRs, for their unique capability to recognize specific motifs on the *M.tb* cell surface (these motifs are also known as pathogen/microbial-associated molecular patterns, PAMPs/MAMPs)] involved in *M.tb* recognition and the subsequent inflammatory response attending in our discussion to the fact that *M.tb* recognition simultaneously includes multiple receptors.

3.1 Phagocytic receptors

The encounter of *M.tb* with the host triggers the phagocytosis process. This process depends of two important factors; one is the constitution of the cell wall of *M.tb* (which is strain dependent) and the surface receptor repertoire present on the phagocyte (which is host cell dependent). Mainly *M.tb* infections occur by airborne transmission of droplet nuclei containing few viable bacilli. The first contact between *M.tb* and the human host cell is within the alveolar space of the lung. When *M.tb* reaches the alveolar space, resident alveolar macrophages (AMs), and alveolar epithelial cells together with recruited monocytes, neutrophils, lymphocytes and fibroblast represent the array of immune cells that participate in host defense. Phagocytic receptors involved in *M.tb* recognition by the host mainly are: the mannose receptor (MR), DC-SIGN, and complement receptors (CRs). *M.tb* uptake by these receptors leads to the formation of an *M.tb* containing phagosome with different outcomes as noted below (Table 1).

3.1.1 The mannose receptor

The cell wall of certain *M.tb* strains has been characterized to be heavily mannosylated with molecules exposing their $\alpha(1\rightarrow2)$-Man*p* termini on the bacterial surface acting as ligands for

host cell receptors contributing to *M.tb* pathogenesis (Torrelles & Schlesinger, 2010). These molecules are ManLAM, LM, PIMs, arabinomannan, mannan, and mannosylated glycoproteins. The MR is conceived as a homeostatic receptor, whose main function is the recycling of endogenous highly *N*-mannosylated glycoproteins normally generated during inflammation (Martinez-Pomares *et al.*, 2001). Studies by Schlesinger and colleagues have suggested that *M.tb* may be capable of using its surface mannose coating to gain entrance and survive within the host cell by associating with the MR. In this context, we demonstrated that *M.tb* can use two of its mannosylated cell wall components, ManLAM and higher-order PIMs, to associate with the MR, leading to a pathway of intracellular survival within the host by blocking phagosome acidification (Kang *et al.*, 2005;Torrelles *et al.*, 2006). Association with the MR has also been shown to reduce microbicidal activities by down-regulating the generation of pro-inflammatory cytokines, nitric oxide, oxygen radicals and by blocking *M.tb*-induced Ca^{2+}-depending apoptosis[reviewed in (Torrelles *et al.*, 2008a)]. In particular, *M.tb* ManLAM has been shown to interact with the MR triggering an anti-inflammatory response by blocking the production of inflammatory cytokines such as TNF and IL-12, and inducing the generation of IL-10 and TGF-β (Astarie-Dequeker *et al.*, 1999;Chieppa *et al.*, 2003;Nigou *et al.*, 2001)(Table 1). Recently, studies by Schlesinger and colleagues showed that engaging of the MR by ManLAM and/or virulent *M.tb* upregulates the peroxisome proliferator-activated receptor-gamma (PPAR-γ, a transcription factor showed to be important in regulating the inflammatory response) leading to a simultaneous increase in the generation of CXCL-8 (or IL-8), expression of cycloxygenase 2 (COX_2), and production of prostaglandin 2 (PGE_2) (Rajaram *et al.*, 2010). Moreover, this study depicts how *M.tb* negatively regulates protective inflammatory modulators through the MR, where engaging of the MR down-regulates TNF levels via PPAR-γ (Rajaram *et al.*, 2010). In addition to *M.tb* ManLAM blocking the generation of TNF via the MR, Schlesinger and colleagues also identified a novel molecular and cellular mechanism underlying the ability of another major *M.tb* cell wall component, the LM, to block TLR2 induced biosynthesis of TNF in human macrophages, thereby allowing *M.tb* to subvert the host immune response and potentially increase its virulence (Rajaram *et al.*, 2011).

3.1.2 DC-SIGN

M.tb is shown to associate with dendritic cell–specific ICAM-3-grabbing non-integrin (or DC-SIGN) (Geijtenbeek *et al.*, 2000) through is cell surface cell wall components ManLAM, LM and PIMs [reviewed in (Ehlers, 2009)](Table 1). Recently, α-glucan was also described as another *M.tb* cell wall ligand for DC-SIGN (Geurtsen *et al.*, 2009). Binding of *M.tb* to DC-SIGN in DCs leads to bacterial killing by acidification of the *M.tb* phagosome (Geijtenbeek *et al.*, 2003). However, the implication of DC-SIGN in triggering the immune response is still controversial. On one hand, engaging of *M.tb*, mannosylated cell wall components, or α-glucan has been shown to induce generation of anti-inflammatory modulators such as IL-10 (Ehlers, 2009;Geurtsen *et al.*, 2009). These findings were supported by *in vivo* studies using mice expressing human DC-SIGN homologues (McGreal *et al.*, 2005;Park *et al.*, 2001;Powlesland *et al.*, 2006) or transgenic mice expressing human DC-SIGN, showing that DC-SIGN may act damping the immune response, and thus, promote host protection by limiting tissue damage (Schaefer *et al.*, 2008;Tanne *et al.*, 2009;Wieland *et al.*, 2007). On the other hand, another study concluded that the ManLAM-PIM/DC-SIGN pathway may not

be significantly involved in regulating cytokine secretion using an engineered *M. marinum* strain lacking essential mannosylated components (Appelmelk *et al.*, 2008).

3.1.3 Complement receptors

Complement receptors are described on the surface of all mononuclear phagocytes. In *M.tb* phagocytosis CR1, CR3 and CR4 have been implicated (Fenton *et al.*, 2005). Several studies have established the role of the complement component 3 (C3) in *M.tb* opsonization. C3 deposition in the form of C3b and C3bi happens quickly via covalent linkages with cell wall components located on the *M.tb* surface (Ferguson *et al.*, 2004). As C3 opsonization depends of serum levels in the tissue, it is still unknown how C3 opsonization varies in form (classical and/or the alternative pathways) and amount among different stages of *M.tb* infection or tissue sites. *M.tb* surface-exposed lower-order PIMs (*i.e.* PIM_2) and specific polysaccharides have been shown to directly interact with the lectin domain of CR3 (Cywes *et al.*, 1997;Hoppe *et al.*, 1997;Villeneuve *et al.*, 2005) and thus, presumably mediate *M.tb* uptake by macrophages (Table 1). PGL-TB from *M.tb* may also interact with CR3, as this is the case for the structurally related PGL-1 from *M. leprae* (Tabouret *et al.*, 2010). Although CR3 seems to drive *M.tb* uptake under opsonic and non-opsonic conditions, *in vitro* and *in vivo* studies using wild type and CR3-deficient mice did not show differences in lung pathology and bacterial burden (Hu *et al.*, 2000), and thus CR3 role in *in vivo* infections remains unanswered. Less attention has been put into CR4, which together with the MR, is highly expressed on AMs and other cells involved in *M.tb* uptake (Hirsch *et al.*, 1994;Zaffran *et al.*, 1998;Schlesinger *et al.*, 2008), and thus may be playing a major role in the uptake of *M.tb* by the naïve host in early stages of infection.

3.2 Signaling receptors

Apart from the phagocytosis process, *M.tb* also is shown to signal through specific signaling receptors located on the host cell surface and/or cytosol. The main signaling receptors for *M.tb* are Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain-like (NODs) receptors.

3.2.1 Toll-like receptors

TLRs are a set of PPRs expressed on many cell types but their function on phagocytes is particularly important [reviewed in (Kawai & Akira, 2010)]. On macrophages TLRs are either expressed on the surface (like TLR2 and 4) or inside cell compartments (like TLR8 and 9) (Kawai & Akira, 2010). TLRs detect a wide range of PAMPs on *M.tb*, which activates the innate immune response and enhance adaptive immunity by mediating the secretion of various pro-inflammatory cytokines along with other anti-bacterial modulators (Table 1). TLRs shown to be key players in triggering immunity against *M.tb* infection are TLR2 (alone or as a heterodimer with TLR1 or TLR6), TLR9, and probably TLR4 (Harding & Boom, 2010). TLR2 alone or dimerized with TLR1 or TLR6, is shown to trigger a strong pro-inflammatory response by recognizing *M.tb* 19 KDa lipoglycoprotein, lower- and higher-order PIMs, LM and TDM [reviewed in (Jo *et al.*, 2007)]. This pro-inflammatory response via TLR2 is shown to be mediated through its adaptor protein myeloid differentiation primary-response protein 88 (MyD88)(Quesniaux *et al.*, 2004), triggering a nuclear factor kappa-light chain-

enhancer of activated B cell (NFκB) signaling cascade through the recruitment of MyD88 and TIRAP (toll-interleukin 1 receptor [TIR] domain containing adaptor protein) (Kawai & Akira, 2010). Of no surprise the intensity of the immune response observed via TLR2 depends on the *M.tb* ligand and the nature of the host cell studied (Thoma-Uszynski *et al.*, 2001;Underhill *et al.*, 1999). Surprisingly, prolonged TLR2 signaling can also benefit *M.tb.* Studies implicating a prolonged stimulation of TLR2 showed an inhibition of antigen presentation due to the down-regulation in the expression of the major histocompatibility complex (MHC) class II in macrophages infected with *M.tb* (Harding & Boom, 2010). Other studies also show that *M.tb* is capable of inhibit MHC-I antigen cross processing and presentation to $CD8^+$ T cells via TLR2 signaling (Harding & Boom, 2010). TLR2-dependent inhibition of TLR9-dependent IFN-α/β expression, thus leading to a decrease of IFN-α/β-dependent MHC-I cross processing is also shown in DCs (Simmons *et al.*, 2010). In this regard, it is unknown if these described mechanisms of MHC-I and -II inhibition via TLR2 will be beneficial for *M.tb* by passing the host immune response, or will be beneficial for the host by limiting the harmful effects of excessive inflammation. Thus, it is not unreasonable to search for a regulatory mechanism(s) among TLRs signaling networks necessary to control inflammation during *M.tb* chronic infection (Drennan *et al.*, 2004;Simmons *et al.*, 2010). TLR9 recognizes unmethylated CpG (cytosine phosphate guanosine motif) found in *M.tb* DNA (Kawai & Akira, 2010). Activation of TLR9 induces IFNα/β and MHC-I antigen cross processing (Simmons *et al.*, 2010). The role of TLR4 in *M.tb* infection is unclear as only a few *M.tb* ligands for TLR4 have been described. Recently, recombinant *M.tb* heat shock protein (hsp) 65 was shown to induce the generation of TLR4-dependent NFκB via MyD88-, TIRAP-, TRIF- (TIR-domain-containing adapter-inducing interferon-β) and TRAM- (TRIF-related adaptor molecule)-dependent signaling pathways (Bulut *et al.*, 2005).

3.2.2 Cytosolic receptor: NOD2

Cytosolic regulators known as NODs receptors (Franchi *et al.*, 2008) are known to participate in the induction of pro-inflammation during *M.tb* infection. Specifically Nod2, which is found in epithelial cells and antigen presenting cells (Gutierrez *et al.*, 2002;Inohara and Nunez, 2003;Ogura *et al.*, 2001), is shown to regulate the production of inflammatory mediators in response to *M.tb* PG components such as muramyl dipeptide (MDP) (Brooks *et al.*, 2011;Franchi *et al.*, 2008;Sirard *et al.*, 2007) (Table 1). Nod2 polymorphism studies in humans are linked to susceptibility to mycobacterial infection (Austin *et al.*, 2008;F.R. Zhang *et al.*, 2009). Studies done *in vitro* using different models and *in vivo* using the mouse model dispute the significance of Nod2 in controlling *M.tb* growth during infection (Divangahi *et al.*, 2008;Gandotra *et al.*, 2007). However, recent studies using human macrophages align with the human polymorphism studies showing that Nod2 plays a role in controlling pro-inflammation and *M.tb* intracellular growth (Brooks *et al.*, 2011). How Nod2 intersects with signaling/trafficking networks starts to be uncovered (Pandey *et al.*, 2009). Although, Nod2 can synergize with TLR-signaling pathways enhancing pro-inflammation (Ferwerda *et al.*, 2005), its capacity to interfere/associate with phagocytic receptor trafficking networks is not well established. As cytosolic Nod2 appears to be associated with intracellular vesicles (Brooks *et al.*, 2011), its role in triggering pro-inflammation may depend on vesicular fusion events controlled during *M.tb* phagocytosis and phagosomal maturation (Sasindran & Torrelles, 2011).

3.3 Other phagocyte receptors

Collectins such as surfactant protein –A and –D, and mannose binding protein, and their specific receptors have been shown to be important in *M.tb* recognition by the host; and their contribution in *M.tb* pathogenesis is discussed elsewhere (Sasindran & Torrelles, 2011;Torrelles & Schlesinger, 2010;Torrelles *et al.*, 2008a). Other receptors involved in the recognition of *M.tb* and inflammation are CD14, scavenger receptor-A, Fcγ-receptor, Mincle, and Dectin-1 (Sasindran & Torrelles, 2011). CD14 (Khanna *et al.*, 1996) and the scavenger receptor SR-A (Zimmerli *et al.*, 1996), are shown to participate in the uptake of non-opsonized bacilli by tissue-specific macrophages; where their role in inflammation varies depending on the species-specific cell type used. Dectin-1 (dendritic cell-associated C-type lectin 1), a β-glucan receptor, in combination with TLR2 has also been shown involved in the immune response against *M.tb* (Yadav & Schorey, 2006). Recently, Mincle (macrophage-inducible C-type lectin) (Yamasaki *et al.*, 2008) on the macrophage surface, has been shown to specifically recognize *M.tb* TDM, inducing a pro-inflammatory response by working together with the Fcγ receptor transmembrane segment (Ishikawa *et al.*, 2009;Schoenen *et al.*, 2010)(Table 1). Conversely, Fcγ receptors do not play a role in the phagocytosis of *M.tb* in the absence of specific antibody (Schlesinger *et al.*, 1990).

In this type of studies we should carefully consider differences between model systems used. There are multiple examples of contradictions when comparing studies performed *in vivo vs. in vitro* and/or when comparing cells from an animal model *vs.* human primary cells. In this context, it is plausible that depending of the model used (*i.e.* primary alveolar macrophage *vs.* THP-1 cell; or human *vs.* another mammalian host cell), a host cell may differentially express the targeted receptor on its surface, or this targeted receptor may be involved in triggering additional or different signaling and/or trafficking network(s). The same concept can be attributed when studying different strains of *M.tb.* A clear example is the variable degree of mannosylation observed on the *M.tb* surface among different strains (*i.e.* less ManLAM and PIMs and more triglycerides, PGL-TB and PDIM on the cell wall of *M.tb* clinical isolates *vs. M.tb* laboratory strains $H_{37}R_v$ and Erdman) (Torrelles *et al.*, 2008b), and how this may impact the infection outcome (Torrelles & Schlesinger, 2010). In light of these findings, we need to be careful in considering which cell wall components are heavily present on the surface of the *M.tb* strain(s) studied and their implications in the host cell phenotype observed.

4. *M. tuberculosis*-Host relationship with the alveolar environment(s) found during infection

It is thought that initial interaction between *M.tb* and the host dictates the pathway and outcome of infection. When *M.tb* infection occurs by airborne transmission, bacilli are deposited in the alveolar spaces of the lungs. The traditional view is that *M.tb* is somewhat "static" during initial infection, does not induce an immune response, and it is taken up by non-activated AMs that serve as an important reservoir for infection. However, we envision that upon deposition in the alveolar space *M.tb* may enter a dynamic phase where it encounters pulmonary surfactant that contains homeostatic and antimicrobial enzymes (Hawgood & Poulain, 2001;van Golde, 1985) (called hydrolases) which alter the *M.tb* cell wall. Due to the dynamics of *M.tb* infection (Chroneos *et al.*, 2009), when *M.tb* is initially

deposited in the terminal bronchioles and alveoli, as well as, following release from lysed macrophages and in cavities in reactivated TB, *M.tb* bacilli are in intimate contact with lung surfactant hydrolases. We recently demonstrated that hydrolases present in the human lung surfactant (Mason, 2006;Williams, 2003), at their relevant concentrations *in vivo*, dramatically alter the cell wall of *M.tb* during infection (Arcos *et al.*, 2011). As a result of these cell wall modifications, a significant decrease in association of *M.tb* with human macrophages was observed followed by an increase in phagosome-lysosome fusion (35%), which translated to a significant decrease in *M.tb* intracellular survival within these cells and an increase in inflammatory cytokine production leading to better control of infection (Arcos *et al.*, 2011). Importantly, we demonstrated that minimal contact time (15 min) with human lung surfactant hydrolases significantly reduced the cell surface exposure of two major *M.tb* virulence factors, ManLAM and TDM (Arcos *et al.*, 2011). As mentioned above, both, ManLAM and TDM have been shown to play important roles in the intracellular survival of *M.tb* in the host by blocking the phagosome maturation process (Axelrod *et al.*, 2008;Kang *et al.*, 2005). Thus, below we will address which are the sources of these hydrolases in the alveolar space.

4.1 The *M. tuberculosis* infection pathway and the alveolar environment: The potential role of human lung surfactant hydrolases

The first interaction between *M.tb* and the human host takes place in the lung. The respiratory epithelium is actively involved in inflammation and host defense in multiple ways: providing a physical barrier, constituting the structural basis of mucociliary clearance aimed at the physical removal of inhaled bacteria; recognizing PAMPs/MAMPs by PPRs expressed on epithelial and myeloid cells, and secreting a variety of pro- and anti-inflammatory mediators, including a large variety of hydrolases (Nicod, 2005). When *M.tb* bacilli reach the alveolar space, AMs, monocytes, and neutrophils represent the array of innate immune myeloid cells that will participate in host defense.

4.1.1 The alveolar macrophage

The AM is the first professional phagocyte to encounter inhaled *M.tb* bacilli. AMs are placed in a unique location within the alveolar surfactant film, the latter of which is produced by type II alveolar epithelial cells and is composed of phospholipids and proteins (Jonsson *et al.*, 1986). AMs are at the interface between air and lung tissue, and represent the first line of defense against inhaled *M.tb* found in the air (Lohmann-Matthes *et al.*, 1994). AMs possess a high phagocytic and clearance potential. In a normal healthy individual, they represent more than 90% of the cells in bronchoalveolar lavage fluid (Reynolds, 1987).

Many studies have demonstrated that resident AMs can phagocytose large numbers of microbes through both opsonic and non-opsonic receptors (Fels & Cohn, 1986;Lohmann-Matthes *et al.*, 1994;Palecanda & Kobzik, 2001;Serrano-Gomez *et al.*, 2004;Stephenson and Shepherd, 1987;Tailleux *et al.*, 2005;Taylor *et al.*, 2002;F.X. Zhang *et al.*, 1999). Though AMs have high phagocytic activity, their microbicidal capacity is less well-defined. Efficient microbial phagocytosis followed by slow intracellular killing may be sufficient to control infection with many routinely encountered extracellular pathogens. Intracellular pathogens like *M.tb*, however, may take advantage of the reduced microbial activity of the AM by residing and multiplying within these cells (Ferguson & Schlesinger, 2000). The

participation of AMs in host defense, inflammatory processes and immune mechanisms has been amply documented (Schlesinger, 1997). In general, their primary function is the intracellular breakdown and disposal of particulate elements. In this regard, they contain a wide variety of hydrolases such as abundant lipase, acid phosphatase, cathepsin, lysozyme, esterase, acid ribonuclease, and β-glucuronidase activities [on a specific activity basis] (Cohn & Wiener, 1963;Sorber *et al.*, 1973). Interestingly, dead BCG-stimulated AMs exhibited up to a 4-fold increase in the activities for lipases, acid phosphatases and lysozyme compared to control (Cohn & Wiener, 1963) indicating an up-regulation of these hydrolases within the AMs in the presence of antigen. This observation was corroborated in AMs obtained from live BCG-vaccinated rabbits, where acid phosphatase, lipase and lysozyme activities increased up to 40-fold when compared to AMs from control rabbits (Sorber *et al.*, 1973). How these hydrolases are regulated during *M.tb* infection and their role in redecorating the cell envelope of *M.tb* are currently unknown.

4.1.2 Monocytes and neutrophils in the alveolar space

Mononuclear phagocytes enter the lung both constitutively to maintain AM and dendritic cell populations, and during lung inflammation (Srivastava *et al.*, 2005). The role of monocyte accumulation in the lung in acute and chronic pulmonary inflammation is largely unknown, although these cells are accessible by bronchoaleolar lavage (Maus *et al.*, 2001). In the mouse, alveolar deposition of a stimulus provoked a significant influx of monocytes into the interstitium of the alveolar compartment along with a characteristic recruitment of neutrophils (Gunn *et al.*, 1997;Li *et al.*, 1998;Ulich *et al.*, 1991). This was confirmed by studies showing that circulating leukocytes could be recruited across the endothelial and epithelial barriers into the alveolar space under both non-inflammatory and highly inflammatory conditions (Li *et al.*, 1998;Maus *et al.*, 2001). Given the capacity of monocytes to produce hydrolases, reactive oxygen species, or inflammatory cytokines (Van Furth, 1988), their accumulation has been implicated in several inflammatory diseases in the pulmonary system (Antoniades *et al.*, 1992). As is the case for AMs, how monocytes and their secreted products affect the cell wall of *M.tb* within the alveolar space remains unknown.

Neutrophils may play an important role in controlling *M.tb* infection (Pedrosa *et al.*, 2000;Seiler *et al.*, 2003). In the bronchoalveolar lavage fluid, they normally represent less than 2% of all cells, however, during inflammation a massive influx of neutrophils occurs (Mizgerd, 2002;P. Zhang *et al.*, 2000). Neutrophils eliminate microbes by a number of oxidative and non-oxidative mechanisms (P. Zhang *et al.*, 2000) including secreted hydrolases such as *N*-acetyl-β-glucosaminidase, β-glucuronidase, α-mannosidases, and lysozyme. Neutrophils can kill *M.tb* through both oxidative and non-oxidative processes (Brown *et al.*, 1987;Jones *et al.*, 1990). Although neutrophils may interact with *M.tb* cell wall components during alveolar deposition [such as the 19-KDa lipoglycoprotein, SL-1 and PGLs (Faldt *et al.*, 1999;Neufert *et al.*, 2001;L. Zhang *et al.*, 1991)], there is no information regarding how alveolar neutrophil-derived hydrolases affect the integrity of the cell wall of *M.tb* during infection.

4.1.3 Alveolar epithelial cells

Alveolar epithelium lines the alveoli air sacs of the lung and is comprised predominantly of two specialized cell types (type I and type II). Alveolar type I cells function in gas exchange.

These cells have an extremely thin cytoplasm extending away from the nuclear body and contain a large number of plasmalemmal invaginations termed caveolae (Gil *et al.*, 1981; Williams, 2003). Caveolae regulate removal of endogenous and exogenous particulates from the alveolar space by regulating activities of receptors, hydrolase secretion to the alveolar lumen and signaling molecules (Gumbleton, 2001;Marx, 2001;Razani & Lisanti, 2001). Importantly, these caveolae contain lipid phosphate phosphohydrolase, a critical enzyme in hydrolyzing a variety of phospholipids to produce diacylglycerol (Nanjundan & Possmayer, 2003). Type I cells (as well as AMs) also produce carboxypeptidase which increases during bacterial deposition in the alveolar space and functions in the processing of many peptides (Skidgel & Erdos, 1998). Other epithelial cell enzymes are related to the regulation of ion transport to the alveolar space (Johnson *et al.*, 2002).

Alveolar type II cells are considerably smaller than the type I cells and are richly endowed with organelles and microvilli on their apical membrane. These cells are located in the corners of the alveolus where their physiological functions include surfactant production, secretion and recycling (Fehrenbach, 2001). Surfactant is released to the alveolar space by exocytosis from intracellular storage organelles termed lamellar bodies which contain the majority of the components of the surfactant. During active secretion of contents from the lamellar bodies to the alveoli and during the surfactant recycling process, a variety of hydrolases have been related to these organelles (de Vries *et al.*, 1985; DiAugustine, 1974; Edelson *et al.*, 1988; Gilder *et al.*, 1981; Hook & Gilmore, 1982; S.L. Young *et al.*, 1993), many of which have lysosomal-type degradative functions. The presence of hydrolases within the lamellar bodies implies that if the contents of these structures are secreted, then hydrolases should also be secreted with surfactant. It has been shown that surfactant contains substantial quantities of hydrolases (Hook, 1978), where some hydrolases are highly active (*i.e.* α-mannosidase and β-*N*-acetylglucosaminidase) while others much less so (*i.e.* β-glucuronidase and arylsulfatase). Thus, the lamellar bodies provide a vehicle for the release of hydrolases into the alveoli, and their influence on host defense is being to be elucidated by our laboratory. In general, studies on epithelial cells have focused on phospholipases as second messengers in signaling, however, these same phospholipases are also degradative hydrolases with great potential to redecorate any microbial cell wall. For example, phospholipase A_2 can release the fatty acids from phospholipids (Dennis, 2000). The action of phophatidylinositol phospholipase C provides both diacylglycerol and inositol trisphosphate. Other hydrolases include secreted and extracellular membrane-associated phosphatidic acid phosphatases which act on 1,2-diacyl,*sn*-glycerol phosphate to produce diacylglycerol and inorganic phosphate (Brindley & Waggoner, 1998), however, the membrane-associated hydrolases also act on a variety of phospholipids to generate anionic and/or neutral lipids (Nanjundan & Possmayer, 2003). These hydrolases may also be active against the rich phospholipid content of the cell wall of *M.tb.* (*i.e.* PIMs, cardiolipid, phosphatidylethanolamine, etc.) (Arcos *et al.*, 2011). To what extent *M.tb* bacilli directly interact with epithelial cells and their secreted products remains unknown. However, the location of the alveolar epithelium, as well as, the relatively large alveolar epithelial surface area estimated at 100 to 140 m^2, makes it likely that *M.tb* will interact with components of the alveolar space prior to and following its residence in the AM. The low alveolar fluid volume relative to the alveolar epithelial surface area (7- 20 ml per 100 m^2) likely increases the local concentration of released hydrolases when compared to other tissue compartments.

This, in turn, increases the probability that secreted hydrolases will impact the *M.tb* bacillus in the alveoli, altering its cell envelope and metabolism.

4.1.4 Pulmonary surfactant

Pulmonary surfactant prevents alveoli from collapsing at low lung volumes by reducing the surface tension in the alveolar space. Dipalmitoylphosphatidylcholine comprises almost 50% of total surfactant and is its major surface-active component (van Golde, 1985). The exact function of the remaining lipid components, such as unsaturated phosphatidyl-cholines, phosphatidylglycerols, phosphatidylethanolamines, phosphatidyl-inositols, and cholesterol are still uncertain (King, 1982;van Golde, 1985). The surfactant protein fraction comprises a highly variable amount of serum proteins, a wide variety of specific hydrolases (Griese, 1999) and four apoproteins (the surfactant proteins termed SP-A, -B, -C, and –D) that contribute to its specific function (Weaver & Whitsett, 1991). As mentioned above, surfactant contains several products secreted by alveolar myeloid and epithelial cells, some of them already defined in host defense, such as the bacteriolytic lysozyme (Haller *et al.*, 1992;Singh *et al.*, 1988). The wide variety of hydrolases secreted into surfactant resemble but are distinct from lysosomal enzymes (Hook and Gilmore, 1982). The surfactant resident hydrolases alter the *M.tb* cell wall (Arcos *et al.*, 2011).

There remain many fundamental questions about how the alveolar environment influences *M.tb* pathogenesis. We recently showed that secreted hydrolases involved in surfactant homeostasis affect the *M.tb* cell wall (Arcos *et al.*, 2011) and/or trigger *M.tb* endogenous hydrolases to modify its own cell wall prior to contacting its natural niche, the AM, or after its lysis from the cell. Exposure of '*de novo*' motifs on the surface of *M.tb* after alveolar enzymatic processing will provide insight into the real nature of the *M.tb* cell envelope during infection. Since components of the *M.tb* cell envelope dictate the innate immune response against the bacillus via their interaction with surface receptors on myeloid cells, the identification of hydrolases that shape the surface of the *M.tb* cell envelope will enable more predictive *in vitro* models to be developed and novel drug targets to be identified.

5. Conclusions

The interface between *M.tb* and the host depends of many factors. *M.tb* strains differ in their cell wall components exposed on their surface. Even within the same strain, it is likely that some bacilli differ, and thus bacilli may interact different with the host. Thus, at a given infection we may find a mixture of events. On one hand, and depending on the cell wall components exposed on their surface, we may find bacilli that interact with a receptor inducing pro-inflammation and promoting *M.tb* killing, and on the other hand, we may find bacilli interacting with another receptor inducing anti-inflammation and *M.tb* intracellular survival. From the host perspective, genetic predisposition and living conditions dictate the predilection for *M.tb* infection. Even in the context of the same infected person and within the same host cell population, differences amongst cells in the expression of cell surface receptors, signaling, trafficking and innate and adaptive function exist. These differences in the host get enhanced even more when we compare host cells from different model systems. Our current studies on the impact of the alveolar space in the *M.tb* infection outcome also

indicate that environmental host factors, such as alveolar hydrolases, play important roles in the establishment of the infection. With this in mind, what are the necessary elements that result in a succesful *M.tb* infection? Is *M.tb* infection just chance or a perfect combination of bacterial and host elements? Upon the successful establishment of *M.tb* infection, the ultimate goal of the host is to reduce inflammation and tissue destruction and in this scenario *M.tb* has learned to evolve, adapt, and survive.

What do we know about the *M.tb* cell wall adaptation to the host? The most revealing attribute of the *M.tb* cell wall is its complexity. Studies have been focused on depicting the composition, structure, biosynthesis, and the spatial conformation of the *M.tb* cell wall and its components for decades. Currently, researchers are focusing to reveal how the cell wall of *M.tb* is during infection (*in vivo* and *in vitro*). The development of new technologies and/or the use of known technologies already successfully applied in other fields (such as cancer research) are moving fast into the field of TB. Thus, studies using scanning electron and atomic force microscopy revealed that the *M.tb* cell wall from MDR- and XDR-strains differs from susceptible strains (Velayati *et al*., 2009a, 2009b, 2010). Experiments analyzing infected granulomas by using high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS-NMR) showed that we are capable of analyzing the cell wall of *M.tb* when inside of the granuloma without further manipulation (Somashekar *et al*., 2011). The introduction of novel reporters that can be used for selective labeling of the cell wall of *M.tb* during infection *in vitro* and *in vivo* is already allowing us to see how the cell wall of *M.tb* is remodeling during infection (Backus *et al*., 2011). Efforts in improving purification techniques are also allowing us to be able to purify *M.tb* directly from infected tissues. The use of novel *state-of-the art* mass spectrometry techniques such as LC/MS/MS (Sartain *et al*., 2006, 2011), ESI/MS (Barry *et al*., 2011) and MALDI-Tissue Imaging (Prideaux *et al*., 2011) in drug discovery will allow us to obtain new information about the cell wall composition from a single *M.tb* bacillus isolated from tissue and also to see how *M.tb* cell wall components, and other biomarkers and drugs are distributed within the infected tissue. These are few of many other novel biotechniques that are starting to be applied in the field of TB. But this is only the beginning, more efforts improving the protocols and the development of new technology will allow us to move quickly to solve the "mystery" involving how *M.tb* adapted to the host and became such a successful infection currently affecting one third of the world population and taking away ~2 million lives every year.

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5

For Host Factors Weddings and a Koch's Bacillus Funeral: Actin, Lipids, Phagosome Maturation and Inflammasome Activation

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1. Introduction

When a particle or microorganism enters the host, is readily phagocytosed by the local macrophages and dendritic cells. If the microorganism is a non-pathogenic bacteria such as *Mycobacterium smegmatis*, the bacteria containing phagosomes will fully mature. Maturation refers to a multi-dimensional and complex process that integrates biochemical alterations on the phagosomal membranes, resulting as a direct response from the external stimuli and indirectly from the immediate response at the gene expression level in the form of transcriptional programs, as well as from its regulation. This, in turn, leads to modifications at the intracellular architecture, with the reorganization of the organelle dynamics, dependent on the assembly of actin, and resulting ultimately in the fusion of phagosomes with lysosomes. Not only several biochemical pathways have been implicated, but also physical and mechanical events will determine the faith of the bacteria containing phagosome. At the biochemical level, acidic hydrolases and the proton ATPase will reach the phagosomes while maturing and promote the lowering of the intraphagosomal pH, thereafter contributing to the killing of the bacteria. The production of nitric oxide (NO) by the inducible nitric oxide synthase (iNOS) and the reactive oxygen species (ROS) by phagosome oxidases (phox) is also known to be important for the bacterial killing. NO and cytokines are an important part of the inflammatory response which is downstream of transcription factors such as nuclear factor-κB (NF- κB).

When the pathogenic *Mycobacterium tuberculosis* enters the macrophage phagosome, the maturation process is blocked. We have shown that at least three distinct processes are targeted: actin assembly, fusion with lysosomes and acidification (Anes et al., 2003, Anes et al., 2006, Castandet et al., 2005). It has been suggested that specific *M. tuberculosis* lipids such as the sulfolipids (Goren et al., 1976), and phenolic glicolipids (Rhoades et al., 2003), and secreted peptides (Walburger et al., 2004) have been implicated as effectors involved in blocking the phagosome maturation to phagolysosome. These factors, which have been reported to be transferred to different host membranes in infected cells, are likely to be the cause of the well-described inhibition of pro-inflammatory responses, including the inhibition of TNF- α secretion, and the restriction of phagosome motility.

These blocking mechanisms, which are still yet poorly understood, can somehow, be reversed. Our published data show that phagosome maturation is directly linked to actin assembly on membranes (Defacque et al., 2000). This will consequently lead the organelles to aggregate before they fuse with lysosomes (Jahraus et al., 2001). We also described the addition of several pro-inflammatory lipids to *M. tuberculosis* infected cells with surprisingly strong killing effects (Anes et al., 2003, Gutierrez et al., 2009, Jordao et al., 2008). These selected lipids can manipulate the phagolysosome maturation by interfering with the actin machinery. In addition to these signaling lipids, ATP, the P2X7 receptor and cAMP were shown to be involved in actin assembly and the killing/survival of pathogenic mycobacteria (Kalamidas et al., 2006, Kuehnel et al., 2009, Kuehnel et al., 2009b). Furthermore, some lipid effectors for actin assembly also control NF-kB (Gutierrez et al., 2009). Using microarray analysis, we linked NF-kB in the regulation of many lysosomal enzymes and membrane-trafficking regulators, including cathepsins, LAMP-2 and Rab34, during infection (Gutierrez et al., 2008).

Important classes of lipids that are effectors for actin assembly and for NF-kB control include eicosanoids. Eicosanoides produced from arachidonic acid such lipoxin X4 (LX4) as well ATP, and the P2X7 R are all additionally involved in a necrotic-programmed cell death (Chen et al., 2008). Distinct molecules were found to play opposite roles either inducing apoptosis or necrosis during infection. In fact while virulent *M. tuberculosis* promotes necrotic cell death and inhibits apoptosis, the non-virulent strain H37Ra induces apoptosis that results in lower bacterial viability. Pyroptosis in the context of tuberculosis is controversial: from one side it leads to potent pro-inflammation that drives tuberculosis; from other side it allows the release of intracellular bacteria and their escape to hydrolytic digestion. This type of programmed cell death depends on the assembly of a specific inflammasome leading to caspase 1 activation and IL-1β and IL-18 secretion. Some groups claimed that *M. tuberculosis* might block the inflammasome activation (Master et al., 2008). We have shown that *M. tuberculosis* in human macrophages, not only activates caspase 1 and IL1-β secretion but also that different inflammasomes are assembled during infection (Mishra et al., 2010).

So we propose in this chapter to present an overview of host factors that may be manipulated in order to reverse phagosome maturation arrest, NF-kB translocation, inflammasome activation and therefore boosting the macrophage killing abilities to Koch's bacilli.

2. The disease

Tuberculosis is usually a lung infection caused by inhalation of Koch's bacilli within microdroplets, but it can affect any organ. In the lung, bacilli are envisaged to be engulfed by cell patrolling the alveolar surface such as alveolar macrophages and tissue dendritic cells (DC). These cells transport the pathogens to the lung interstitium and draining lymph nodes. At both sites, primary lesions develop that rarely cause disease. These professional phagocytes that are devoted to maintain the lungs cleaned from particles, produce a series of bacterial insults designed to kill pathogens. However *M. tuberculosis* developed capacities to subvert the killing mechanisms of phagocytes to allow their intracellular survival and/or

replication. The first contact with these phagocytic cells will display a series of innate immune mechanisms that may lead to the complete clearance of the bacilli. These include the release of NO, ROS, the fusion of the phagosome with the lysosome with the simultaneously bacteria digestion by acidic hydrolases and antimicrobial peptides (Cooper et al., 2000, Liu et al., 2007, MacMicking et al., 1997). Ultimately the programmed cell death of the infected host cell will kill the reminiscent infecting bacteria (Park et al., 2006).

Furthermore the initially infected cells release pro-inflammatory cytokines which leads to recruitment of more DC, monocytes and neutrophils from the blood stream and the infected DC become mature and migrate to the local lymph node where they activate specific T cells. The cytokines IL-12 and IL-18 from the infected cells induce natural killer cell (NK) activity, and the NK cells in turn produce IFN-γ, which activates the macrophages to produce TNF-α and more microbicidal effectors (Korbel et al., 2008). In fact resting phagocytic cells are designed to uptake and clear bacteria by phagolysosome fusion with full bacteria digestion, while activated macrophages are reprogrammed to produce more free radicals, and decrease the extend of bacteria digestion in order to produce antigens for immune cross presentation. Through cytokine and chemokine signaling, other immune cells are recruited and the pathological hallmark of TB, the granuloma, is formed. In the granuloma, macrophages differentiate further into epitheloid cells or foamy macrophages, or fuse to form giant cells, and become surrounded by lymphocytes and an outer cuff of fibroblasts and extracellular matrix proteins. At this stage infected people may complaint for symptoms that may resemble a simple cold.

Thereby, the bacilli are contained until the granuloma fails due to immunosuppression (Russell, 2007). For a long time, the granuloma was viewed as beneficial only for the host – it coincided with the onset of adaptive immunity and reduction of bacterial growth in the lung – but recent studies in zebrafish embryos infected with a close relative of *M. tuberculosis*, *Mycobacterium marinum*, have indicated that mycobacteria also use the granuloma for their benefit upon initial infection, recruiting new macrophages to allow spread between host cells (Davis & Ramakrishnan, 2009). This stage were the centre of the granuloma is formed by infected macrophages usually is referred as a latent infection without disease symptoms and according to the World Health Organization (WHO reports 2008) corresponds to one third of the world infected people. From these only 5-10% will develop disease during their lifetime (Kaufmann & Parida, 2007).

When the granuloma centre becomes caseous containing necrotic macrophages following necrotic programme cell death, latent infection becomes in active disease, which in advanced TB form cavities in the lung. Historical texts identify the disease as "consumption," "wasting away," "king's evil," "lupus vulgaris," "the white plague" or "phthisis" based on its clinical manifestations (Donoghue, 2009). Although mycobacteria are able to survive and proliferate within phagocytes, it should be noted that during active pulmonary tuberculosis they are preferentially found in the extracellular space. The necrotic centers of infection sites during active disease contain liquefied, so-called caseating, debris. These lesions are rich in cellular detritus, membrane phospholipids and cholesterol, which serve as an important carbon source for mycobacteria. Spillage of infectious bacilli into the airways occurs when the structure ruptures, and this allows spread to new individuals (Russell, 2007).

The present overview of host innate immune response to *M. tuberculosis* highlight the relevance of the phagolysosome fusion, host programmed cell death and inflammation as important mechanisms whose possibility of control by molecular effectors may provide alternative therapies for TB.

3. Phagosome maturation blockade into phagolysosome by *M. tuberculosis*: targeting phagosomal actin assembly, fusion and acidification

Phagocytosis — the engulfment of particles by unicellular organisms and higher eukaryotic cells — was first observed and documented by Metchnikoff around the beginning of the last century. His observations with macrophages ("big eater" in Greek) persuaded him that these professional phagocytes were the primary defence against microbial invaders. Although this view garnered little support at the time, the central role of phagocytes in immune protection is now universally accepted. The successful completion of these protective activities requires maturation of the phagosome into an acidic, hydrolytically competent, organelle that can fuse with lysosomes, which contain a battery of degradative enzymes. In fact we were able to show that pH acidification renders mycobacteria more susceptible to kill with the highest susceptibility observed in BCG at a pH bellow 6.3 when compared to *M. bovis* (Jordao et al., 2008).

Upon uptake of a pathogen by receptor-mediated phagocytosis into a macrophage, the resulting phagosome undergoes a series of fusion and fission events with the endocytic pathway. One of the important functional characteristics of the maturation process is that newly formed phagosomes, like early endosomes, mature to a state where they no longer fuse with early (or maturing) endosomes; only then can they fuse with late endosomes and lysosomes (Jahraus et al., 1998).

Two theories of how the communication between the phagosome and the endosomal network and lysosomes occurs have been postulated – the kiss and run-hypothesis where the vesicles interact through transient fusion and fission events via a fusion-pore-like structure (Desjardins, 1995), and the fusion hypothesis where the phagosome completely fuses with pre-existing endosomes (Flannagan et al., 2009). It has been argued that the kiss and run-hypothesis is more probable since phagosomes do not acquire endosomal proteins and solutes simultaneously, and since molecules of different size are recruited from the same endosomal type at different time points (Tjelle et al., 2000). In addition to acquisition through interaction with endosomes and lysosomes, phagosomes can acquire proteins and lysosomal hydrolases from the trans Golgi network (Rohde et al., 2007).

The ageing process of phagosomes is tightly regulated, although several pathogens have the capacity to subvert this process. During the late 1960s and early 1970s, D'Arcy Hart reported that pathogenic mycobacteria, including *M. tuberculosis*, are maintained in vacuoles that are not accessible to tracers known to be delivered to lysosomes, and that this 'non-fusogenic' phenotype correlates with the viability of the infecting organisms (Armstrong & Hart, 1975, Hart & Armstrong, 1974, Hart et al., 1972). These data marked one of the earliest applications of modern cell biological techniques to an intracellular bacterial infection. Indeed these seminal studies brought cell biology and microbiology together.

The demonstration that vacuoles containing *M. tuberculosis* contain both transferrin receptor and major histocompatibility complex (MHC) class II molecules indicates that they communicate with the cell plasma membrane (Clemens & Horwitz, 1995, Clemens & Horwitz, 1996). Although these vacuoles fail to fuse with lysosomes, they remain fusion-competent, acquire some 'lysosomal' proteins from the synthetic pathway of the host cell, and undergo fusion with other vesicles of the early endosomal system. The consensus that has emerged in the literature is that pathogenic mycobacteria have evolved a strategy to arrest the normal maturation process of phagosomes after uptake by macrophages. Because of the paucity of the vesicular proton ATPase on the membrane of mycobacterial phagosomes, *M. avium* and *M. tuberculosis* phagosomes fail to acidify in the way that phagosomes with inert particles do (pH 4.5-5) and maintain a pH of around 6.3 (Sturgill-Koszycki et al., 1994). In addition mycobacteria express a urease generating ammonia, which could buffer the lumen to counteract proton influx (Gordon et al., 1980). Moreover, mycobacterial phagosomes carry only limited amounts of lysosome-associated membrane proteins such as LAMP1, and have limited hydrolytic enzyme activity such as cathepsin B/L. Cathepsin D, which requires proteolytic and auto catalytic processing at low pH lysosomes to generate the enzymatically active form, is only present in its high molecular weight pro-form in mycobacterial phagosomes (Sturgill-Koszycki et al., 1996). This indicates that mycobacterial phagosomes are restricted from fusion events with downstream late endosomal/lysosomal compartments.

Once an appreciation of the physiology of these vacuoles was attained, groups started to probe for proteins known to regulate the membrane fusion events associated with endosome–phagosome maturation. It is well established that small GTPases of the Rab family are present on certain organelles and take part in specific fusion events. For example, Rab5, which functions during early endosome homotypic fusion, is normally recruited to nascent phagosomes and dissociates from these vesicles as they acquire Rab7, another GTPase that functions during fusion of late endosomes and lysosomes. Rab5 is associated with, and retained by, phagosomes containing live *Mycobacterium bovis* (bacillus Calmette-Guérin or BCG) or *M. tuberculosis* (Via et al., 1997). Rab5 is associated with phagosomes immediately after phagocytosis and facilitates the recruitment of Rab5 effector proteins, EEA1 and class III phosphatidylinositol-3-phosphate kinase which drives the synthesis of the early endosomal lipid phosphatidylinositol 3-phosphate (PI(3)P) (Vieira et al., 2001). PI3P interacts with Rab5 and EEA1 to promote endosome maturation. Membrane bound Rab5 is rapidly dissociated from the phagosome after its activation. Rab7 appears on the phagosome membrane after Rab5 dissociation and resides on the membrane during phagosome maturation (Vieira et al., 2003). After acquisition of Rab7, phagolysosome biogenesis is accelerated by the recruitment of Rab7-interacting-lysosomal-protein (RILP) to the phagosome (Harrison et al., 2003). Recently it was show that more Rab GTPases are recruited during progression of phagosome maturation and that their release and/or dissociation from *M. tuberculosis*-containing phagosomes has the relevance for the *M. tuberculosis*-induced inhibition of phagolysosome biogenesis. (Seto et al., 2011). Finally, the early endosomal v-SNARE cellubrevin is degraded on mycobacterial phagosomes, making it unavailable for fusion events and delivery of transport vesicles from the trans-Golgi (Fratti et al., 2002).

Rab GTPases also mediate a microtubule-based transport system that is likely to be responsible for providing the scaffold for endosome movement . Microtubules together with actin cytoskeleton are important for phagosome processing, for recycling from the phagosome and for phagosome intracellular transport and fusion with endocytic organelles (Southwick et al., 2003). In fact during maturation phagosomes move from the cell periphery to the perinuclear region where phagolysosome fusion occurs (Talaat et al., 2004).

Manipulation of Rab and SNARE proteins is a key component of the survival strategy of *Mycobacterium* (Kumar et al., 2010). Moreover, expression of Rabs is down-regulated in patients with tuberculosis compared with healthy *M. tuberculosis*-infected donors (Jacobsen et al., 2005). We found that the synthesis of Rab34 was elevated in a NF-κB dependent manner in both *M. smegmatis*- and *M. avium*-infected macrophages. However, the regulation of this protein was the opposite in the two systems. In *M. smegmatis*-infected cells, NF- κB was a positive regulator, whereas in *M. avium*-infected cells it behaved as a negative regulator. Although the mechanism of this difference is open, these data suggest that *M. avium* has the ability to switch the transcriptional response linked to NF-κB to a state more conducive for its survival (Gutierrez et al., 2008). Rab34 has been shown to regulate lysosome positioning within the cell (Wang & Hong, 2002). Since lysosome positioning may be required for mycobacterial killing, we are testing the hypothesis that the regulation of Rab34 expression controls the ability of phagosomes to be positioned close to lysosomes.

In addition to microtubules several studies have shown that actin filaments increase the uptake of ligands and their delivery to the degradative compartments downstream of the region where microtubules are required (Durrbach et al., 1996). The actin filaments also are involved in the motility and distribution of early endosomes via Rho D protein (Murphy et al., 1996). However, to what extent the actin filament network is required for interaction of phagosomes with early and/or late organelles of the endocytic pathway?

It is clear that manipulation of actin's fate by intracellular pathogens within the host cytoplasm is a key event that determines bacterial survival. The roles of actin in host-microorganism interactions can be used to categorize pathogens into four groups: 1) some use actin to enter non phagocytic cells; 2) some use it to avoid uptake by phagocytic cells; 3) some use it to promote attachment to the host plasma membrane forming pedestals; 4) some escape from the phagosome and use the actin to move within cells and travel from cell to cell. In addition to the pathogens that have an extraphagosomal way of life, it is clear that some pathogens can modify the actin cytoskeleton from within the phagosome.

Pioneer work by de Chastellier and co-workers indicates that intracellular *Mycobacterium avium* disrupt the macrophage actin filament network (Guerin & de Chastellier, 2000). Griffiths group showed that actin assembly by isolated latex beads phagosomes (LBP) correlates with their maturation status (Defacque et al., 2000). This *in vitro* assay monitors the polymerization of rhodamine-actin by phagosomal intact membranes in the presence of ATP and thymosin-β4 and in the absence of cytosolic extracts. The actin nucleation machinery, which includes the ezrin, radixin, moesin (ERM) protein family and phosphatidylinositol phosphates PIP and PIP2, are part of the membrane. Thymosin-β4 is used to prevent spontaneous actin nucleation at a concentration such that the phenomenon only occurs if a stimulator effector will induce the system. The standard assay works better at low ATP concentrations (0.2 mM) and the percentage of phagosomes that present red

actin dots in the LBP system is around 10-30% (Figure 1.A). At physiological concentrations of ATP (5mM) the system is blocked unless a stimulator will activate actin assembly (Defacque et al., 2000). Since LBP make cAMP at high ATP but not at low, via protein kinase A and cAMP is an inhibitor of actin assembly this may provide an explanation why the system is blocked at physiological concentrations of ATP (Kalamidas et al., 2006).

Intriguingly *in vitro*, phagosomes containing live pathogenic *Mycobacterium* failed to induce actin assembly, whereas phagosomes containing avirulent or dead mycobacteria nucleated actin readily depending on the age (as for the latex beads system)(Anes et al., 2003) (Figure 1.B).

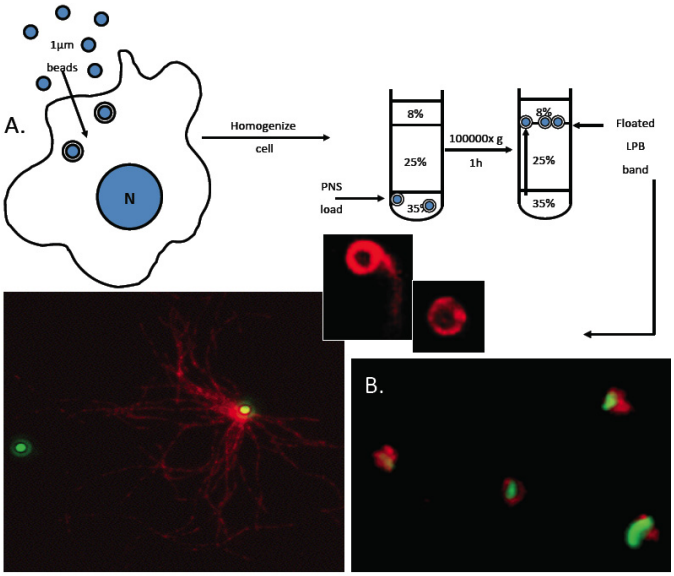

Fig. 1. Actin nucleation/assembly assay (red). (A) latex beads containing phagosomes. (B) *Mycobacterium smegmatis*-containing phagosomes

We have accumulated a large set of correlative observations such that all conditions we have identified as being stimulating in the in *vitro actin* assembly assay also stimulated actin accumulation around phagosomes *in vivo*, phagosome fusion with late endosomes and lysosomes and the killing of mycobacteria, a consequence of phagolysosome fusion (Anes et al., 2003, Anes et al., 2006) (Figure 2).

Conversely, compounds that inhibited *in vitro* actin assembly do not increase F-actin on phagosomes fail to promote phagolysosome fusion and favour growth of intracellular mycobacteria. Models have been developed to explain how actin filaments nucleated from the surface of a membrane organelle could "attract" other bound organelles toward it (Kjeken et al., 2004) (Figure3). Therefore we found conditions were phagosome maturation blockade by *M. tuberculosis* could be reversed to some extend by treating host infected cells with effectors that induced actin assembly and phagolysosome fusion with concomitant lumen acidification.

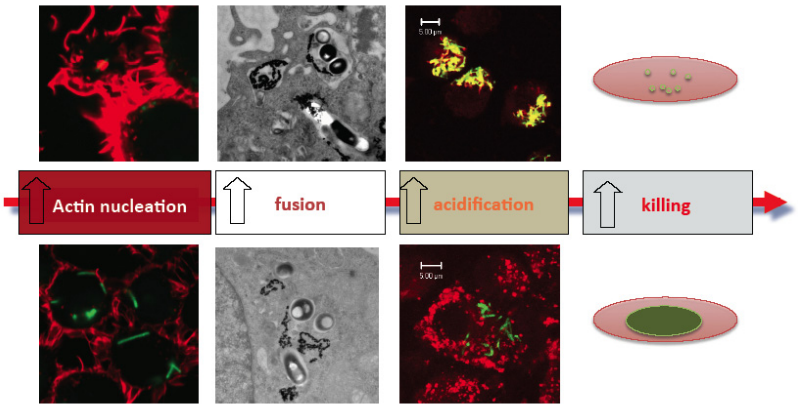

Fig. 2. Actin assembly *in vivo*: conditions that increase actin in phagosomal membranes lead to increase membrane fusion with lysosomes, acidification and mycobacteria killing. Left panel: confocal microscopy; (red) F-actin rhodamin-phaloidin , (green) Mtb-GFP. Middle panel: EM of gold-containing lysosomes and fusion with phagosomes. Right panel, acidotrophic lysotracker red vesicles and phagosomes containing heat killed Mtb (green).

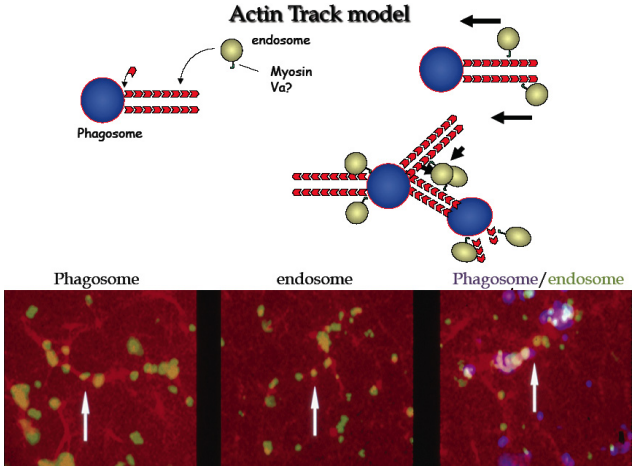

Fig. 3. The actin track model. Actin filaments nucleated from the surface of a membrane organelle could "attract" other bound organelles toward it helping fusion events. Confocal microscopy showing aggregation of phagosomes with endosomes along actin filaments (red).

4. Reversion of phagolysosome blockade by host effectors: Role of lipids, ATP, P2X7 receptor on actin assembly and on mycobacteria phagosome maturation

Phagosome maturation is known to be influenced by the lipid species present on its membrane, although studies published have focused mostly on the kinase that generates phosphatidyl i n o s i tol-4-phosphate (PIP), and on PtdIns(4,5)P2 (PIP2), which binds actin

nucleation proteins (Vieira et al., 2002). The key role of PIP and PIP2 opened the door for the analysis of all lipids that interconnected with these phosphoinositides in the actin assembly process, as well as sphingolipids and fatty acids (Anes et al., 2003).

The notion that *M. tuberculosis* may control phagosome maturation through the modulation of host lipids has been suggested by previous studies (Fratti et al., 2003), but the possible lipid mediators were not characterized. We showed that the enrichment of certain lipid, to mycobacteria-phagosome membranes stimulates the nucleation of actin and therefore phagosome maturation boosting the killing ability of macrophages to mycobacteria (Anes et al., 2003, Jordao et al., 2008). The F-Actin Stimulatory Factors (ASF) in the above experiments are the eicosanoide omega 6 arachadonic acid (AA), ceramide (Cer) and sphingosine-1-phosphate (S1P).

An important role of ATP here is to be used by kinases to produce PIP, PIP2, S1P, ceramide-1-phosphate (Cer1P) and phosphatidic acid (PA). All these phosphorylated lipids are stimulatory of F-actin at physiological concentrations of this purine. The incorporation of these phosphorylated lipids into the phagosomal membrane in the presence of ATP and ADP led to the translocation of ADP, but not ATP across the phagosomal membrane into the lumen. Once there, this ADP is converted to ATP by adenylate kinase activity. We propose that luminal ATP accumulates in response to selected lipids and activates the purinergic receptor P2X7R that signals across the phagosomal membrane to trigger actin assembly on the cytoplasmic membrane surface. (Kuehnel et al., 2009). P2X7R is a cationic channel, identified in the J774 macrophage latex bead phagosomes proteome (M. Desjardins, personal communication) that is able to bind via its cytoplasmic domain to a complex of 12 proteins, including actin. Whereas LBP prepared from wild-type P2X7R- positive bone marrow macrophages could assemble actin and was stimulated by S1P or PIP at physiological concentrations of ATP, the equivalent LBP from P2X7R-knock-out mice macrophages failed to be stimulated by these lipids. This argues for a model in which PIP and S1P induce ATP accumulation in the phagosome lumen. This ATP then activates the P2X7R that signals downstream of the phagosome actin assembly machinery (Kuehnel et al., 2009, Kuehnel et al., 2009b). From all these studies described above, its is now becoming clear that phagosomes are not passive vesicles that only acquire proteins and lipids via vesicular transport, or (for proteins) directly from the cytoplasm, and lose these components by recycling or dissociation. These organelles are also themselves capable of a plethora of biochemical activities, for example the enzymatic synthesis of different lipids. A dynamic description, including a predictive model of the interactions of lipids linked to PIP2 has recently been performed. This includes a systems level analysis of these lipids and other molecules on phagosomal membranes (Kuhnel et al., 2008).

The inhibitors (AIF: for F-Actin inhibitor factors) include the eicosanoides omega 3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Anes et al., 2003) and phosphatidylcholine (PC) (Treede et al., 2007). This group of lipids enriched in the membranes of infected cultures of macrophages protected the bacteria inducing the intracellular growth of *M. tuberculosis* and other mycobacteria.

4.1 Eicosanoides, important immunoregulatory and host defence mechanisms in TB

The omega 3 and omega 6 ratio are important for immunoregulatory and host defence functions. A striking effect on phagosomal-actin assembly, late fusion and killing of

mycobacteria was seen when a number of pro-inflammatory lipids, especially the omega-6 fatty acid, arachidonic acid (AA), were added to J774 macrophages infected with the non-pathogenic *M. smegmatis* or with virulent strains of *M. tuberculosis*. In contrast, the anti-inflammatory omega-3 fatty acid eicosapentaenoic acid (EPA) induced an increase in mycobacterial growth in macrophages. These results were exciting because they fitted nicely into a general pattern seen with these groups of lipids in whole organisms. In support of this, three earlier studies showed that diets rich in omega-3 fatty acids led to significant increases in growth of *Salmonella* in mice (Chang et al., 1992) and *M. tuberculosis* in guinea pigs (Mayatepek et al., 1994, Paul et al., 1997).

Indeed during the last years we have followed the effects of these lipids and their molecular effectors on the outcome of the infection in more complex systems than the macrophage cell culture model. Collectively, our data suggests that a high omega-6 fatty acids diet might be beneficial against mycobacteriosis, while a high omega-3 fatty acids diet might be detrimental. However, depending on the molecular effectors and, on the time during infection that the lipid is supplemented, opposite signaling effects were observed. The data with Koch's bacilli raise an important public health question: are those individuals latently infected with *M. tuberculosis*, but asymptomatic at increased risk of reactivating the disease upon omega-3 fatty acids supplementation?

Given this increasing tendency to advise and use omega-3 dietary supplements, which have beneficial effects among uninfected individuals it is important to evaluate their effect on patients suffering from tuberculosis and other infections since their impact on disease evolution is unknown. A literature search on the effects of omega-3 and -6 lipids on a variety of different pathogens, especially in animal models, the conclusion suggest that polyunsaturated fatty acids are not generally beneficial and are often detrimental. In a critical evaluation of all the experiments done to test the effects of omega-3 lipids in the context of infectious diseases Anderson and Fritsche concluded that there were "an equal number of papers published that report an adverse effect of omega-3 fatty acids on host infectious disease resistance as those that do not show an effect or show a beneficial effect"(Anderson & Fritsche, 2002).

The results obtained by our group were surprising in that the opposite effects were seen in the animals, compared with observations in macrophages. For mycobacteria infected macrophages the omega-3 fatty acid, EPA, enhanced intracellular survival of *M.tuberculosis* while in infected mice an omega-3 fatty acid enriched diet promoted bacteria killing. When we looked at the effects of an omega-6 fatty acid enriched diet on the intracellular survival of intracellular pathogens we found no effect on *M.tuberculosis* infected mice while for salmonella infected animals the bacteria survival was improved (Jordao et al., 2008). From the perspective of actin assembly, our results were in agreement with the salmonella model because AA, by stimulating actin assembly will create an even higher actin meshwork around the salmonella containing vacuole preventing membranes contact and therefore fusion with lysosomes, thus protecting the bacteria. For the mycobacteria model the results, in infected macrophages, also agree with our model as AA, by increasing actin around *M.tuberculosis* phagosomes will help bacteria killing. In animals it may be the case that downstream breakdown products of the omega-6 fatty acid added to the diet could play opposite and more complex effects thus reflecting the lack of agreement regarding the outcome of the infection.

Eicosanoids are lipids responsible for many of the effects found in acute inflammation. Depending on the nature of the stimulus and the local pulmonary environment, the alveolar macrophage can be triggered to selectively release AA from membranes (Cochran et al., 1987). Free AA thus becomes the substrate for the ciclooxygenase (COX) and 5-lipoxygenases (5-LO) enzyme cascades that convert AA to a complex array of prostanoids, leukotrienes and lipoxins. The inflammatory response play a key role in shaping the adaptive response in large part through the secretion of an array of mediators such as interleukin 1β (IL-1β), IL-18, IL-12, tumor necrosis factor α (TNF-α), prostaglandin E2 (PGE2), leukotriene B4 (LTB4) and lipoxin A4 (LXA4) in response to the pathogen. EPA, another major source of eicosanoids present in membranes, inhibits these conversions and has a general anti-inflammatory role. However the enrichment of a membrane with EPA will transiently induce AA release that will become free for COX and 5-LO enzyme cascades. The long time enrichment of membranes with EPA will lead to AA depletion and to anti-inflammation by preventing AA conversion into the group of potent pro-inflammatory modulators. Furthermore it will generate a class of PGs of the 3-series that are far less pro-inflammatory than those of the 2-series (Calder, 2002).

Recently was found that the membrane of latex bead containing phagosome has the enzymes required to convert AA to a number of prostaglandins (PGs) (Griffiths G., personal communication). Moreover, some of these PGs, such as PGE2 inhibit actin assembly by LBP. In contrast, AA the precursor of PGE2, is a potent stimulator of phagosomal actin assembly. Here depending on whether there is accumulation of the precursor (AA) or the ending product (PGE2) opposite effects will be observed on the ability of phagosomal membranes to assemble actin. AA stimulating actin assembly will induce phagosome maturation and lysosomal pathogen killing while its product, with an inhibitory effect on actin assembly, will contribute to pathogen survival.

5. Control through the transcription factor NF-κB on phagosome maturation and macrophage activation by signaling lipids

A critical regulator of genes involved in inflammation is NF- κB (Caamano & Hunter, 2002). This transcription factor consists of two subfamilies: the 'NF-κB' proteins and the 'Rel' proteins which are present in the cytoplasm as hetero-dimers, in a complex with an inhibitor, IκB. When pro-inflammatory signaling occurs via activation of cell surface receptors such as the Toll-like receptors (TLR), IκB becomes phosphorylated. This releases the active subunits that enter the nucleus, where they up-regulate the transcription of hundreds of genes, a reflection of the complexity of this part of the inflammatory response (Natoli et al., 2005). In a recent study we showed that NF-κB is transiently activated early after infection of J774 cells and primary bone marrow derived macrophages with *M. smegmatis*. This activation is essential for mycobacterial killing since when NF-κB is blocked *M. smegmatis* survives. Using microarray analysis, we identified many lysosomal enzymes and membrane-trafficking regulators, including cathepsins, LAMP-2 and Rab34, were regulated by NF-κB during infection (Gutierrez et al., 2008). Our results argue that NF-κB activation increases the synthesis of membrane trafficking molecules, which may be rate limiting for regulating phagolysosome fusion during infection. The direct consequence of NF-κB inhibition is the impaired delivery of lysosomal enzymes to *M. smegmatis* phagosomes and reduced killing.

Whether or not pathogenic mycobacteria activate NF-κB is not clearly established, with different groups reporting conflicting results. Using confocal microscopy and ELISA, we observed a low and transient activation of NF-κB by *M. avium*. Similar results were reported before (Lee & Schorey, 2005). In contrast, others observed that high and low virulence strains of the *M. avium*-intracellular complex (MAC) activated NF-κB (Giri et al., 1998). There are similar reports of NF-κB activation in macrophages infected with *M. tuberculosis*. However, many studies argue that the pathogens strongly block the proinflammatory response in general (Beltan et al., 2000). Based on our observations, we suggest the model in which pathogenic mycobacteria induce NF-κB activation upon contact with the macrophage. As soon as the mycobacteria start the synthesis and secretion of virulence effectors, the system may be repressed. A hitherto unappreciated benefit to the pathogen in inhibiting the NF-κB system is the consequence that the phagolysosome fusion events would also be blocked. In summary, our results pinpoint a novel role of the NF-κB system to allow (directly or indirectly) the synthesis of molecules involved in intracellular trafficking. This regulation is linked to phagolysosome fusion, which facilitates killing of intracellular mycobacteria. In addition, our microarray screen identified a large number of potential new regulators of phagolysosome fusion; NF-κB is thus a key regulator of factors that facilitate the intracellular killing of mycobacteria.

Many links have been described between lipids and NF-κB activation in different systems. For example, in Caco-2cells AA activates NF-κB whereas EPA had no effect (Ramakers et al., 2007). Moreover, in the same cells phosphatidylcholine, which inhibits LBP actin assembly, inhibited NF-κB activation induced by TNF- α (Treede et al., 2007).

In macrophages, sphingomyelin (SM), PIP and AA enhanced NF-κB activation and the cell surface expression of CD69, a macrophage activation marker regulated by NF-κB. Sph, S1P, EPA and PC failed to activate either NF-κB or CD69. Cer activated CD69 expression without activating NF-κB. In *M. smegmatis*-infected macrophages, SM, PIP and AA transiently activated NF-κB in a manner that was enhanced. In contrast *Mycobacterium avium* mostly repressed NF-κB activation and only SM and AA could induce its partial activation. While lipids that activate NF-κB in uninfected cells tend to kill mycobacteria in macrophages Sph and S1P failed to activate NF-κB under most conditions but nevertheless enhanced killing of *M. smegmatis*, *M. avium* and *M. tuberculosis* H37Rv. Our results argue that both NF-κB-dependent and -independent mechanisms are involved in macrophage killing of mycobacteria and that both mechanisms can be enhanced by selected lipids (Gutierrez et al., 2009).

As stated above, NF-κB signaling could be also induced by TNF-α. TNF-α signaling involves binding to members of TNF receptor super-family and recruitment of a complex of adapter proteins. Among these, TNF-receptor associated factors (TRAFs) activate several intracellular signal transduction pathways, in particular NF-κB and, Map kinases (MAPKs) that lead to modulation of gene expression by different transcription factors. Pathogenic mycobacteria tends to inhibit all these pathways (Gutierrez et al., 2008, Schorey & Cooper, 2003).

MAPKs are central players in cell signaling and much of their activities are localized on membranes. There are three different classes of these kinases, namely ERK, JNK and p38. A

number of studies have shown that these kinases are activated upon infection with mycobacteria (Schorey & Cooper, 2003). Moreover, p38 and ERK are activated more during infection with non-pathogenic compared with pathogenic mycobacteria, implying that the pathogens inhibit these kinases (Roach & Schorey, 2002). The kinase p38 has been implicated in early endosome fusion; Fratti and colleagues also associated p38 activation with an inhibition of phagosome maturation in cells infected with *Mycobacterium bovis* (BCG) (Fratti et al., 2003). We additionally provided evidence for its importance in regulating phagosomal actin assembly (Anes et al., 2006). By inhibiting this kinase, a block on phagosome actin assembly *in vitro* was observed. *In vivo* p38 inhibition, blocked phagosome maturation and, increased survival of *M. smegmatis* within J774 macrophages. We also found that in *M.tuberculosis*-infected macrophages TNF-α secretion was stimulated by treatment with AA, whereas EPA inhibited this process (Jordao et al., 2008b). In line with this, AA strongly activated the pro-inflammatory MAPK p38 in uninfected cells but *M.tuberculosis* infected cells blocked the ability of AA to activate p38 leading us to conclude that AA-dependent killing is therefore independent of p38.

6. Control through programmed cell death and inflammation: Inflammasome activation by Koch's bacilli

Several intracellular pathogens, including Leishmania, Coxiella, Salmonella, Chlamydia and Yersinia, induce host-cell apoptosis as a way of minimizing the inflammatory response and avoid detection (Bergsbaken & Cookson, 2007, John & Hunter, 2008, Peters et al., 2008). In some of these cases, the induction of host-cell necrosis, sometimes by pyroptosis, leads to lower bacterial viability and is beneficial to the host (Haimovich & Venkatesan, 2006). Other acute bacterial pathogens, such as Pseudomonas, Neisseria and Streptococcus, are cleared by host-cell apoptosis, and these pathogens evade innate immunity by inhibiting apoptosis (Tunbridge et al., 2006). Virulent strains of *M. tuberculosis* induce necrosis of both human and mouse macrophages, whereas attenuated *M. tuberculosis* strains or other nonpathogenic mycobacterial species generally do not (Divangahi et al., 2009). In addition, there is accumulating evidence that apoptosis, whether induced by the pathogen itself, pharmacologically or by cytotoxic lymphocytes results in lower viability of *M. tuberculosis* (Duan et al., 2001, Gan et al., 2008, Oddo et al., 1998).

One strategy used by *M. tuberculosis* to avoid apoptosis is the subversion of host eicosanoid biosynthetic pathways. The finding that *M. tuberculosis* infection significantly increases the membrane release of AA via activation of cPLA2 γ (Duan et al., 2001) and that mice knock-out for 5-LO are more resistant to *M. tuberculosis* infection (Bafica et al., 2005) raised the question of whether eicosanoid production is involved in macrophage necrotic programme cell death and inhibition of apoptosis induced by virulent *M. tuberculosis*. The attenuated *M. tuberculosis* strain H37Ra induces the production of prostaglandin E2 (PGE2), which protects the mitochondrial inner membrane and induces the repair of plasma membrane microdisruptions inflicted by the pathogen (Keane et al., 2000). These events protect the host macrophages against necrosis and instead promote apoptosis. In contrast, intra-cellular infection with the virulent H37Rv strain of *M. tuberculosis* induces the production of lipoxin A4 (LXA4), which inhibits cyclooxygenase production and PGE2 biosynthesis. In a PGE2-poor microenvironment, macrophages cannot prevent the mitochondrial damage or repair the plasma membrane disruptions caused by *M. tuberculosis*, and this leads to

necrosis. Virulent *M. tuberculosis* in pre-necrotic macrophages continues to replicate and to spread to uninfected macrophages after the cells are lysed. Thus, the balance of PGE2 and LXA4 regulates the relative amounts of apoptosis and necrosis after *M. tuberculosis* infection, with important functional consequences for innate control of the intracellular infection.

Pyroptosis in the context of tuberculosis is controversial: from one side it leads to potent pro-inflammation that drives the active disease; from other side allows the release of intracellular bacteria and their escape to hydrolytic digestion. At one point in the life of an intracellular Koch's bacillus, there comes the time to exit the cell and infect the next one. In the larger scale of the human infection, mycobacteria need to access a novel host organism. In the infectious stage bacilli are coughed up and aerosolized within microdroplets. Dissemination of the infection therefore requires leaking of bacilli out of caseating necrotic foci or caverns into the alveolar space, which is promoted by exacerbated inflammation and massive cell death. Intranasal lipopolysaccharide treatment simultaneously to *M. tuberculosis* infection in mice induced necrotic lesions, otherwise not seen in this animal model for TB, indicating the importance of inflammatory stimuli for caseating necrosis.

This necrotic programme of cell death results from the assembly of inflammasomes, a complex of pattern recognition cytosolic receptors (NLRs: NOD-like receptors) (reviewed by (Sirard et al., 2007)) and adaptor proteins containing caspase recruitment domains (CARD) which are able to recruit and activate pro-caspase-1. Mature Casp-1 then activates the isoforms of pro-IL-1β and pro-IL-18 and the active pro-inflammatory interleukins are then secreted through exosomes, concomitant or not with induction of pyroptosis. In humans, the NLR family has at least 20 members. Structuraly, NLRs are characterized by their nucleotide-binding oligomerization domain (NOD), a leucin-rich domain that recognizes pathogen associated molecular patterns (PAMPs) and a signalling domain that either triggers NF- κB, or other signaling platforms such as MAPK, type 1 interferon pathways or induces inflammasome activation.

Different NLRs associate with each other and CARD-containing protein adaptors to form diverse inflammasome complexes in response to different bacterial elicitor molecules or endogenous danger signals. For example, the *Salmonella* and *Legionella* flagellin proteins are sensed by an NLRC4/CARD12/CLAN inflammasome (Mariathasan et al., 2004) whereas muramyl dipeptide (MDP) is sensed by NLRP1 and NLRP3 inflammasomes (Martinon et al., 2004). Various microbial toxins have also been shown to activate the inflammasome (Nour et al., 2009). The NLRP3/CIAS1/Cryopyrin inflammasome is activated either by uric acid crystals formed during gout, silica, asbestos or cathepsin B as well extracellular ATP released from necrotic cells (Cassel et al., 2008, Mariathasan et al., 2006).

M. tuberculosis resides largely within a phagosome-like compartment of host macrophages during infection (Jordao et al., 2008). Inflammasome activation it is postulated to occur only if these effectors or products from a signalling cascade reach the cytosol and gain access to NLRs. Despite its apparent sequestration from the cytosol, *M. tuberculosis* has been shown to trigger NLR pathways that induce type I IFN expression. This recognition event depends on the ESX-1 specialized protein secretion system of the bacterium (Pandey et al., 2009). ESX-1 has been shown to be involved in the perturbation of host cell membranes, and has therefore been hypothesized to promote type I IFN production by facilitating the delivery of bacterial products into the cytosol including those responsible for secretion of the virulence-

associated antigens ESAT-6 and the CFP-10 (Stanley et al., 2007). Indeed this is in agreement with recent findings that support the escape of *M. tuberculosis* from the phagosome to the cytosol at latter times post-infection in human primary macrophages and DCs (van der Wel et al., 2007). Some groups claimed that *M. tuberculosis* blocks inflammasome activation (Master et al., 2008). However part of the experiments were performed using BCG and because this attenuated strain had lost the complete RD1 region that codifies for ESX-1 and ESAT6 is unable to secrete important PAMPs to the cytosol that may be envolved in inflammasome activation. The same logic is applied for the use of the attenuated *M. tuberculosis* H37Ra, which has a mutation in PhoP which inhibits ESX-1 function, predominantly prevents necrosis and leads to sequestration and decimation of the intracellular bacteria (Chen et al., 2006). It has previously been shown that the secreted mycobacterial protein ESAT-6 in its purified form induces cell death in macrophages (Derrick & Morris, 2007) and that the protein has membrane-lysing activity (Smith et al., 2008). This makes ESAT-6 an attractive candidate as the bacterial factor responsible for necrosis. Lerm group found that deletion of the gene encoding ESAT-6 or of the RD1 region led to abrogation of the necrosis-inducing effect of *M. tuberculosis* as well as diminished IL-1β secretion by the host macrophage (Welin et al., 2011).

Recent studies have demonstrated that casp-1 activation and subsequent IL-1β synthesis are strongly upregulated in *M. tuberculosis*-infected macrophages (Montero et al., 2004), and inflammasome activation occurs upon *M. tuberculosis* infection (Netea et al., 2006). Finally, *M. tuberculosis* infection was proved to be sensed by NOD2, a cytosolic NLR that recognizes a component of peptidoglycan found in mycobacterial cell wall (Ferwerda et al., 2005). Another mycobacterial protein, a metalloprotease was so far the unique demonstrated to suppress the IPAF/NLRC4 inflammasome (Master et al., 2008).

Our group hypothesized that inflammasome activation is triggered by *M. tuberculosis* components that cross the phagosomal barrier where they are sensed by cytosolic pattern recognition receptors (PRRs) to activate the inflammasome and thereby promote IL-1β maturation. It was demonstrated that casp-1 activation depends on live intracellular bacteria that express a functional ESX-1 system. The contribution of ESX-1 appears to be attributable to the ESAT-6 protein, which facilitates the diffusion of other bacterial products such as AG85 into the cytosol. Casp-1 activation was found to be increased after co-challenge of individual *M. tuberculosis* PAMPs such as AG85, PIM1/2, mAGP, Man-LAM and PGN (Mishra et al., 2010).

To identify the components of the inflammasome most important during *M. tuberculosis* infection, a shRNA-based screen was carried out to silence individual NLR and CARD proteins selectively in THP-1 monocytes. These cells were transduced with a panel of lentivirus-encoding shRNAs that targeted 22 NLRs and 14 CARD domain-containing proteins in the human genome. We further identify an NLRP3 and ASC-containing complex as the major inflammasome activated in macrophages by *M. tuberculosis* infection, and implicate a number of other NLR and CARD proteins in this process. Nine genes were considered to be either positive or negative regulators of inflammasome function during *M. tuberculosis* infection, six genes played a role upon ESAT-6 stimulation, and 14 genes were important upon dual stimulation with ESAT-6 and AG85. Most of the shRNAs that inhibited IL-1β secretion in ESAT-6 pulsed cells also inhibited this response when ESAT-6 was added together with AG85. Most importantly, knock-down of NLRP3/CIAS1, PYCARD/ASC and

CARD6 resulted in a similar inhibition of IL-1β secretion in all the three conditions tested (Mishra et al., 2010).

In addition to NLRP3 and ASC, we found that depletion of CARD6 also impaired IL-1β secretion in response to all stimuli. CARD6 has been shown to positively regulate NF-kB activation (Dufner & Mak, 2006). Although CARD6 was important for IL-1β secretion in response to both live bacteria and isolated components, we were not able to detect a physical interaction between CARD6 and the other inflammasome components including casp-1. Thus, we speculate that CARD6 may not be involved directly in inflammasome assembly during *M. tuberculosis* infection, but may promote IL-1β production by regulating the synthesis of proIL-1β by activating NF-kB family of transcription factors. Consistent with the previously published reports, we identified NOD2 as one of the sensors for *M. tuberculosis* infection.

IL-1β secretion was also impaired upon depletion of CARD12/NLRC4 when cells were challenged with ESAT-6 plus AG85, which might suggest the involvement of more than one inflammasome platform in the regulation of IL-1β release during *M. tuberculosis* infection. The mycobacterial cord factor Trehalose-6, 6-dimycolate (TDM; also called cord factor) is a mycobacterial cell wall glycolipid that may be the most studied immunostimulatory component of *M. tuberculosis*. Macrophages detect TDM through a recently identified C-type lectin receptor-'Mincle' and produce inflammatory cytokines (Ishikawa et al., 2009). This activation most likely proceeds through a Syk-CARD9-Bcl10-Malt1 signalling pathway to induce a specific innate activation programme that is distinct from the response to TLR ligands (Werninghaus et al., 2009).

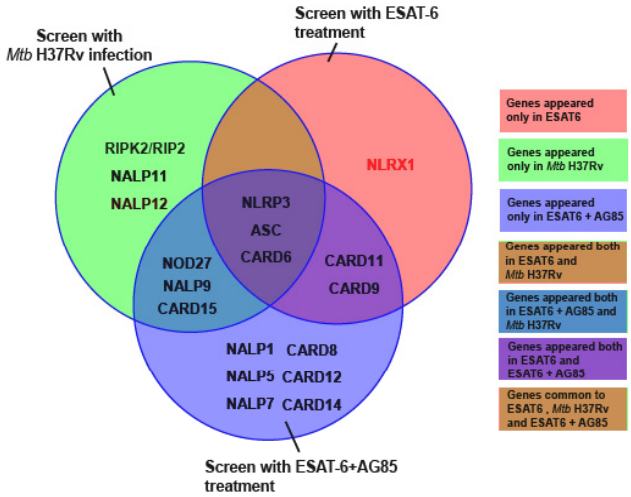

Fig. 4. NLR's and CARD-containing protein adaptors that responds to Koch's infection and to major PAMPs ESAT-6 and ESAT-6 co-challenged with AG85.

Recent reports have identified CARD9 as a central integrator of various PRR signaling pathways (Colonna, 2007). We observed that a component of the IL-1β secretion in macrophages challenged with ESAT-6 and with ESAT-6 plus AG85 depended on CARD9.

Together these data support an important role for CARD9 in the innate immune response triggered by *M. tuberculosis* infection, ESAT-6, AG85 and TDM. In conclusion our data suggest a previously unrecognized role for ESAT-6 in aiding the access of mycobacterial PAMPs such as AG85 to the macrophage cytosol eliciting casp-1 activation and IL-1β secretion. The observation that IL-1β and NLRP3 mRNA is induced in macrophages derived from patients with active TB indicates that a similar inflammatory pathway is likely to be important for the pathogenesis of human TB (Mishra et al., 2010).

Targeting cytosolic receptors or their adapters and therefore blocking the most relevant inflammasomes to be assembled during latent infection may prevent the active disease by decreasing pro-inflammatory responses that drives intracellular bacteria release from macrophages and the necrosis of the granuloma.

7. Conclusion

From the above review of the literature, it is evident that the interaction between the Koch's bacillus and the host macrophage is essential in determining the outcome of infection. However, the way in which the bacteria subvert the response of the macrophage originally designed to kill is still not completely understood. There are gaps in the knowledge, including how the bacteria inhibit phagosomal maturation and how the bacilli induce cell death. Furthermore, although a lot of effort has gone into studying phagosomal maturation in the context of *M. tuberculosis*, it is still not clear what determines the outcome of infection. The deciding factors for overcoming the block in phagosomal maturation, the crucial determinants in controlling the bacilli and disabling their replication, and the activation factors necessary to enhance macrophage function to remove the infection are not fully known. The dogma that inhibition of phagolysosomal fusion is the determinant factor that allows mycobacterial growth has not been well substantiated in a human system. Furthermore, methods designed to study these factors are needed to enable a better understanding. Many pieces of information come from studies of mouse or other animal models, and are based on results obtained with avirulent species of mycobacteria and the extrapolation of these to the human *in vivo* situation is often difficult.

The most pressing questions that emerge from the current body of data on *M. tuberculosis* fall into two discrete categories. First, how does the bacterium modulate the endocytic network of its host cell? Although the answer to this question is important for our understanding of the bacterium's success as a pathogen, it is unlikely to give rise to a treatment for infection because reversal of this phenotype would probably be difficult to achieve locally, and systemic modulation of endosome–lysosome fusion does not strike me as a feasible solution. Second, how can our appreciation of the environment in which the bacterium finds itself be exploited to result in new or improved ways to fight infection? Understanding the position of the vacuole within the endosomal continuum could lead to improved drug delivery. Most important, however, is the need to increase our knowledge of the intralumenal environment within the vacuole. If we want to screen for, and isolate, new drugs against relevant targets, we have to develop a more detailed understanding of the environment within the lumen of the endosomal vesicles. A huge amount of activity has been devoted to membrane fusion and its modulation by cytosolic mediators. A much greater understanding of the content of the endosomal–lysosomal milieu is needed. What are the concentrations of metal ions? What is the form and abundance of lipids and fatty

acids? These questions are key to our appreciation of both intracellular infection and to our understanding of the physiology of the endosomal system.

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6

The Role of Non-Phagocytic Cells in Mycobacterial Infections

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1. Introduction

Tuberculosis is one of the infectious diseases with great impact in the world. It affects mainly to young adults in the productive stage of the life and most of the deaths due to this disease occur in developing countries. All the cities are affected, but 85% of the cases occur in Africa (30%) and Asia (55%). In 2009, the WHO reported 9.4 million new cases, 14 million prevalent cases and 1.7 million deaths; in addition, there is an estimate that one third part of the world-wide population lodges the latent infection. These data place tuberculosis like the third cause of death at world-wide level, after HIV/AIDS and cardiac diseases (WHO, 2010).

Mycobacterium tuberculosis, the causal agent of tuberculosis, is a slow growth, acid-fast resistant bacillus, has a complex cellular wall constituted by complex lipids and carbohydrates. It is a facultative intracellular pathogen able to infect and to survive within the hosts cells. *M. tuberculosis* has developed numerous strategies to evade the immune response, so it is considered one of the most successful pathogens.

M. tuberculosis reach the human body through small drops expel by individuals with active disease. Once within the respiratory tract *M. tuberculosis* reaches the alveolar space and bounds to specific receptors of alveolar macrophages, dendritic cells and monocytes. For years the paradigm has been that alveolar macrophages are the main cells responsible of endocytosis, recognition and handling of *M. tuberculosis*. However, actual evidence demonstrates that non-phagocytic cells interact, harbour and respond to *M. tuberculosis*. In this work we will present and discuss some of the most relevant information on the interaction of non-phagocytic cells-*M. tuberculosis*, but first a brief revision of the main receptors described on macrophages and their role in the recognition of *M. tuberculosis* will be presented.

2. *Mycobacterium tuberculosis* interaction with macrophages

The lung is a highly susceptible organ for the invasion and establishment of microorganisms that are transmitted by aerosols. To guarantee control and elimination of those microorganisms that skip physical and chemical barriers of respiratory system, the lung requires activation coordinated and regulated by cells from the immune system located at

the bronchoalveolar space, like macrophages, lymphocytes, mast cells, neutrophils, etc. Macrophages play an important role in the innate and adaptive immune response towards pathogenic microorganisms, and are implied in their recognition and phagocytosis, in their processing and antigen presentation, in the antimicrobial metabolite production and in the production of cytokines that contribute to recruitment and activation of other cellular lineages that amplify and control the immunological response. Due to their anatomical location in the interface of the alveolar space, the alveolar macrophages are the first line against environmental particles or microorganisms that reach the lung (Schneberger et al., 2011). Alveolar macrophages express several immune receptors like the FC-γ receptors and complement receptors (CR1, CR3 and CR4), and high levels of molecules know as "Pattern recognition receptors" or PRRs, like mannose receptor (MR), Dectin-1 (β-glucan receptor), scavengers receptors, Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Fels & Cohn, 1986; Means et al., 2001; Palecanda & Kobzik, 2001; Srivastava et al., 2007; Stephenson & Shepherd, 1987; Taylor et al., 2002). Although alveolar macrophages have been attributed with pro-inflammatory functions (Schneberger et al., 2011), numerous reports indicate that alveolar macrophages rather exhibit an anti-inflammatory phenotype, known as "alternative activation", which includes an altered cytokine response enriched of IL-10 and TGF-β (Takabayshi et al., 2006), a reduced production of oxygen metabolites in response to stimuli and a decreased antimicrobial activity (Gordon, 2003).

Initial encounter of phagocytic cells with mycobacteria is a complex event and relays on several factors including those of the host and those of the bacteria. It seems that *M. tuberculosis* interaction do not depend on a single ligand-receptor bound (Schäfer et al., 2009). Apparently, during interaction, several receptors maybe involved each one with different roles. Final result of cell receptors-mycobacteria ligands engagement will be dependent of the type and number of cell receptors expressed by each cell lineage, and the intracellular signaling response triggered during those interactions. Next, the main receptors involved in the macrophage-*M. tuberculosis* recognition will be discussed.

2.1 C-type lectins

C-type lectins play an important role in M. *tuberculosis* recognition and in the inflammatory response. C-type lectins include a big family of proteins that bound carbohydrates (Anderson et al., 2008) and can be classified in soluble lectins and lectins associated to the cellular membrane (transmembrane lectins). The relevant soluble C-type lectins for mycobacterial infection are proteins A and D of the pulmonary surfactant (SP-A and SP-D). SP-A and SP-D are secreted into the alveolar space mainly by the type II pneumocytes. SP-A promotes *M. tuberculosis* phagocytosis through direct interaction of SP-A with the macrophages (Gaynor et al, 1995), which then over-express mannose-receptor (Beharka et al., 2002). In contrast, SP-D boosts mycobacteria agglutination and decrease mycobacteria phagocytosis by reducing mycobacterium recognition by mannose receptor (Ferguson et al., 1999; Torreles et al., 2008).

The most important transmembrane C-type lectins for *M. tuberculosis* recognition are mannose-receptor (MR), DC-SIGN and Dectin-1. *M. tuberculosis* recognition by human macrophages is primarily carried out through MR and it is associated to an anti-inflammatory response (Chieppa et al., 2003; Schlesinger, 1993). MR is a C-type lectin expressed by tissue macrophages, alveolar macrophages and dendritic cells, but not by

monocytes (Stahl & Ezekowitz, 1998). MR is a PRR that recognizes mannose-capped lipoarabinomannan (ManLAM), the most abundant mycobacterial lipoglycan (Schlesinger et al., 1994). *M. tuberculosis* interaction to MR depends of length, exposure and abundance of the ManLAMs on mycobacterium surface, in addition mycobacteria recognition by MR are limited to virulent species of *M. tuberculosis* complex (Schlesinger, 1993). The bound of *M. tuberculosis* to MR through ManLAM triggers an anti-inflammatory signaling pathway interfering with IL-12 production induced by lipopolysaccharide (Nigou et al., 2001), which suggests that this interaction hamper macrophage response towards *M. tuberculosis* promoting then mycobacterial infection (Jo, 2008). In addition, it has been demonstrated that engagement of MR by ManLAM during phagocytosis is crucial to delay phagosome maturation and for inhibition of phago-lysosome fusion (Astarie-Dequeker et al., 1999; Kang et al., 2005), initiating then the building of a secure niche for *M. tuberculosis* survival (Kang et al., 2005).

DC-SIGN (Dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin or CD 209) is a transmembrane C-type lectin expressed mainly by dendritic cells, but is also expressed by some macrophage subpopulations (Soilleux et al., 2002); 70% of alveolar macrophages from individuals with tuberculosis express this receptor (Tailleux et al., 2005). DC-SIGN presents a carbohydrate recognition domain (CRD) that recognizes structures with high mannose content and blood group antigens containing fucose (Appelmelk et al., 2003; Mitchell et al., 2001). *In vitro* studies showed that in dendritic cells, DC-SIGN is the main phagocytic receptor for *M. tuberculosis* (Tailleux et al., 2003). Activation trough DC-SIGN in the mycobacterial infection depends on agonists for this receptor expressed by each mycobacteria strain. Seven mycobacterial molecules recognized by DC-SIGN include lipomannan, mannose capped arabinomannan, two mannoside glycoproteins (Pitarque et al., 2005), phosphatide-inositide mannosides (Torrelles et al., 2006), ManLAM (Geijtenbeek et al., 2003) and α-glucan (Geurtsen et al., 2009). DC-SIGN can differentiate mycobacterial species through selective recognition of these mycobacterial molecules, especially ManLAM, present in *M. tuberculosis* but absent in non-tuberculosis mycobacteria like *M. smegmatis*, *M. chelonae* and *M. fortuitum*. Intracellular fate of *M. tuberculosis* inside dendritic cells differs from that into macrophages, whereas in macrophages *M. tuberculosis* may survive and replicate, into the hostile environment developed by dendritic cells *M. tuberculosis* growth is inhibited (Herrmann & Lagrange, 2005). It has been suggested that this difference is due to uptake features and intracellular trafficking of *M. tuberculosis* into each cell type and to the balance and relative activity of MR and DC-SIGN on each cell surface. Macrophages express high levels of MR whereas dendritic cells express high DC-SIGN activity; it has been speculated that this difference explains why macrophages are the main intracellular niche for *M. tuberculosis* (Kang et al., 2005).

Dectin-1 is a type II transmembrane receptor, which contains a single extracellular CRD and a tyrosine-based activation motif (ITAM) involved in cellular activation (Herre et al., 2004a; McGreal et al., 2005). This receptor is expressed predominantly by myeloid cells like macrophages, dendritic cells, neutrophils, and a sub-type of T cell; its expression is influenced by cytokines and several microbial products (Taylor et al., 2002; Willment et al., 2003). Dectin-1 acts as a PRR and recognizes particulate and soluble β-glucans (Herre et al., 2004a). Activation of Dectin-1 induces numerous cellular responses, like pathogens uptake, production of reactive oxygen species, and cytokines and chemokines production, making

Dectin-1 a link between the innate and the adaptive immune response (Brown et al., 2003; Taylor et al., 2007). Most of the studies have been focused to establish the role of Dectin-1 on fungi infections; however, the importance of this receptor in bacterial infections is less known. Recently, some investigators have suggested that Dectin-1 has an important role in the proinflammatory response against mycobacterial infections (Rothfuchs et al., 2007; Yadav & Schorey, 2006). It has been reported that TNF-α production by macrophages infected with virulent strains of *M. avium* and *M. tuberculosis* is Dectin-1 dependent, the opposite occurred in macrophage infections with avirulent or attenuated mycobacterial strains like *M. smegmatis*, *M. phlei* or *M. bovis* BCG; the minimal proinflammatory response elicited by macrophages infected with virulent mycobacteria has been attributed to Dectin-1 engagement (Yadav & Schorey, 2006). It has been demonstrated that during fungal pathogens recognition by macrophages, TLR2 and Dectin-1 have a synergic effect (Rothfuchs et al., 2007; Shin et al., 2008; Yadav & Schorey, 2006), a similar situation has not been reported for mycobacterial infections. On the contrary, a recent study showed that *M. tuberculosis* recognition by Dectin-1 triggers a Th1 and Th17 immune response against mycobacteria and is independent of TLR2 recognition (van de Veerdonk et al., 2010). The mycobacterial component recognized by Dectin-1 is unknown, the presence of β-glucans in the mycobacteria has not been reported yet, some hypotheses suggest that α-glucans presents in *M. tuberculosis* and BCG could be the molecules recognized by this receptor (Dinadayala et al., 2004; Lemassu & Daffe, 1994).

Other C-type lectin receptor involved in mycobacterial PAMPs recognition, include Mincle (macrophage inducible C-type lectin), which is expressed by macrophages expose to inflammatory stimuli like LPS, IL-6, etc. (Matsumoto et al., 1999). Ishikawa and collaborators demonstrated that Mincle bounds the mycobacterial glycolipid Trehalose-6-6´-dimycolate (TDM) also known as cord factor (Ishikawa et al., 2009), which is one of the most studied mycobacterial constituent with immunostimulatory activity.

Complement receptor 3 (CR3), also known as CD11b/CD18 or Mac-1, is a cell membrane heterodimeric receptor, belonging to the integrin superfamily, and in spite of this presents a lectin domain that interacts with *M. tuberculosis* components (Schäfer et al., 2009). Neutrophils, monocytes, natural killer cells and alveolar macrophages express this receptor, although it has been reported opsonic bound of *M. tuberculosis* to alveolar macrophages is inefficient because these cells express low amounts of CR3 (Stokes et al., 1998). It has been described that the CR3 is the main complement receptor in the opsonic mycobacteria phagocytosis, 80% of the opsonized mycobacteria is uptake trough this receptor. Interestingly, CR3 can bind a wide variety of ligands and since it presents multiple bounding sites, CR3 may promote opsonic and non-opsonic *M. tuberculosis* endocytosis (Velasco-Velazquez et al., 2003). Most of the evidence indicates that CR3 is responsible of mycobacterial bounding and internalization into the macrophage, however, lack of CR3 expression does not have a significant effect on the production of reactive oxygen species or nitric oxide by macrophages (Rooyakkers & Stokes, 2005; Velasco-Velazquez et al., 2003).

2.2 Scavenger receptors

The cell membrane glycoprotein CD36 (scavenger receptor class B) is found in macrophages, dendritic cells, endothelial cells and other cell types (Murphy et al., 2005). CD36 has been implied in several cellular functions like fatty acid transport, angiogenesis regulation,

inflammation and as PRR participates in the innate immune response against several pathogens including mycobacteria (Febbraio et al., 2001). The role of CD36 in protection against mycobacteria is controversial, recently Hawkes and collaborators (2010) demonstrated that CD36-deficiency confers resistance to mycobacterial infection; macrophages from CD36-/- mice allowed a reduction in the mycobacteria intracellular survival, but the mechanisms responsible of mycobacterial growth containment were not clear. They suggest that the better response of the knock-out mice could be due to the impairment of mycobacterial immune evasion strategies that take advantage of CD36. Detailed structural studies of the mycobacterial cell wall lipomannans have demonstrated that diacylated lipomannans inhibit LPS-induced inflammation by murine macrophages (Doz et al., 2007). Intriguingly, CD36 is a sensor of diacylglycerides (Hoebe et al., 2005) and may be the host receptor through which diacylated mycobacterial lipomannans suppress macrophage function. Another possible explanation is that through its association with the TLR2/6 heterodimer, CD36 may participate in immune-suppressor responses dependent of TLR2 as was reported by Noss and collaborators (2001). The family of scavenger receptors also includes macrophage receptor with collagen structure (MARCO) and scavenger receptors class A type I and II (SR-A). They are expressed in alveolar macrophages and dendritic cells (Areschoug & Gordon, 2009; Pearson, 1996). MARCO and SR-A bound acetylated-low density lipoproteins and bacteria; they promote inhaled particles and bacteria uptake (Arredouani et al., 2005; Palecanda et al., 1999). A recent report describe that macrophages expressing MARCO and bounding TDM respond producing proinflammatory cytokines, but this response required the concomitant participation of TLR2 and CD14 (Bowdish et al., 2009). With respect to SR-A, this receptor can also bound TDM, nevertheless, this recognition diminishes TNF-α/MIP-1-α production by activated macrophages, suggesting that SR-A have an important role in the suppression of the excessive inflammatory response presented during mycobacterial infection (Ozeki et al., 2006). Recent evidence suggest that scavenger receptors along with C-type lectins, cooperate to maintain controlled the immune response established *in vivo* against the mycobacterial infection (Court et al., 2010).

2.3 Toll like-receptors and NOD2

Toll like-receptors (TLRs) are not directly implied in microbial uptake; however, bacterial recognition by TLRs triggers an innate and adaptive immune response through the activation of monocytes, macrophages and dendritic cells. TLRs engagement by *M. tuberculosis* or its components is one of the first events in the *M. tuberculosis*-host cell interactions, resulting in activation of signaling cascades that culminates in production of proinflammatory responses. TLRs are expressed on immune cells like macrophages, dendritic cells, B lymphocytes, some types of T cells and some non-immune cells also express TLRs, like endothelial, fibroblasts and epithelial cells (Akira et al., 2006). The TLRs involved in *M. tuberculosis* recognition are TLR2, TLR4, TLR9 and TLR1/TLR6 that forms a heterodimer with TLR2 (Jo, 2008; Jo et al., 2007; Ryffel et al., 2005). Several studies have reported a number of mycobacterial lipids and proteins implied in TLR recognition and signaling, Jo and colleagues in 2007 presented an excellent review of these findings. Most of the studies oriented to explain the role of TLRs in mycobacterial infection, have been made in TLR knock-out mice, in this way, the protective role of TLR2 and in lesser extend of TLR4 was demonstrated, especially in the acute phase of the infection (Tjärnlund et al.,

2006). In addition, the finding that macrophages from mice TLR2-/- are not able to control mycobacterial growth, correlates with a reduction in TNF-α production by this cells (Tjärnlund et al., 2006). TNF-α is clue cytokine to contain mycobacterial infection (Roach et al., 2002); TLR2 engagement triggers an excellent TNF-α production, making this receptor an important element against mycobacterial infection. The role of TLR4 in the protection against *M. tuberculosis* infection is not absolutely accepted, some evidences demonstrate that TLR4 plays a protective role in the defense against pulmonary tuberculosis, since mice expressing a non-functional TLR4 had higher mortality and higher bacillary loads in the lungs (Branger et al., 2004). However, other reports demonstrated that mice with deficient TLR4 receptors are equally susceptible to *M. tuberculosis* or *M. avium* infections that the wild type mice (Feng et al., 2003; Reiling et al., 2002). Jo and coworkers suggested that these differences may depend of the signaling route involved, whereas the molecules that engage TLR2 will involve only MyD88 (myeloid differentiation primary response protein 88), those molecules that engage TLR4 may generate signals MyD88 dependent and independent (Jo et al., 2007). Another TLR with an important role in the antimycobacterial response is TLR9; mice TLR9-deficient were more susceptible to *M. tuberculosis* and mice with a combined TLR2 and TLR9 deficiency, were much more susceptibility to the infection (Bafica et al., 2005), pointing towards the need of the combined presence of these receptors for a better protection against tuberculosis. Nevertheless, other studies demonstrated that none of these TLRs (2, 4 and 9) are required to induce an adaptive immune response cellular, but the single MyD88 deficiency favored the unrestricted growth of *M. tuberculosis*, indicating the crucial role of this molecule for the generation of efficient effectors mechanisms by the macrophage (Hölscher et al., 2008). The role of other TLRs like TLR1 or TLR6 is not clear yet, it has been reported that mice TLR6-deficient are resistant to *M. tuberculosis* infection at high doses (Sugawara et al., 2003) and some clinical studies looking for associations between the mycobacterial infection and polymorphisms of a single nucleotide (SNPs) in the TLRs genes demonstrated that TLR1 SNP 1602S regulates the innate immune response towards the triacyl-lipopeptide and towards mycobacterium extracts (Hawn et al., 2007).

TLRs and C-type lectin receptors are molecules present on the plasma or endosomal membranes that recognize microbial components, whereas NOD (nucleotide binding oligomerization domain) receptors are expressed in the cytoplasm. Several evidences indicate that NODs family has an important role in the recognition of intracellular bacteria like *M. tuberculosis* (Takeda & Akira, 2005). NOD2 is a member of the Nod like receptors (NLRs) that recognizes muramyl-dipeptide (MDP), a key component of the peptidoglycan of Gram negative and Gram positive bacteria; on the contrary NOD1 recognizes only peptidoglycan from Gram negative bacteria. The intracellular location of NOD2 and *M. tuberculosis*, and the high content of peptidoglycan in mycobacterium cell wall allowed to suggest NOD2 as candidate for mycobacterium recognition. A recent study reported that NOD2 deficient mice (-/-) had high bacterial load in their lungs after *M. tuberculosis* infection, and a deficient cytokine production, suggesting that NOD2 participates in the resistance against mycobacterial infection, favoring the innate and adaptive immunity (Divangahi et al., 2008). Ferwerda and coworkers reported that NOD2 along with TLR2 represent a non-redundant system for *M. tuberculosis* recognition, and demonstrated that mycobacterial ligands for TLR2 and NOD2 synergize for proinflammatory cytokine production (Ferwerda et al., 2005).

Numerous are the receptors expressed by the macrophage and numerous are the receptors described for *M. tuberculosis* recognition (Table 1), nevertheless is obvious that *M. tuberculosis* recognition and internalization will not depend solely of a single receptor-ligand interaction. Additionally, mycobacterium surface displays many molecules, which may bound diverse receptors and hence activate different signaling routes. For these reasons during mycobacteria-macrophage interaction the possibility of multiple receptor-ligands interaction is high, so the first question arose, which of the multiple interactions will be the predominant? Then, which of the multiple signaling pathways activated in the cell will be executed? Presented in this way it looks like a chaotic scenario, so it is probable that the ultimate response, will be the final result of the multiple individual responses, for instance if during the interaction, many TLRs are engaged, a robust intracellular signaling response leading towards an inflammatory response will be building up, but if at the same time some lectin C-type receptors like mannose-receptors are engaged, favoring an anti-inflammatory response, then the final response will be a weaker inflammatory response. On the other side, the mycobacteria will have an active role for unbalance the host-response for their benefit, for instance, virulent mycobacteria over-express and expose for their easy recognition, those ligands with anti-inflammatory activity like ManLAM (recognized mainly by MR), whereas less virulent mycobacteria expose phosphoinositide-capped lipoarabinomannans that are considered as proinflammatory molecules. In summary, the studies briefly described here, suggest that the final outcome of the intracellular mycobacteria (survival or elimination) into the phagocytic cell, will be depending in much by the first encounter with the host cell, the mycobacterial components recognized in the encounter and the type and amount of receptors involved in the recognition.

Receptor			Mycobacterial components	Reference
C- Type lectins	Soluble lectins	SP-A	LAM, ManLAM	Sidobre, et al., 2000
		SP-D	LAM	Ferguson, et al.,1999
	Lectins associated to the cellular membrane	Mannose receptor	ManLAM	Schlesinger, et al., 1994
		Dectin-1	Particulate and soluble β-glucan	Herre, et al., 2004
		DC-SIGN	Man LAM	Geijtenbeek, et al., 2003
			Lipomannan	Pitarque, et al., 2005
			Mannose-capped arabinomannana	Pitarque, et al., 2005
			Two mannositide	Pitarque, et al., 2005
			Phosphatide-inositide mannosides	Torrelles, et al., 2006
			α-glucan	Geurtsen, et al., 2009
		Mincle	Glycolipid Trehalose-6-6´-dimycolate	Ishikawa, et al., 2009
Complement		CR3	LAM	Thorson, et al., 2001
			Ag 85C	Hetland and Wiker, 1994
Scavenger		SR-B	Sulfolipids	Ernst JD, 1998
TLR		TLR2	19 kDa,27 kDa, lipoprotein	Brightbill, et al., 1999, Hovav, et al., 2004
			Lipomannan	Vignal, et al., 2003
			Phosphatidyl-myo-inositol mannoside	Gilleron, et al., 2003
			LprA y LprG lipoprotein	Pecora, et al., 2006
				Gehring, et al., 2004
		TLR2, TLR4	HSP70	Bulut, et al., 2005
		TLR4	HSP65	Bulut, et al., 2005
		TLR1, TLR6 y TLR2	Soluble tuberculosis factor	Bulut, et al., 2001
NOD		NOD2	Muramyl dipeptide	Saiga, et al., 2011

Table 1. Phagocytic cell receptors and molecules involved in *M. tuberculosis* recognition.

3. *Mycobacterium tuberculosis* interaction with non-phagocytic cells

As it was stated earlier, interaction between intracellular pathogens and their host cells is a highly complex process, conformed by mechanisms that during bacterial and host cell evolution have guaranteed their nowadays survival. For the host cell, diverse strategies are displayed to contain the infection. Most of the strategies elicited by phagocytic cells are known, but bacterial containment does not rely only on phagocytic cells, other cell types participate also, these cells in conjunction are known as non-phagocytic cells. From the host cells side, phagocytic or non-phagocytic cells employ diverse mechanisms for bacterial containment, beginning with bacterial uptake up to the establishment of an adequate cellular activation state, which includes the display of effective antimicrobial mechanisms, apoptosis induction, autophagy or bacterial Ag presentation for recognition and death induction by activated cytotoxic lymphocytes, among others. From the pathogen perspective, these have developed numerous strategies to overcome the defense mechanisms elicited by the host cells. Actual evidence of the ability of intracellular pathogens to induce their entrance into non-phagocytic cells, have supported the hypothesis that these cells may be reservoirs of intracellular pathogens favoring their survival and persistence.

3.1 Epithelial cells infection by *M. tuberculosis*

Among the cells that comprise the pulmonary epithelium, alveolar epithelial cells type II are the non-phagocytic cell most studied, their participation in the immune response is acknowledged by their capacity to produce surfactant proteins A, C and D and a great variety of cytokines and chemokines in response to certain stimulus (Lin et al., 1998). By their location and distribution, these cells are exposed to all type of pathogenic agents that can reach the alveolar space, being then susceptible to the infection. In fact, the possibility that a microorganism finds an epithelial cell is much greater than finding an alveolar macrophage, since comparatively the number of pneumocytes type II surpasses up to 30 times the number of macrophages (Bermudez et al., 2002). In addition, pneumocytes type II may participate as antigen presenting cells, since they express histocompatibility molecules class I and class II, they also express adhesion molecules like ICAM-1, VCAM, LFA-3 and B7 (Corbière et al., 2011; Cunningham et al., 1994).

The ability of *M. tuberculosis* to invade non-phagocytic cells was initially described by Shepard, who described the susceptibility of HeLa cells to *M. tuberculosis* infection (Shepard, 1955). Years later Mapother and Songer described the intestinal epithelium cell invasion by *M. avium*, emphasizing the active role of mycobacteria to induce its uptake (Maphoter & Songer, 1984). Later, Bermudez and Goodman demonstrated the invasion and intracellular replication of *M. tuberculosis* into epithelial cells, using a pneumocytes type II cell line as model (Bermudez & Goodman, 1996). The presence of *M. tuberculosis* DNA into pulmonary non-phagocytic cells including epithelial, endothelial and fibroblast from individuals that died by causes other than tuberculosis, confirmed the ability of *M. tuberculosis* to infect epithelial cells *in vivo* (Hernandez-Pando et al., 2000). After recognition and acceptance of the ability of *M. tuberculosis* to infect human pneumocytes type II, the next studies were carry out to determine the mechanism responsible of *M. tuberculosis* internalization into the host cell, as well as the mycobacteria ligands involved in the recognition and triggering of

the endocytosis mechanisms. Nowadays the antimicrobial mechanisms exerted by the non-phagocytic cells to contain *M. tuberculosis* growth are been revealed.

The first studies achieved to explain the invasion mechanism, were made by Bermudez and Goodman, who reported the invasion and replication of *M. tuberculosis* into a cell line of human pneumocytes type II (A549 cells), they described that the internalization process was dependent of actin microfilaments and microtubules, and they suggested that vitronectin receptor and β-1 integrin were the membrane receptors responsible of *M. tuberculosis* recognition by the epithelial cells (Bermudez & Goodman, 1996). Later, Reddy and Kumar reported cell membrane projections and membrane ruffling formation during *M. avium* interaction with cells from the respiratory epithelium (Hep-2), and identified two *M. avium* proteins (31 and 25 kD), as responsible of this interaction (Reddy & Kumar, 2000). Our group reported that *M. tuberculosis* entrance into the A549 cells was carried out by an endocytic process known as macropinocytosis, which involves the participation of actin filaments and membrane ruffling formation, in this study we suggested that the viability of the mycobacteria was an indispensable requirement for membrane projections formation and consequently for bacterial entry (Garcia-Perez, et al., 2003). Later, we also reported that entrance of the low-pathogenic *M. smegmatis*, into the A549 cells was induced by macropinocytosis as well, and we suggested that this endocytic route was not a virulence factor for the mycobacteria (Garcia-Perez et al., 2008). Diverse studies have postulated macropinocytosis as the endocytic pathway responsible of pathogenic microorganisms entrance into non-phagocytic cells (Kerr et al., 2009). It is also known that the establishment of the intracellular signaling for macropinocytosis induction requires an initial stimulation with growth factors, hormones, phorbol-esters and some bacterial products (Swanson, 1989; Swanson & Watts, 1995; Patel & Galan, 2006). Some microorganisms, like *Salmonella enterica*, presents a highly specialized organelle known as secretion system type III (SSTIII), through which proteins responsible of the pathogenicity of this microorganism are transferred into the host cell (Patel & Galan, 2006). Many of these proteins act imitating functions from the host cell especially those responsible of cytoskeleton rearrangements and macropinocytosis induction. Although *M. tuberculosis* does not count with a proper SSTIII, a related system known as ESX-1, modulates the early events occurred during mycobacterium infection (DiGiuseppe Champion & Cox, 2007). Different groups of investigators have reported the production of several molecules that can mediate *M. tuberculosis* interaction with non-phagocytic cells. Arruda and coworkers reported the production of an "invasive like" protein, although they did not show conclusive results on its role in tuberculosis infection (Arruda et al., 1993). The role of the Mce1 protein (one of the members of the Mce protein family) in the entrance of *M. tuberculosis* into the non-phagocytic cells, was demonstrated by using polystyrene microspheres covered with recombinant protein, Chitale and coworkers reported that Mce1 protein was able to induce the formation of "membrane disturbances" in non-phagocytic cells (Chitale et al., 2001). Recently, another group of investigators, reported that Mce3A protein bound to latex particles facilitated uptake by HeLa cells, suggesting that this protein also has a role in *M. tuberculosis* interaction with host cell (El-Shazly et al., 2007). Also, it has been reported that Mce4A protein facilitates the invasion of HeLa cells by a non-pathogenic *E. coli* strain expressing Mce4A. Since the Mce4A protein is expressed at the late stage of mycobacterial growth, it has been postulated that this protein besides favoring *M. tuberculosis* entrance, it can play an active role in mycobacterium survival and persistence (Saini et al., 2008).

Another protein of *M. tuberculosis* involved in the interaction with the non-phagocytic cells is the heparin binding hemagglutinin (HBHA), which has been reported that induces membrane projections and bacteria internalization into Hep-2 cells (respiratory epithelium) (Reddy and Hayworth, 2002). Additionally, HBHA has been implied in extrapulmonary dissemination of *M. tuberculosis*, *hbha* gene disruption has deep effects on *M. tuberculosis* interaction with epithelial cells, but does not affect interaction with macrophages. On the other hand, the mutant strain of HBHA expressed a reduced capacity to colonize spleen, although lung colonization was not affected. These data emphasize the importance of HBHA in extrapulmonary dissemination and remarks the importance of non-phagocytic cells-*M. tuberculosis* interaction in tuberculosis pathogenesis (Pethe et al., 2001). Menozzi and coworkers reported that HBHA induce endocytosis mediated by receptor through the recognition of proteoglycans containing heparan sulphate (Menozzi et al., 2006). Like other bacterial proteins, mycobacterial HBHA can mimic the function of some cellular proteins being responsible of the modification of diverse cellular activities, including entrance, survival and pathogen dissemination. Recent studies support this theory, Verbelen and collaborators reported that HBHA has a sequence similar to proteins that bound actin, like tropomiosin-1, ezrin-1 and the heavy chain of miosina-9, and their results demonstrated the specific and stable union of HBHA to actin (Verbelen et al., 2008). Another research group showed that HBHA was able to bind to G-actin without altering their nucleation but obstructing actin polymerization; these authors suggest that like profilin, HBHA can affect polymerization-depolymerization dynamics of F-actin facilitating *M. tuberculosis* mobility into the cytoplasm (Esposito et al., 2011). This report emphasize the importance of HBHA in *M. tuberculosis* dissemination, since it has demonstrated that HBHA induces membrane protrusions formation, is possible to infer that HBHA may be one of the mycobacterial molecules that can act as "signals for internalization" hence inducing mycobacteria entrance by macropinocytosis, as previously we suggested (Garcia-Perez et al., 2008). Other molecules of *M. tuberculosis* responsible of the invasion of epithelial cells are the mycobacterial DNA-binding protein 1 (MDP1), which promotes A549 cells infection through hyaluronic acid (Aoki et al., 2004); and a group of high active bound peptides (HABPs) of different hypothetical proteins from *M. tuberculosis* (Rv2301, Rv0180c, Rv0679c, among others), which have shown that facilitates the bound and internalization of latex particles to A549 cells (Cáceres et al., 2011; Cifuentes et al., 2010; Ocampo et al., 2011).

The knowledge of the molecules that participate in *M. tuberculosis* invasion to non-phagocytic cells is still scarce, basically two surface proteins, Mce and HBHA are the most studied. The great complexity of the mycobacterium cell wall and the external lipids "wall" composition allows us to suggest that most probably not only proteins are involved in mycobacterium invasion, but also some other constituents like mycobacterium-lipids may have an important role. We described that the morphological changes and the cytoskeleton modification of the A549 cells infected by *M. tuberculosis* were observed in cells with bound or internalized bacteria, but also were observed in cells without bacteria, this made us to suggest that those changes could be induced also by secretion molecules (Garcia-Perez et al, 2003; Garcia-Perez et al., 2008). Chopra and colleagues speculated that the nucleotide diphosphate kinase (Ndk), a *M. tuberculosis* secretion protein, which acts as a GTPases protein activator may contribute to internalization of *M. tuberculosis* into the host cell by

inducing cytoskeleton reorganization (Chopra et al., 2004). Still there is a need to elucidate and explain the role of *M. tuberculosis* secretion molecules that could be related to invasion and survival of mycobacteria into the non-phagocytic cells.

M. tuberculosis entrance into the non-phagocytic cells is not a circumstantial event, it must be induce by mycobacterial product(s), the best candidates could be mycobacterial actively secreted products that could reach many cells in a short time, and these molecules can be recognized as the "first signal". This first signal initiates the intracellular signaling cascades that leads to the formation of membrane protrusions necessary to internalize the bacillus, but this event apparently is not enough to assure mycobacterial internalization. During the non-phagocytic cell infection event, only few cells will be finally infected; if we infect simultaneously, with the same mycobacterial cell suspension, during the same time, a monolayer of macrophages and a monolayer of non-phagocytic cells (for instance lung epithelial cells), up to 90% of the macrophages will be infected compared to 10% of epithelial cells (Garcia-Pérez et al., 2003). So, most probably another signal, the "second signal" is needed to assure that mycobacteria will be internalized by the non-phagocytic cell, and most probably this "second signal" will depend on the physical interaction of some bacterium molecule (PAMP) with a receptor molecule on the host cell (PRR). For the case of macrophages, already we described the variety of receptors involved in the interaction mycobacteria:phagocytic cell. With respect to the non-phagocytic cells, there are few studies of cell receptors involved in mycobacterium invasion. Given the frequency and ubiquitous distribution of glycosaminoglycans on the epithelial cell surface, it has been proposed that these molecules are good candidates for the union and penetration of pathogenic microorganisms in the tissues (Menozzi et al., 1996). The evidences indicate that cells from the respiratory epithelium express PRRs, specifically TLRs, making them able to contribute to the establishment of the pulmonary innate immune response. In the pulmonary epithelium, 11 TLRs are expressed, along with CD14, an important element for TLR4 function, these molecules have been involved in the recognition of pathogens by these epithelial cells (Bals & Hiemstra, 2004; Gribar et al., 2008). Interestingly, Lee and collaborators reported that *M. tuberculosis* actively induce Dectin-1 receptor expression by A549 cells; Dectin-1 is a classical receptor for fungi, and is expressed mainly by myeloid cells. They established that for Dectin-1 expression, the participation of TLR2 and ROS were crucial. In addition, the authors demonstrated that Dectin-1 and TLR2 were equally responsible of the early activation of Src in the epithelial cells stimulated by *M. tuberculosis*, and suggest that Dectin-1 in the epithelial cell contribute to mycobacterial survival (Lee et al., 2009) (Fig.1).

3.2 Endothelial cells infection by *M. tuberculosis*

The endothelium is composed of a single layer of thin flattened cells known as endothelial cells, that lines internal body cavities and the lumens of vessels, endothelial cells had a mesodermal origin. Endothelial cells participate in several cellular functions like vasoconstriction and vasodilation (blood pressure control), angiogenesis, inflammation, etc., so endothelial cells contribute to the whole homeostasis of the organism. In cases of bacteraemia or viraemia, endothelial cells are among the first cells to be in contact with microbial pathogens and are also among the first cells that respond to endogenous

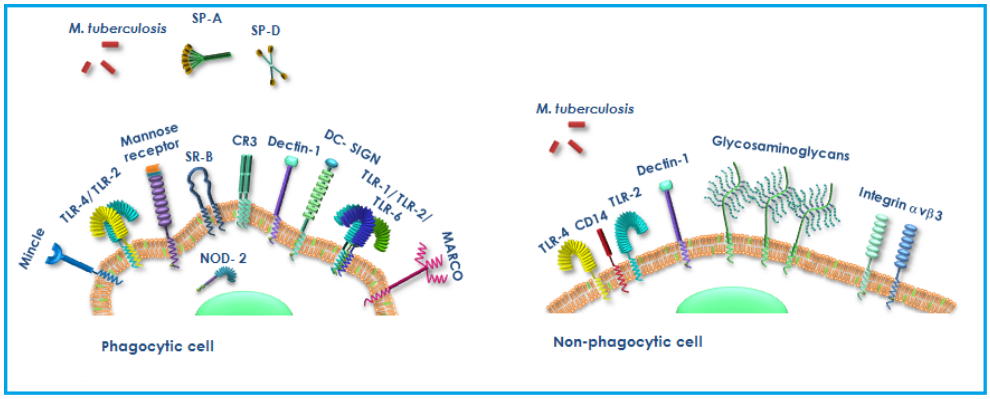

Fig. 1. Non-phagocytic and phagocytic cell receptors involved in *M. tuberculosis* recognition.

molecules released in events of tissue damage (Opitz et al., 2009). Different research groups have demonstrated *M. tuberculosis* interaction with the endothelial cells; in the first study, Birkness and coworkers used a bilayer model of epithelial and endothelial cells, and they demonstrated adhesion, internalization and passage of *M. tuberculosis* through the bilayer (Birkness et al., 1999). Later, the study of Bermudez and collaborators established that *M. tuberculosis* crosses the alveolar wall to reach the endothelial cell, hence suggesting the ability of *M. tuberculosis* to disseminate (Bermudez et al., 2002). The presence of *M. tuberculosis* DNA in lung endothelial cells of human samples from cadaveric donors from countries with high TB incidence, confirmed the infection of endothelial cells in *in vivo* situations (Hernandez-Pando et al., 2000). The first oriented study to establish some features of endothelial cell infection by *M. tuberculosis* was performed by Metha and coworkers; in this work, authors describe the interaction of *M. tuberculosis* with two endothelial cell lines, one from human lung (HULEC) and the other from human foreskin (HMEC-1). The authors determined that *M. tuberculosis* may bound, enter and survive better into the pulmonary cell line (HULEC), than in the skin cell line HMEC-1, suggesting that lung cells are a better niche for mycobacterial replication (Metha et al., 2006). Recently, our group reported the innate response of human umbilical vein endothelial cells (HUVEC) against *M. tuberculosis*, *M. abscessus* and *M. smegmatis* infection. The study demonstrated that although the three different mycobacterium species were internalized, the intracellular fate of each strain was different. The mechanism responsible of mycobacteria entrance into the endothelial cells is still unknown, but we found that during mycobacteria internalization the endothelial cells display cytoskeleton rearrangements and the magnitude of those changes was different for each mycobacterium strain, for instance, the more virulent mycobacteria induced the most dramatic rearrangements. It is necessary to determine if macropinocytosis is also the mechanisms responsible of mycobacterial uptake by endothelial cells, as it occurs for epithelial cells (Garcia-Perez et al., 2003). Contrary to other cell models, intracellular *M. tuberculosis* did not replicate, or was eliminated by HUVECs, resembling a "latency stage", that could be triggered by the high NO levels induced at early times of the infection (Garcia-Perez et al., 2011).

Up today there are not conclusive studies on the molecules responsible of *M. tuberculosis* recognition by endothelial cells, however is known that endothelial cells express a great variety of PRRs through which they interact with different pathogens (Opitz et al., 2009). The few reports on mycobacterial molecules involved in endothelial recognition indicate that phosphatidylinositide mannoside (PIMs) can act as adhesins that mediate *M. tuberculosis* bounding to non-phagocytic cells (Hoppe et al., 1997). Another study indicates that HBHA induces acting filament reorganization in a confluent endothelial cells monolayer, which suggests that this protein may also be involved in the recognition of *M. tuberculosis* by endothelial cells (Menozzi et al., 2006).

3.3 Interaction of *M. tuberculosis* with other non-phagocytic cells

M. tuberculosis interaction with other non-phagocytic cells like adipocytes or fibroblasts has been reported, although few reports exist on the matter. A recent study demonstrated that adipocytes are susceptible to *M. tuberculosis* invasion but in these cells, *M. tuberculosis* persists in a non-replicative state. Mycobacterial recognition by adipocytes was mediated by scavenger receptors. The authors suggest that due to the abundance and wide distribution of fatty tissues in the human body, adipocytes may represent an ideal reservoir for persistent tuberculosis bacilli (Neyrolles et al., 2006).

Fibroblasts and epithelial cells constitute two of the main structural cell lineages of the lung, in tuberculosis, fibroblasts are present in the periphery of granulomas; they synthesize extracellular matrix proteins that are required for the maintenance and repairing of the lung; in addition they produce cytokines and chemokines that allow leukocyte recruitment and activation (Suzuki et al., 2008). In spite of the physiological relevance of the fibroblasts, its interaction with pathogens, specifically with *M. tuberculosis* has been scarcely explored. The first study of the matter, performed by Rastogi and coworkers, demonstrated that *M. tuberculosis* and *M. avium* can infect a murine fibroblast cell line, and in response to the infection, fibroblasts secreted several chemical mediators that promote mycobacterium elimination by macrophages (Rastogi et al., 1992). Up today, two additional studies demonstrated the role of the fibroblasts in the mycobacterial infection. In the first study, Hernandez-Pando and coworkers reported the presence of mycobacterial DNA in lung fibroblasts (among other cells) from cadaveric donors that die for causes different to tuberculosis; these individuals lived in countries with high tuberculosis prevalence. These data suggest that a previous *in vivo M. tuberculosis* infection was established and controlled in those individuals and reveal the importance of lung fibroblast in the response (Hernandez-Pando et al., 2000). The second study confirms that fibroblast are susceptible of *M. tuberculosis* infection; and describes the multiplication features of several strains and mutants of *M. tuberculosis* into human lung fibroblast and rat lung fibroblasts, although they found that human fibroblast are more permissive to mycobacterial growth. Contrary to several studies with other cell lineages like epithelial or endothelial cells, these authors report that in fibroblast, *M. tuberculosis* internalization is actin and tubulin independent, and conclude that mycobacteria do no requires cell host cytoskeleton reorganization to be internalized (Ferrer et al., 2009). Preliminary results of our group demonstrate that infection of mouse lung fibroblasts (MLg cell line) by *M. tuberculosis* induce actin mobilization and membrane ruffling contrary to those observations described by Ferrer and coworkers (Fig. 2). The discrepancy in these results forces to make new studies to clarify and precise the

mechanism responsible of *M. tuberculosis* internalization into fibroblasts, and to determine the mycobacterial molecules and the cell receptors involved in the recognition and triggering of this event. In addition, it is necessary determine the defense mechanisms elicited by the fibroblasts to contain the mycobacterial infections.

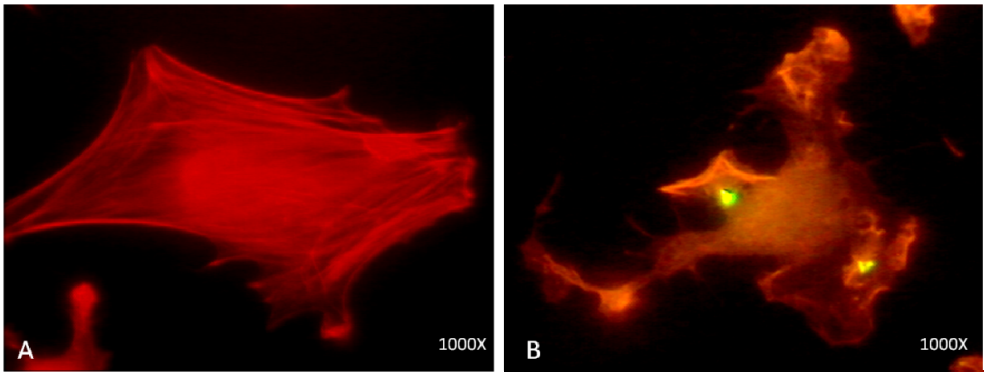

Fig. 2. Interaction of *M. tuberculosis* with fibroblasts. Cells were infected during 1 h with *M. tuberculosis*. Before the infection, bacilli were stained with fluorescein isothiocyanate (FITC, green color). After infection, actin filaments were labeled with rhodamine-phalloidin (red). A) Uninfected cell showing actin filaments longitudinally distributed. B) Infected cell showing membrane protrusions and membrane ruffling in contact with mycobacteria.

4. Innate immune response of non-phagocytic infected by *M. tuberculosis*

Phagocytic cells like macrophages, neutrophils and monocytes have a crucial role in the defense against infections; they are responsible of bacterial phagocytosis and their elimination. They count with well adapted systems for bacterial recognition and exhibit sophisticate and highly effective mechanisms to eliminate the pathogenic microorganisms. Production of reactive oxygen and reactive nitrogen species, cytokines and chemokines production, degradative enzymes production, and antimicrobial peptide production are some of the elements displayed by the phagocytic cells to contain and kill pathogens. On the other hand, pathogenic microorganisms have developed numerous mechanisms to evade the antimicrobial arsenal and to guarantee their survival and persistence. In particular, *M. tuberculosis* has developed several mechanisms to avoid the hostile environment of the macrophages and to persist within them: a) inhibition of the phago-lysosome fusion, b) inhibition of the phago-lysosome acidification, c) recruitment and retention of TACO protein (tryptophan- aspartate containing coat protein) on phagosomes to prevent fusion with lysosome and d) expression of proteins and lipids for protection against the oxidative stress (Meena & Rajni, 2010). Also the ability of mycobacterium to enter into non-phagocytic cells is another mechanism of immunological evasion, since mycobacteria "hided" in these cells could skip hostile environment of the macrophages and create an ideal niche for its replication and establishment. Nevertheless, recent evidences suggest that non-phagocytic cells have an important role in the immune response against mycobacteria and these cells have the potentially to exert potent antimycobacterial mechanisms (Garcia-Perez et al., 2011; Kwon et al., 1998; Sharma et al., 2007). Besides, we demonstrated that the intracellular

destiny of *M. tuberculosis* will be dictated by the cell type infected, hence while in epithelial cells *M. tuberculosis* survives and replicates, in the endothelial cells tends to persist without showing replication (Garcia-Perez et al., 2011). We consider that non-phagocytic cells can actively respond to *M. tuberculosis* infection, but the magnitude and diversity of their response can be modulated by the bacillus to guarantee its survival, persistence and/or dissemination. Kwon and George demonstrated that epithelial cells are able to produce nitric oxide (NO) in response to cytokines like IL-1 and IFN-γ (Kwon & George, 1999) and also in response to *M. tuberculosis* (Kwon et al., 1998). Later, Roy and collaborators established that direct infection of epithelial cells by *M. tuberculosis*, stimulates *the novo* production of NO via iNOS, although they demonstrated that the low levels of NO produced during the direct-mycobacterial stimulation, were not sufficient to kill the bacilli, however NO production by cells infected and simultaneously stimulated with cytokines was sufficient to kill the mycobacteria and to reduce the bacterial load (Roy et al., 2004). We reported that endothelial cells produce NO in response to mycobacterial infection and we found that NO concentrations correlated with the capacity of infective bacteria to induce modifications in the actin cytoskeleton. We demonstrated that a high NO production does not correlate with the diminution of the bacterial load, on the contrary, the mycobacterium with the greater intracellular replication (*M. abscessus*), induced the greater amount of NO. In comparison, the mycobacterium that induced the lower NO levels was eliminated (*M. smegmatis*), but induced the higher ROS production, which allowed us to suggest that ROS have a more important role for mycobacterial elimination than NO. Interestingly, in the endothelial cells, *M. tuberculosis* exhibited a non-replicative state, resembling the latency state characteristic of tuberculosis. In these cells, *M. tuberculosis* triggered intermediate levels of NO compared with the other two mycobacteria, but at very early stages of infection, a "burst of NO" production was observed, and a rapid diminution of this metabolite proceed to this event (Garcia-Perez et al., 2011). Based on these results, we can suggest that the ability of the mycobacteria to modulate NO production by the endothelial cells will determine, in great extent, its final intracellular fate.

The importance of ROS in the innate immunity was recognized initially in the phagocytic cells, which under certain stimuli generate the "respiratory burst". ROS production is related to the activity of the NADPH oxidases, although ROS may be produced by other mechanisms (West et al., 2011). In the phagocytic cells, the NADPH oxidase responsible of ROS production is NOX2. In recent years, 7 isoforms of NOX/DUOX enzymes have been described, and their distribution and function in different cells or tissues is been recognized (Krause, 2004). ROS have an ample variety of biological activities, participating not only as antimicrobial agent (which is one of the least sophisticated activities). ROS participate in hormone biosynthesis, intracellular signaling, blood pressure control, etc. (Krause, 2004). At the moment it has been recognized that some non-phagocytic cells express some members of this oxidase family; one of the most studied cells are the pneumocytes type II A549 cells, which has been reported that express NOX1, NOX2, DUOX 1 and DUOX2 (Kolářová et al., 2010), and although in these cells the role of ROS for pathogens elimination has not been studied, there are reports that indicate the importance of ROS generation in the modulation of the immune response. In the infection of epithelial cells by *M. tuberculosis*, Lee and collaborators reported that ROS are produced via the activation of Src, after Dectin-1 engagement, Dectin-1 is a crucial receptor that contributes to mycobacterium

internalization, the expression of pro-inflammatory mediators and the establishment of an effective antimicrobial environment, constituted in part by ROS (Lee et al., 2009). In the endothelial cells, Van Buul and coworkers, reported the differential expression and location of NOX2 and NOX4, whereas NOX4 was located in the endoplasmic reticulum, the regulating proteins of NOX2 were located and associated to actin present at membrane protrusions and ruffles; in this work the authors correlated ROS scavenging activity with an impair of cytoskeleton rearrangement and the formation of confluent monolayers (Van Buul et al., 2005). We reported that endothelial cells produce ROS in response to the mycobacterium infection, and we correlated a high ROS production with mycobacterium elimination (Garcia-Perez et al., 2011). The role of ROS as direct *M. tuberculosis* effectors is still controversial, however ROS indirectly, may contribute to *M. tuberculosis* elimination, for example the signaling route dependent of ROS and NOX2, is crucial for the achievement of the antimycobacterial effect of cathelicidin, an antimicrobial peptide induced by vitamin D3 in macrophages (Yang et al., 2009).

The participation of antimicrobial peptides or defensins in *M. tuberculosis* infection is a current issue. Defensin production by some non-phagocytic cells infected with mycobacteria has been reported; Rivas-Santiago and collaborators, found that *in vitro* infection of pneumocytes (A549 cells) by *M. tuberculosis*, induced human β-defensin 2 (HBD-2) production, immunolocalization with colloidal gold demonstrated HBD-2 associated to damaged bacillus, suggesting the antimicrobial effect of this peptide (Rivas-Santiago et al., 2005). Later, the same group reported the expression and production of another antimicrobial peptide cathelicidin LL37, by epithelial cells infected by *M. tuberculosis* (Rivas-Santiago et al., 2008). In a mouse model of chronic pulmonary tuberculosis and latent tuberculosis, defensin expression was analyzed; the results demonstrated that β-defensin-3 (MBD-3), β-defensin-4 (MBD4) and CRAMP (the mouse equivalent of the cathelicidin LL37) were expressed by different cell types including lung epithelial cells. In the progressive tuberculosis model, initial production of MBD3 and MBD4 correlated with the transitory control of mycobacterial growth, although their expression diminished at the late stage of the disease. In the latent infection, MBD3 and MBD4 were expressed continuously, but after disease reactivation, their production was abrogated. CRAMP expression was high in the progressive phase of the disease and the protein was observed in different structures of the mycobacteria. The authors of these studies conclude that both defensins and cathelicidin participate in mycobacterial containment, not only by the direct activity against the bacteria, but by their recognized chemotactic activity (Rivas-Santiago et al., 2006; Castañeda-Delgado et al., 2010). Another antimicrobial peptide involved in the defense against *M. tuberculosis* is hepcidin. Recently, the production of this antimicrobial peptide by the epithelial cells stimulated with ManLAM and PIMs from *M. tuberculosis* was reported, and proposed that the antimicrobial activity was mediated by a reduction in the iron available at the alveolar space (Sow et al., 2011). We recently reported that *M. tuberculosis* infection of endothelial cells stimulated HBD-1 production, and we proposed that this defensin could have an important role in the mycobacterial growth control observed in this model (Garcia-Perez et al., 2011). The direct antimycobacterial role of HBD1 is not determined so far.

One of the actual hypotheses is that the environment surrounding the non-phagocytic cells is crucial for their activation and preparation for bacterial elimination. Greco and collaborators reported that the enriched lysophospholipid pulmonary microenvironment

protects pneumocytes type II from cytotoxicity induced by *M. tuberculosis,* and increases the antimycobacterium response by promoting acidification of endosomal compartments (Greco et al., 2010). Desvignes and Ernst demonstrated that epithelial and endothelial cells after IFN-γ stimulation express a better response for *M. tuberculosis* growth control. These authors established that one of the mechanisms involved in the response induced by IFN-γ is expression of indoleamine-2,3-dioxygenase (IDO) and regulation of the expression of IL-17 (Desvignes & Ernst, 2009). The capacity of epithelial cells to improve their response after IFN-γ exposure is supported by the observation that cells infected by *M. tuberculosis* express elevated levels of the receptor specific for this cytokine (Sharma et al., 2007).

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Epithelioid Cell: A New Opinion on Its Nature, Parentage, Histogenesis, Cytomorphogenesis, Morphofunctional Potency, Role in Pathogenesis and Morphogenesis of Tuberculous Process

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1. Introduction

It is known that during an embryogenesis form a well-defined for each type of organism the number of cell types, each of which has its own specific morphophysiological characteristics. Some cells operate only at certain stages of embryonic development and then disappear as a result of apoptotic death, while others are unique for adult organism. However, in researching of granulomas formed in some granulomatous diseases have been described cells are not found in a healthy organism. To such cells referred «basophilic hystiocytes» in rheumatic disease, Mikulich cells in scleroma, epithelioid cells (ECs), formating ECs granulomas in a number of infectious, including tuberculosis, as well as some allergic and autoimmune diseases. It was shown that ECs forming in nidus of the inflammation in granulomatousis diseases of different etiology. Suppose that ECs do not enter number of differentiated cell-like types neither embryonic not adult organism; they occur only at particular pathological statuses and forming ECs-granulomas. This granulomas determine clinicomorphologic essence of many granulomatousis diseases in man. Moreover, the ECs-granulomas form in different groups animals, relating to different branches of "phylogenetic tree". Thus, ECs formation in the nidus of inflammation can be related to one of most ancient mechanisms of cell-like response on imbalance of the "antigenic-structural" homeostasis in organism. The concept of ECs origin from cells of macrophages (Mphs) family till now is considered conventional, which some theoretical fundamentals were placed in workers of Ashoff L. (1924) and Maksimov A. (1926). Affirms that ECs transform from Mphs located in the nidus where the pathological process flows past and under some conditions - directly from monocytes of a blood. This concept is based on the hypothesis that in a basis of the differentiation resulting in to derivation ECs from Mphs in reply to particular pathogenic stimulus lie the changes of genic activity and that in a basis of this transformation resulting in to formation ECs lie epigenetic changes, and the phenomenon can be considered as “intra-tissue transdetermination” (Shvemberger, 1976). Important thus to mark that till now is not obtained sufficiently convince facts touching not only the

mechanisms of transformation of Mphs into ECs, but also process of series transformation of Mphs into ECs. It is explained to that indicated the concept and hypothesis based mainly on the results of classic morphological researches, in which as well as in many modern morphological works, registered only the fact of appearance of ECs in populations of cells of macrophage type without the analysis of the transition forms from Mphs to ECs. It is necessary thus to underline that in none of works dedicated search of ECs metastructure, is not obtained of enough convincing and indisputable evidences of existence of the legible transition forms between Mphs and ECs. Moreover, there are no convincing facts which would testify that differentiated Mphs can undergo dedifferentiation that is switch on in the process being a basis of possible conversion cell-like phenotype. At usage of cell-like technologies in learning ECs cytomorphogenesis was obtained the in essence new facts, which have forced to refuse the concept of origin of ECs from Mphs (Arkhipov, 1995). The application of different cell-like technologies (cultivation in vitro, explantation of cells of granulomas in cultures etc.) allows to place that among peritoneal cells (PCs), mononuclear blood cells and bone marrow exist low-differentiated cells - ECs-precursors (pre-ECs), distinguishing from cells of macrophage series on number of cytomorphologic identifier, registered in vitro. Obtained data allow to confirm the hypothesis that exist unipotent precursors ECs (pre-pre-ECs), which differentiate only into ECs at defined conditions combined in the nidus of chronic inflammation (Arkhipov, 1996). All stages of differentiation of pre-ECs into mature cells of epithelioid type possessing about proliferative activity are defined. On the basis of the obtained data lay down the new conception of origin and differentiation of ECs (Arkhipov, 1997). Is was shown that of ECs-germ forming in norm quantitatively restricted population of low-differentiated monocytoid blood cells being committed cells precursors of ECs. In cronic inflammation the pool of pre-ECs in organism increases. By cytomorphologic characteristics pre-ECs were referred to the class of reticular cells. To the present time obtained the new experiment data indicating the existence a genetic determinancy of a datum basal level ECs reactivity concerning different inductors of an inflammation. Set a question on correlation between function and phenotypic variation of ECs. Data obtained directing that the morphogenesis of ECs granulomas might determine by the several factors: initial genetically determinate level of a pool pre-pre-ECs, inflow pre-ECs, committed into ECs, trended to differentiation in the nidus of inflammation, and also intensity of processes of their proliferation and differentiation. The data obtained allow in a new fashion to formulate a hypothesis about a probable origin and early stages of a histogenesis ECs, namely that ECs might the descendants of mesenchymal stem cells of a bone marrow parentage, out of which differentiate some stromal cells of organism. The clearing up of early stages of ECs histogenesis will allow to answer the question not only about biological essence of ECs formating in different chronic granulomatous processes, including tuberculosis, but also more precisely to spot their function assignment in an organism in pathology.

2. The morphofunctional characteristics of epithelioid cells, generated in the foci of granulomatous inflammation in different granulomatous diseases, including tuberculosis

Morphological manifestation of many granulomatous diseases is forming epithelioid-cell-like granulomas (Alamelu, 2004; Epstein, 1991; Kojima, 1996; Shkurupy, 2007; Strukov & Kaufman, 1989). ECs will be derivated in the centers of a chronic inflammation in

granulomatous diseases of different etiology. ECs-granulomas or the separate clumps of ECs will be derivated in many infectious diseases, including in tuberculosis (Malik et al., 1999; Russell et al.,2009; Shkurupy, 2007), some rheumatic (Chadarevian et al., 1993), autoimmune (Ren, 1992), lymphoproliferative diseases (Takeshita et al., 1993), histiocytoses (Goerdt et al., 1993), in development of tumors of a different histogenesis (McCartney, 1995), granulomatous diseases of a unknown etiology (Kaneishi et al., 1995; Tozman, 1991), hit in an organism of salts of some metals (Haley et al., 1994; Kelly, 1993), foreign bodies (McCarthy et al., 1993), allergens of a different nature (Yamanaka et al., 1994). The issue of granulomatous process largely depends on dynamics of epithelioid-cell-like cytomorphosis in the centers of an inflammation that apparently is specified by morphofunctional features of ECs, permitting by it to fulfill simultaneously functions of boundary conditions (Hasegawa et al., 1994; Noga et al., 1989), exocellular damage of pathogen agents by products of secretion (Baba et al., 1992; Myatt et al., 1994; Tanaka et al., 1996), modulation of Mphs function activity (Mariano, 1995; Miyazaki et al., 1992; Shigenaga et al., 1995), lymphocytes (Abe et al., 1990; Chensue et al., 1992) and fibroblasts (Allen, 1991; Limper et al., 1994). Now is generally acknowledged that ECs are transformed from Mphs in an organ or tissue, where pathological granulomatous process is developed (Dastur et al., 1995; Moraes & Moraes, 1993; Russell, 2009; Takahashi et al., 1994), and under certain conditions - from monocytes of a blood (Facchetti et al., 1989; Williams & Williams, 1983). When is spoken about transformation of monocytes into ECs it is supposed that the monocytes have equal potencies to differentiation into Mphs and ECs (De Vos et al., 1990; Kowalewsky, 1976; Noble et al., 1989). In the concept of a parentage of ECs from Mphs to different subpopulations of lymphocytes tapped the role of controllers of transformation Mphs into ECs, and Mphs appear in a role of acceptors of differentiating signals of lymphocytes (Haley et al., 1994; Horvath et al., 1993). Thus, the accumulation ECs in the center of granulomatous inflammation is reduced to stochastic (on probability of an induction) and continuous transformation Mphs into ECs (Haley et al., 1994; Okabe, 1994). Three types of ECs are now defined: plasmacyte-like, vesicula-like and fibroblast-like (Epstein, 1991; Horiuchi & Masuzawa, 1995; Rasmussen & Petersen, 1993). T. Williams and W. Williams (1983) distinguished two types of ECs: plasmacyte-like, which on structure of a nuclei and development of a granular cytoplasmic reticulum remind plasma cells or plasmacyte (type A), and vesicula-like (type B). Under the data of these researchers ECs such as type A more often meet at early stages of granulomagenesis, ECs such as type B - in more late period. Under the data of Shkurupy and colleagues (Shkurupy et al., 1993), on the contrary, the cells such as type B meet at early stages of generation of ECs-like granulomas. Consider that features of structures of vesicula-like ECs testifies to an expressiveness of their synthetic and secretory function (Shkurupy, 2007; Turk, 1989). It is fixed that the vesicles of ECs advance to cell plasmalemma with the subsequent exhaust of their contents on exocellular medium (Baba et al., 1992). It is exhibited that bactericidal and also the secretory activity in ECs-granulomas is more expressed than in mature macrophage granulomas (Abe et al., 1990; Tanaka et al., 1996). In ECs in the field of Golgi lamellar complex are taped not only zonated, but also sleek vesicles with dense center, and also great many (more than 100) large granulas with diameters up to 340 nm and with finegranular matrix more light than in macrophage granulas, sometimes with perigranular halo (Rhee et al., 1979; Samtsov & Shiliaeva, 1990). The number of lysosomes in ECs enlarged in comparison with their number

in monocytes and Mphs. Some types of such structures are detected: 1) homogeneous lysosomal; 2) with myelinic bodies; 3) having frame multivesicular bodies; 4) with major irregular crystalline inserts. The cell-like center in the majority of ECs more volumetric that than in mature Mphs is marked. Thus, ascending density of filaments in cytoplasm, which have radial orientation and diameter 5-6 nm or about 10 nm is revealed (Epstein, 1991; Horiuchi & Masuzawa, 1995; Turk, 1989). Under the data Rhee (1979) secondary lysosomes and macrophage granula in such cells are not taped. In ECs such as type B the specific composition of enzymes (acidic phosphatase, β-galactosidase, nonspecific esterase, peroxidase, lysozyme, angiotensin converting enzyme), and also factors affecting the activity of fibroblasts and them collagengenerating function are detected (Allen, 1991; Inuzuka et al., 1994; Limper et al., 1994; Miyazaki et al., 1992; Turk, 1980). It is fixed that the angiotensin converting enzyme can brake migration Mphs, that is play a role of the factor inhibiting migration of Mphs, that is important for formation of cell-like aggregate in granulomagenerating process (Mariano, 1995; Williams & Williams, 1983;). At usage enzyme-linked immunoassay methods it is exhibited that ECs produce IL-1, IL-2, IL-4, IL-6, TNF-α (tumors necrosis factor), multifunctional growth factor TGF-β (Limper et al., 1994; Myatt et al., 1994; Toossi et al., 1995). These data specify that ECs play important regulatory function in formation of granulomas and in pathogenesis of granulomatous diseases. In a sarcoidosis on a surface of ECs happens expression HLA-DR (Ia) and HLA-DQ of antigenes (Hoffmann-Fezer et al., 1992). Apparently, it promotes immunological interaction of these cells with T-lymphocytes. The majority of investigators registered that ECs have lower phagocytic activity in comparison with Mphs. In cytoplasm of ECs the lipids, which are surveyed as oddments of the killed and digested micro-organisms, also crystalloid frames, asteroid bodies, formation such as phagosomas and multivesicular bodies, are detected (Horiuchi & Masuzawa, 1995; Navarro et al., 1992). It is necessary to underline that there are contradictory enough data about ability of ECs to phagocytic response. One investigators register absence of phagocytosis for ECs (Momotani et al., 1993; Velge et al., 1994) and other specify existence weak phagocytic activity of ECs, and also on boundedness of a population of ECs, in which the objects of phagocytosis are registered (Desportes-Livage et al., 1996; Hoop et al., 1994). At the same time bactericidal and secretory activity of ECs is expressed more strongly, than for Mphs (Kumar et al., 1989; Turk, 1980; 1989). The inconsistency of the data about ability ECs to an phagocytosis is to some extent explained by the concept "divergent differentiation" of monocytic blood cells, migrating to the center of inflammation, caused by microorganisms. According to this concept it is supposed that one of monocytes specialize on an phagocytosis and other - losing phagocytic potency, only pinocyte particular yields of decay (cytophagous material), and are transformed into ECs (Kowalewsky, 1976). Under the data Baba and colleagues the organizations cytoskeleton of ECs in the center of an inflammation more compatible to cytoskeleton characteristic of epithelial cell than to cytoskeleton of active and movable Mphs (Baba et al.,1992). The three-dimensional metastructure of ECs in usage of methods of prompt freezing, penetrating etching and freeze-substitution was studied (QF-FS-method). The granulomas were caused in rats by injections of muramyldipeptid. It is exhibited that the dense webs of intermediate filaments, bound with cores, mitochondrions and other organellas, are supervised everywhere in cytoplasm of ECs. Some fascicles of actinic filaments were posed in filopodiums below than membranes of the cells. Exact interdigital triping of membranes of

cells between interfacing ECs were clearly demonstrated by QF-FS- method. The so-called coated pits (zonated fossas) in a basis of interdigital filopodiums are identified. The characteristic indication of ECs is their aggregation with formation tight interdigital tripings as a fastener "lightning", which, apparently, can have the important value in differentiation of these cells (Baba et al., 1992; Noga et al., 1989). It is marked that on early terms of generating of granulomas the indicated frames miss. Probably, they ensure particular “localizing” and biochemical barrier for different pathogen agents, and also fastness in relation to proteolytic enzymes. At the present time the monoclonal antibodies IHY-1, which react only with ECs in sarcoid granulomas, are obtained (Ishioka et al., 1990; Ishioka & Yamakido, 1990). These antibodies did not react with erythrocytes, lymphocytes, monocytes, alveolar Mphs, and also with macrophage derivatives - cells of cultures U-973 and KG-1. These monoclonal antibodies also reacted with ECs in granulomas of lymphatic clusters in tuberculous persons (Ishioka et al., 1990). Under the data of the different contributors already it is possible to present immunological phenotype of mature ECs. In the whole series of different operations by the immunological methods identified following antigenic markers on cells with morphological phenotype ECs: RFD-9, IHY-1, CD1, CD4, CD11, CD14, CD25, CD31, CD36, HLA-DR, HLA-DQ, OKDR, MAC-387, OM2, 25F9, KiM1P, ICAM-1 (a5, b2), LFA-3 (Boehncke et al., 1993; Cerio et al., 1990; Hoffmann-Fezer et al., 1992; Ruco et al., 1992; Spiteri et al., 1989). However, now from known markers only antibodies named RFD-9 (Munro et al., 1987) and IHY-1 (Ishioka et al., 1990) allow precisely enough to differentiate ECs from other classes of cells. The functions of proteins, to which the indicated antibodies yet are bound did not fix. It should also be noted that many data obtained by different researchers, should be treated fairly critically and carefully, because the first thing necessary for the immunological identification of cells - is an accurate identification of morphofunctional types of cells. At the same time in many operations dedicated an indicated problem, will be utillized only a few morphological, is legible not of particular tests of identification of ECs, which are very subjective and can give errors at definition phenotype of ECs. Moreover, there are some terminological indeterminacies. For example, often use the term "epithelioid Mphs", «epithelioid histiocytes" (Aguiar-Passeti et al., 1997; Orrell et al., 1992; Westwood et al., 1995). At the same time morphologicaly is not improved, what cells are available in view of typical ECs or activated Mphs, which having any indications that are the characteristic for ECs. It is necessary still pay attention and to that fact that many cell-like structures or products (antigenes, receptors, cytokins) already are detected, which are characteristic not only for ECs or Mphs, but also for cells of other types, for example, dendritic cells, lymphocytes, cells of an endothelium and even of erythrocytes. So, for example, antigenes HLA-DR (Ia) and the molecules of a cell-like adhesion (ICAM-1) are detected not only on Mphs and ECs, but also in cells of an endothelium, dendritic cells, and also interdigitate reticular cells of lymphoid organs and tissues (Giotaki et al., 1992). The protein S100 is detected in ECs, Mphs, dendritic cells, cells of an endothelium, in all types of a macroglia, Schwann cells, and also in small amount in neurones, adipocytes, chondrocytes and lymphocytes (Hachitanda et al., 1990; Momotani et al., 1990; Momotani et al., 1993). Differentiation antigene CD68, characteristic for Mphs, were determined not only in ECs, but also in lymphoblastic leukosis cells, and also in spindle-shaped tumoral cells, which are included in cell-like composition of a fibroxanthoma (Horny et al., 1993; Longacre et al., 1993; Kodelja & Goerdt, 1994). The antigene CD11c is taped on cytoplasmic membranes of

ECs, Mphs and polymorphonuclear leucocytes. Angiotensin converting enzyme is present in Mphs, ECs and cells of an endothelium (Abe et al., 1990; Allen, 1991; Inuzuka et al., 1994), collagenase - in Mphs, ECs, huge multinuclear cells, endothelial cells and fibroblasts (Santavirta et al., 1993). The antigene CD25 is detected on ECs, activated T-lymphocytes and NK-cells; CD1 - on ECs, thymocytes and arborescent cells of a skin (Langergans cells); CR3 - on Mphs, ECs, T-lymphocytes, NK-cells and granulocytes (Horny et al., 1993; Kodelja & Goerdt, 1994). Thus, many facts obtained by the different researchers in study the morphofunctional characteristics of ECs, are contradictory enough and are not completely stacked in the concept of a parentage ECs from Mphs.

3. Criticism of the modern concept of origin of epithelioid cells from macrophages and other cells of the system of mononuclear phagocytes

It is now considered conventional representations that the different forms or types of ECs are derivative of Mphs and will be derivated in the center of granulomatous inflammation, for example in tuberculosis, as a result of transformation Mphs or monocytes (precursors of Mphs) into ECs [Cipriano et al., 2003; Turner et al., 2003]. This concept was formed many decades back on the basis of numerous morphological examinations of tubercular process, and also other granulomatous diseases of infectious and noninfectious etiology. The basis of this concept is that fact that the formation of ECs happens among Mphs in formating granulomas. Thus in generating ECs-like granulomas, for example in tuberculosis, the ascending of amount of ECs in granulomas interface to relative decrease in them Mphs. Thus, was quite logical to assume that ECs will be derivated from Mphs. The analysis of the literature, in which are mentioned ECs, generatored in granulomas in tuberculosis or in granulomatous processes of other infectious etiology, testifies that till now was not conducted of any examinations, in which the convincing data confirming the fact of transformation Mphs into ECs would represent. The majority of the modern contributors, surveying problems touching ECs, and speaking about a parentage of ECs, is written with such phrases: «as is known ECs will be derivated from Mphs», «it is considered that ECs are derivative of Mphs and monocytes». However, at the best they refer to any old operations, in which was not given the convincing proofs indicative of transformation Mphs into ECs. In the scientific work of Sutton and Weiss (1966) was exhibited that in culture of monocytes of a blood of a chicken two types of cells - Mphs and ECs are formed (Sutton & Weiss,1966). However, not any proofs of transformation Mphs into ECs then are represented. The similar data were obtained also by other investigators in other pilot models indicative of that in cultures of monocytes of a blood can be formed Mphs, ECs, and also huge multinuclear cells of two types - "foreign bodies" and cells, similar Langhans cells in tubercular granulomatous inflammation (Levis, 1925; Levis & Levis, 1926; Nakagavara et al, 1981; Sutton, 1967; Zuckerman et al., 1979). However, and in these works the speech went formation of the different cell-like forms in culture of monocytes, the heterogeneity of which cell-like composition now does not call doubts. In the scientific work of Pulford and Souhami (1980) was exhibited that in cultures of cells enriched with Kupffer cells, the formation ECs is recorded (Pulford & Souhami, 1980). However, quality of a fractionating and identification of Kupffer cells, having been available in the 80-s years, does not allow to speak that among explanted in cultures of the Kupffer cells there were also other undifferentiated types of cells. In the review Spector and Lykke (1983) have written that «mononuclear phagocyte origin is not in doubt, but there remains controversy over the

mechanisms by which epithelioid cells are formed, and in particular the role of cell-mediated immunity» (Spector & Lykke, 1983). At the same time they refer to series of operations (Adams, 1974; Turk & Narayan, 1982), in which be not represented of the data proving a position about an opportunity of transformation Mphs into ECs. In the scientific work Rhee et al. (1979) an issue about differentiation of monocytes in Mphs, and then in ECs and multinuclear cells, is regarded. However, of any direct evidences indicative about differentiation Mphs into ECs, is not resulted (Rhee et al., 1979). Only some cytomorphologic and cytochemical features of monocytes, Mphs and ECs, are surveyed, which do not allow uniquely to conclude that ECs will be derivated from Mphs, instead of from any variety of a monocyte or monocytoid form of cells. But they links to other contributors (Adams, 1974; Papadimitriou and Spector, 1971), in which operations nor represented one valued and stipulated facts proving that ECs will be derivated as a result of transformation Mphs. In the scientific work Mariano et al. (2003) the data indicative that peritoneal Mphs, cultured in medium, containing heightened concentration IL-4, gain in their judgement, some morphological features of ECs, characteristic for them in culture, are obtained (Mariano et al., 2003). However, on our view, introduced in this operation of a photo and other data, testify only about major phenotypic variability of Mphs, as in operations of other writers this phenomenon was scored earlier. In the scientific work Stanton et al. (2003) the attempt was made to construct the scheme of differentiation ECs from resident Mphs in lung in tuberculosis at usage of an estimation of several enzymes in cells, including angiotensin converting enzyme (Stanton et al.,2003). However, as writers of this operation, obtained by them, the data recognize do not allow to solve the problem whence take Mphs - precursors ECs. In the logic build-ups they based on a parentage ECs from cells of system of mononuclear phagocytes. Thus, it is possible to make the inference that the concept of a parentage ECs from Mphs and monocytes, as precursors of Mphs, was formed still in 60-80-th years of the last century under effect of operations, dedicated effects of transformation of monocytes into Mphs, ECs and huge multinuclear cells in vitro, started Maximow (1925), Levis (1925), and also other "«more late" classical morphological examinations of granulomatous inflammatory processes (Levis,1925; Maximow,1925). Actually this concept is grounded on a position about existence among “monocytes" only one populations of cells - precursors of Mphs. This circumstance requires treating a problem about a parentage ECs from modern positions. If ECs are differentiated from Mphs, why nobody has described of the legible transition shapes of these cells? If ECs gain any new functions, how there is their becoming? If ECs are differentiated from Mphs or monocytes, whether that can on any measure be spotted phenotype of Mphs or monocytes, which become on a trajectory differentiation in ECs? As the answers to these problems in the scientific literature is not present, it was represented very important and interesting to receive detailed exposition of all stages differentiation of ECs from Mphs in vitro.

4. The results of own experimental researches, indicative that epithelioid cells will be derivated not from macrophages and monocytes (the precursor of macrophages), but from monocytoid cells, committed in a epithelioid-cell-like direction of differentiation

In the series of operations it is exhibited that in cultures of tissues and cells, containing Mphs or monocytes, ECs are taped 2-3 weeks after beginning of cultivation (Arai et al., 1999;

Kodelja & Goerdt, 1994; Rhee et al., 1979). We originally attempted to reveal by morphological criteria a subpopulation of Mphs, possessing particular potencies to differentiation into ECs. It is fixed that for 7 days of cultivating in cultures of PCs of intact mice line BALB/c there are large cells of an epithelioid type distinguished from cells of macrophage type on a lot of morphological indications. In standard colouring by azure-eosine the cytoplasm of these cells gained an acyanotic grey - blue tone, and the nuclei (as against macrophage cells of a type with intensive colouring of nuclei) in light pink colour and had a chromatin with the weakly expressed reticulate structure were coloured. These cells were also expressed by major sizes of nuclei of the oval shape and cytoplasm, which contours, as a rule, gained the polygon shape, and also major nucleolus. On the first investigation phase it was necessary to answer one more question. Whether it is possible to survey cells of an epithelioid type formatived in cultures of PCs of mice, as typical ECs, formatived in the centers of chronic granulomatous inflammation in vivo? It is known that the hypodermic introduction of a Freund's complete adjuvant results in development of granulomas, containing in the composition the ECs. It is known also that the intraperitoneal or intravenous introduction of mycobacteria of BCG vaccine can result in inductions chronic granulomatous inflammation with generating of ECs-like granulomas in a liver (and other organs) of mice (Orrell et al., 1992; Shkurupy, 2007). Thus, it is possible to survey vaccine BCG and Freund's complete adjuvant, containing in the composition heat-killed micobacteria of a tuberculosis, as particular inductors epithelioid-cell-like forming in the center of granulomatous inflammation. Starting from these positions, it was possible to

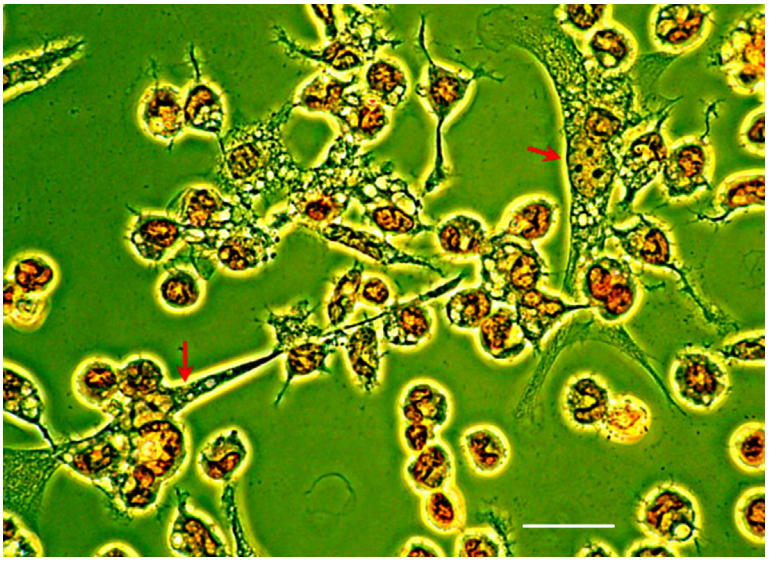

Fig. 1. Culture of PCs of mice of line BALB/c, beforehand stimulated in vivo (7 days prior to an explantation of cells) by Freund's complete adjuvant (intraperitoneal introduction 50 % of emulsion in physiological saline solution), 7 days cultivated. Plasmacytoid EC (right) and fibroblast-like ECs (left) - are marked by arrows. In Mphs and ECs the builders of an adjuvant are visible on the basis of a vaseline oil. A method of colour interferential contrast (cytomorphologic analysis in yellow-green area of a spectrum). Scale bar: 25 μm.

expect that the intraperitoneal introduction of the indicated inductors can give in a stimulation of processes of formation ECs in cultures of PCs.

The legitimacy of the expressed guess was confirmed in following experiment. In 7 days after intraperitoneal introduction into mice of line BALB/c Freund's complete adjuvant, cells of peritoneal transsudate explanted in cultures and cultivated in vitro within 5-7 days. It is fixed that in 5-7 days of cultivating in cultures of PCs, stimulated by Freund's complete adjuvant, the amount of cells of an epithelioid type will increase in comparison with the control at 7-10 of time. Moreover, in such stimulated cultures were shaped not only small epithelioid-cell-like clusters, consisting from 3-5 ECs, but also major monolayer layers consisting of fitting closely to each other mature cells of an epithelioid type, morphologicaly similar to layers of typical epithelial cells that cultivating in vitro. On morphology by using the method colour interferential contrast (Arkhipov, 2002) was chosen 3 forms of ECs formatived in cultures of PCs: fibroblast-like, plasmacytoid and vesicul-like ECs (fig. 1). The cells of a type plasmacytoid forms always dominated. The intravital observations (in microchambers) by process of formating of epithelioid-cell-like clusters and layers have allowed to make the conclusion that the gain value of cells in epithelioid-cell-like clusters and layers is carried out by direct division of ECs (as a result of processes of an endomitosis and amitosis), taking place mainly on a periphery of clusters or cell-like layers, with subsequent cytotomy (fig. 2).

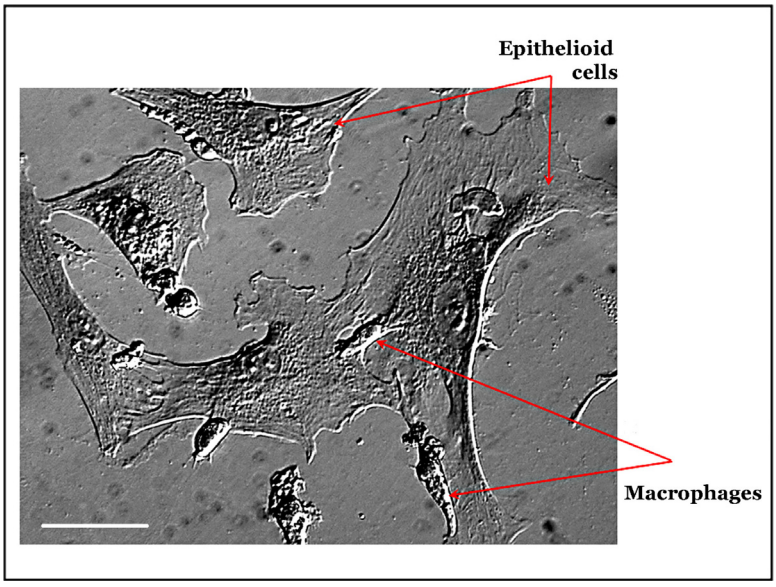

Fig. 2. Epithelioid-cell-like cluster, formed in culture of PCs of mice of line BALB/c, beforehand stimulated in vivo (7 days prior to an explantation of cells) by Freund's complete adjuvant (intraperitoneal introduction 50 % of emulsion in physiological saline solution), 10 days of cultivating. A method of differential interferential contrast. Scale bar: 30 μm.

Thus as a result of disproportionate cytotomy from one EC some “affiliated” (or “child”) cells of the smaller sizes formatived mainly of cones of cytoplasm growth can be formed at

once. The majority ECs, taking place in clusters, gain the polygon shape (fig. 3). Thus, epithelioid-cell-like clusters and the layers formatived in cultures of PCs, gained features of typical cultures of epithelial cells formatived in a monolayer (on morphology, locating in a monolayer and type of cell-like body growth).

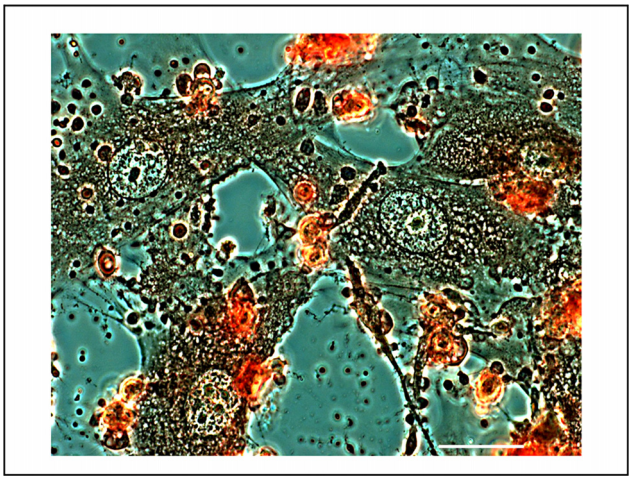

Fig. 3. Epithelioid-cell-like cluster, formed in culture of PCs of mice of line BALB/c, beforehand stimulated in vivo (7 days prior to an explantation of cells) by Freund's complete adjuvant (intraperitoneal introduction 50 % of emulsion in physiological saline solution), 14 days of cultivating. Among ECs are visible numerous apoptotic bodies of macrophages; by red colour are painted the apoptotic changed macrophages. A method of colour interferential contrast. Scale bar: 40 μm.

Most visually, cytomorphologic difference between Mphs and ECs, generatored in culture of PCs, are visible at usage for image analysis of cells of method of color coding of the numeric images of cells. At usage of the method color coding on a brightness of image, consisting in assignment to pixels with particular luminosity of particular colour or a monochrome color tone, contrasting with by other contiguous "cluster" of brightness gradation, the new colour image of mixed culture Mphs and ECs was received, which grows out of colour code translation of the starting image of cells. At polychromatic pseudo-colouring Mphs and ECs, obtained computer transformation of the starting image, it is visible as far as are essential cytomorphologic difference of Mphs and ECs, defining some kind of different «color phenotypes» describing them cytomorphologic differences (fig. 4).

Based on these data were formulated two working hypotheses appear in cultures of peritoneal cells clusters of ECs. According to the first assumption mature macrophage cells, transforming into ECs, can gain proliferative activity. However, it contradicts modern representations that in process of differentiation the proliferative activity of cells of monocytic-macrophagic histogenesis is considerably reduced or is completely lost. Thus, if to proceed from the conventional concept of a parentage ECs from Mphs, then it is necessary to enter more still as a minimum two assumption that mature Mphs can undergo dedifferentiation, start to proliferate, and in dedifferentiated Mphs or undifferentiated

monocytes (precursors Mphs) the new genetic program differentiation, starting purely others, though and in something similar with Mphs, biochemical metabolic pathways of synthesis of new substances resulting in to generation epithelioid-cell-like phenotype, should somehow join.

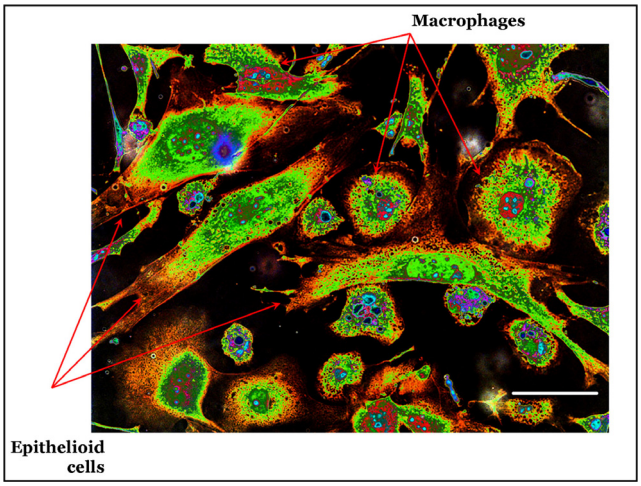

Fig. 4. Culture of PCs of mice of line BALB/c, beforehand stimulated in vivo (7 days prior to an explantation of cells) by Freund's complete adjuvant (intraperitoneal introduction 50 % of emulsion in physiological saline solution), 10 days of cultivating. Mphs and ECs are marked by by arrows. Colouring by azure-II-eosine. A method of color coding of the numeric image (color coding of contrasting color hues on 20 gradation of brightness). Scale bar: 40 μm.

According to the second hypothesis the mechanism of formation of epithelioid-cell-like clusters in culture of cells could be is stipulated by existence some kind of chain reactions (effect "of an impinging domino"), when one mature differentiated EC stimulates processes of an induction neighboring Mphs, including mechanisms differentiation of these cells in an epithelioid-cell-like direction. This hypothesis was discarded after the first experiments in vitro, as in composition of epithelioid-cell-like clusters was not detected of any cell, which could be surveyed as the transition form from Mphs to a cell of an epithelioid type. As a whole cytomorphologic analysis of cultures of PCs beforehand stimulated by Freund's complete adjuvant has shown that on 5-7 days cultivating is possible in cultures to differentiated some types of cells: 1) the cells of a macrophage type, absolute majority from which phagocytized granulas of a zymosan (GZ); 2) ECs (not phagocyting); 3) fibroblast-like cells (not phagocyting); 4) huge multinuclear cells of a macrophage type (phagocyting), huge multinuclear cells of an epithelioid type - similar cells Langhance (not phagocyting), multinuclear cells of a mixed type derivated as a result of fusion Mphs and ECs – “symplast”. By the way opportunity of formation “symplasts” from Mphs and ECs allows to explain - how objects of an phagocytosis (for example, the micobacteria of a tuberculosis) can turn out in cytoplasm of ECs that is not as a result of natural phagocytic activity, but in result mergings with Mphs, which already have imbibed micobacteria. At the total analysis of all cytological preparations of cell-like cultures of PCs, obtained in different experiments in vitro, was not detected of cells, which could be referred to morphological indications

simultaneously both to Mphs and to ECs that is to the transition forms of cells from Mphs to ECs. As ECs did not phagocytize the granulas of a zymosan, is not time-dependent additions of granulas into cultures, was apparent that the cells-precursors of ECs and their transition forms have no phagocytic activity. GZ were detected only in symplasts, generatored as a result of fusion Mphs and ECs. During examination in cultures of intact PCs and stimulated by Freund's complete adjuvant were detected rather large monocyte-like cells, distinguished from typical monocytes, which on the sizes, colouring, morphology of nuclei (and their colouring), shape of spreading it was possible to refer to the transition shapes ECs of a different degree of maturity. These cells differed from typical ECs of epithelioid-cell-like clusters, included in composition, and in ECs-layers, only by small sizes. Conditionally these cells were termed "juvenile" or "young" ECs. Starting from these tentative datas, the new alternate hypothesis epithelioid-cell-like generating in vitro was formulated, which essence consists that ECs in cultures of cells are formed not from Mphs, but of any low-differentiate mononuclear cells – precursors of ECs – the cells of monocytoid type with some cytomorphologic features, distinguishing them from typical monocytes.

5. The characteristic (morphofunctional and cytochemical) of epithelioid cells and its cells-precursors (pre-ECs), contained in abdominal cavity after induction of an experimental peritonitis (intraperitoneal introduction by Freund's complete adjuvant) – Development of cytomorphological criteria of identification of epithelioid cells and pre-ECs in vitro

For targeted searching for low-differentiate mononuclear cells - precursors of ECs, distinguished from cells of macrophage series, it was necessary to spot well-defined criteria of identification ECs by which it was possible to distinguish them from cells of macrophage series, and on which it would be possible to find cells, from which are differentiated ECs. With the purpose of development of a complex scientifically justified of cytomorphological criteria of identification low-differentiate forms of ECs in culture in a following stage of operation the complex comparative examination of cytomorphology of Mphs, phagocyted of GZ, and mature ECs, which formatived clusters in vitro after a preliminary stimulation by a Freund's complete adjuvant (containing micobacterium of a tuberculosis - M. tuberculosis, killed by heat) in vivo (look previous division chapter) was conducted. For detection of cells, which could be surveyed as cells precursors of ECs, the legible morphological criteria of their identification were necessary. It was represented important quantitatively to estimate self descriptiveness separate cytomorphological and morphofunctional features of indications ECs, and also their combinations as criteria of identification of the different transition forms of ECs in culture. For the solution of this problem the multivariate cluster analysis and random principles of process of a discernment of objects utilised. The quantities of absolute probability of a discernment ECs in mixed culture Mphs and ECs on quantitative parameters calculated on the basis of multivariate cluster analysis of mixed sampling from Mphs, possessing of phagocytic activity were retrieved, and ECs, which accessories to epithelioid phenotype did not call doubt. The following absolute probabilities of a discernment of ECs in mixed cultures Mphs and ECs on quantitative parameters are fixed (Arkhipov, 2001a): maximal diameter of a nuclei (P = 0,95), area of a cell (P = 0,73), diameter of a nucleolus (P = 0,76), nucleus-cytoplasm relations (P = 0,52); amount of ruffles (P = 0,53), amount of filopodiums (P = 0,60); amount of lamellopodies (P = 0,55). The padding measure of identification ECs, representing conditional probabilities of a

discernment ECs to quality indications that is probability of identification rated at performance of additionals: spreading cell has the trapezoidal shape (P = 0,86); the ellipse shape of a nuclei (P = 0,87); uniform "mesh" allocation of a bazihromatin in nuclei, with a dominance of small lumps on a rim of nuclei and with "expressiveness" of an euchromatin (P = 0,93); a rounded profile of a regional zones of cytoplasm (P = 0,95); availability of the expressed cones of body growth (P = 0,99); undermembrane fascicles of actinic filaments posed mainly in one direction (P = 0,99); microvillis scarle (P = 0,54) uniformly distributed. The multivariate cluster analysis based on the registration of three different measure of identification (with high probability of a discernment ECs) has shown that usage of several criteria of identification allows to identify all ECs in cultures Mphs with probability to equal unity, coming nearer to quantity. It is established that the criteria of identification of ECs, designed at examination ECs, formatived in cultures of the PCs, stimulate by Freund's complete adjuvant, well "work" in an estimation of cytomorphological measure in the relation of ECs, chosen from granulomas, induced hypodermic introduction by Freund's complete adjuvant or introduction BCG, and explantated in cultures in vitro. It is exhibited that cytomorphological feature of ECs, formatived in cultures of PCs after their "stimulation" in vivo (intraperitoneal introduction 50 % of emulsions Freund's complete adjuvant, prepared in physiological saline solution), and ECs, formed in granulomas, induced adjuvant or BCG, coincide on the majority of estimated parameters (shape of spreading cell, sizes and shape of nuclei, features of a structure of a nuclear chromatin, features of a structure of a regional band spreading cell, features of architectonics of actinic cytoskeleton, and also series of other cell-like performances). For more precise detection of all transition forms of ECs, and also low-differentiate forms cells-precursors, alongside with an estimation of phagocytic activity, it was necessary to explore some other parameters of their manifestation of their function activity. The estimation of ability of ECs was conducted to reduce nitroblue tetrazolium (NBT) to formazan, accumulate acridine orange (AO) and neutral red (NR) in lysosomes, to be coloured by a vital stain Janus green (intravital colouring on a mitochondrion). Besides these the estimation of peroxidase activity of ECs was conducted. As for all ECs of a nuclei not enough "heterochromatin" contained and in the morphological schedule were described as a nuclei with dominance "euchromatin" (as the testimony of high function activity of nuclei) the immunocytochemical colouring of nuclei on expression in nuclei the histone protein H1 was conducted. As a result of these examinations it is fixed following. ECs as well as Mphs were capable to reduce NBT, but the character of allocation of formazan in cytoplasm of Mphs and ECs essentially differed. For Mphs the accumulation by the way large heteromorphic lumps in a perinuclear band of cytoplasm was characteristic. In cytoplasm of ECs finely divided inserts formazan were proportioned rather uniformly. Moreover, through a polarization microscope was fixed that the shaping lumps of formazan can happen and on an exterior surface of a cell-like membrane ECs that testifies to availability for ECs of the mechanism of exocellular production of the free forms of oxygenium. The character of colouring ECs and Mphs by a fluorescent stain of AO and vital stain by NR also differed. The lysosomal structures of Mphs, accumulating lizosomotropny stain NR, were rather heterogeneous on the sizes, AO in ECs was stored in small lysosomes (or secretory granulas?) comparable on quantity. The mitochondrions, painted by Janus green, in ECs were of the mainly prolate shape and the polarizations of a cell are oriented lengthwise axis that probably was stipulated by a locating

of fascicles of undermembrane actinic filaments (stresses - fibers), a defining direction "polarization" of ECs. ECs had no peroxidase activity. The different degree of peroxidase activity was detected for 17,5 % of cells referred on cytomorphological features to monocytes / Mphs. Nuclei of all ECs in a different degree express a histone H1 that testified to different genetic activity of nuclei of ECs. As a result of comparative cytomorphologic analysis Mphs and all transition forms of ECs, formatived in culture, and also designed on its basis of criteria of identification of ECs in culture following cytomorphologic indications, describing low-differentiate cells, referred to cells-precursors of ECs, were established. Pre-ECs are cells about a diameter 15-23 microns (in spreading state), having a major round or oval excentricly posed nuclei (diameter 12-16 microns) with a major nucleolus, nucleus-cytoplasm relation > 1; the surface of nuclei flattened and has no penetrating impressions; chromatin of nuclei is fine-structural , mesh, having characteristic drawing of alternating strips of an enlightenment. Cytoplasm is weak basophilic. In cytoplasm of some cells in small quantity thin eosinophilic and azurophilic graininess are taped. Amounts of lysosomes in cells is major. A topography of a surface pre-ECs by places plaited with small quantity of microvillis. Peroxidase activity is missed. The colouring of cytoplasm in the NBT-test is well expressed. Expression of a H1-histone is brightly expressed (Arkhipov, 1997, 2001a, 2004). After explantation in cultures of cells with phenotype pre-ECs in 1-2 hours after an attachment gain characteristic for ECs the triangular, trapezoidal or polygon shape with cones of growth (Fig. 5).

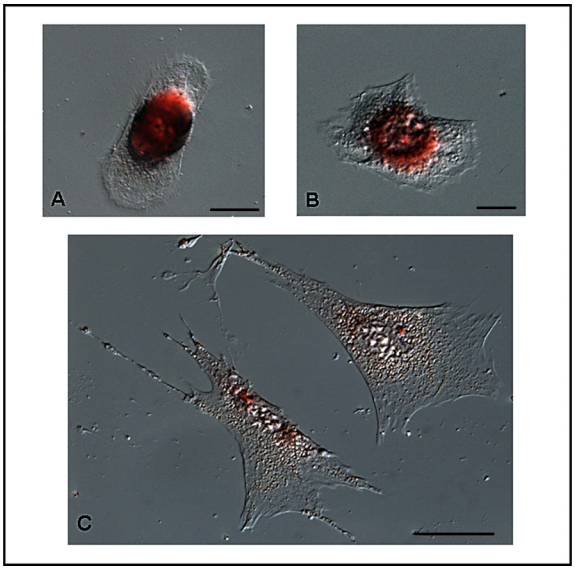

Fig. 5. Culture of PCs of mice of line BALB/c, beforehand stimulated in vivo (7 days prior to an explantation of cells) by Freund's complete adjuvant (intraperitoneal introduction 50 % of emulsion in physiological saline solution). A - pre-EC, B - "«transition" form of pre-EC, C - "«juvenile" form of ECs. Vital colouring neutral red (accumulation of a stain in lysosomes), additional contrasting of cells by a method of differential interferential contrast. Scale bar: 10 µm.

To be sure that the fixed measure of identification pre-ECs are reliable and substantially work, it was necessary to conduct a series of examinations on a statistical validity and proof of a position that everything the cells, detected with phenotype, marked as "«pre-ECs", are differentiated in cells of epithelioid type.

6. Statistical validity and confirmation of existence of a specialized line of cells-precursor of epithelioid cells (pre-ECs)

The statistical validity of existence of an epithelioid-cell-like line in an organism - population of cells, committed to formation of ECs in norm that in the absence of inflammatory response or any pathological process in an organism was conducted. In the fig. 6 the data on the dynamics of accumulation in cultures of all transition forms of ECs, represented in cultivating of PCs of intact mice, liberated from lymphocytes and other nonadhered cells. By cytomorphological study of such cell-like cultures for various periods of cultivation determined that the amounts of cells, identified in the first hour of cultivation as a poorly differentiated pre-ECs, reliablly does not differ from amounts of mature ECs, detected to 7 days of cultivating. As it is visible from the fig. 6 the quantity of all transition forms of ECs in control (intact mice) on the 7-th day of cultivation practically corresponds to starting quantity of pre-ECs in culture, which is registered in the first hours of cultivation. Moreover, it is exhibited that in cultures of cells of intact mice for 14 day cultivation, the total unities of epithelioid-cell-like formating - separately spaced apart of ECs and epithelioid-cell-like clusters, formation which occurs as a result of cell division, it is also statistically identical.

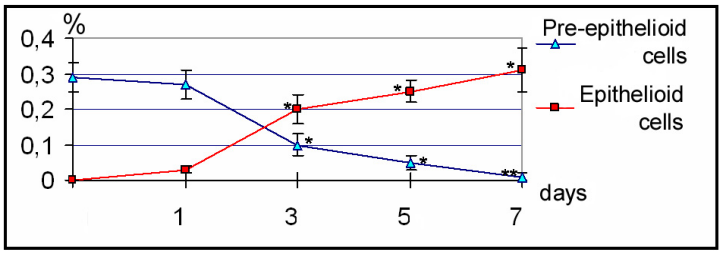

Fig. 6. The dynamics of accumulation of all transition forms of ECs cells in culture of PCs of intact mice of line BALB/c. The results are presented in the form $x \pm s_e$ (%), where x - arithmetic mean (average), s_x - standard error of the mean. Significant differences between groups was assessed using the nonparametric Wilcoxon-Mann-Whitney test (*$p < 0,05$; ** $p < 0,01$).

In the Fig. 7 shows the dynamics of accumulation of all transition forms of Ecs that was registered in examination of PCs of mice, whom at least 7 days prior to explantation of cells in vitro were injected intraperitoneally Freund's complete adjuvant. The intraperitoneal introduction of Freund's complete adjuvant results in augmentation of amounts of cells-precursors of ECs in 5 times in comparison with their quantities in the "control" (intact mice) and reaches in separate cultures to more 1,0 %. "Disappearance" pre-ECs (bound from them differentiation and transformation to more mature forms) in cultures of cells, stimulated by Freund's complete adjuvant, is accompanied in the beginning by augmentation of quantity

of the "juvenile" forms of ECs for 5 day of cultivation. With further cultivation the quantity of the "juvenile" forms of ECs is reduced and the number of mature ECs, including ECs, which are in phases of growth and reproduction, increases exponentially. The phase of a proliferation is characterized by augmentation in culture of quantity of ECs with nuclei having indications of endomitosis (with characteristic "patterns" with more or less discernible "contours" of chromosomes), two-nuclear ECs and clusters of ECs.

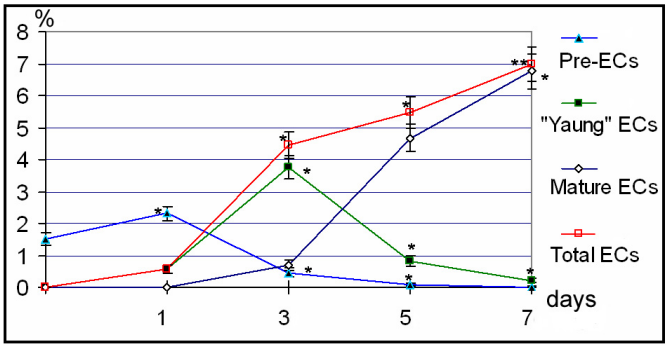

Fig. 7. The dynamics of accumulation of the different transition forms of ECs in culture of PCs of line BALB/c, stimulated in vivo by Freund's complete adjuvant. Transsudate of PCs, obtained in 7 days after intraperitoneal injection of adjuvant. On an axis of ordinates - quantity of ECs (in % from all cells in culture). The results are presented in the form $x \pm s_e$ (%), where x - arithmetic mean (average), s_x - standard error of the mean. Significant differences between groups was assessed using the nonparametric Wilcoxon-Mann-Whitney test (*$p < 0,05$; ** $p < 0,01$).

In "control" (not stimulated cell cultures of intact mice) only simple ECs transferred in a stage of a proliferation to 14 days of cultivation, forming simple clusters of 3-5 ECs. When cultured PCs, activated with Freund's complete adjuvant, marked stimulation (acceleration) of all the processes that determine the differentiation of pre-ECs into ECs - from the beginning of the transformation pre-ECs in ECs to phase of reproduction with the formation of large clusters and 2-layered epithelioid-cell layers. At the same time noted stimulation of Mphs apoptosis, taking place in immediate proximity from ECs, with formation numerous apoptotic bodies, which are likely to use EC as an additional plastic material in active growth and proliferation of ECs (see Fig. 3). The obtained data have allowed to make following findings: 1) in a population of PCs there is a subpopulation low-differentiated cells, morphologically distinguished from cells macrophage line (monocytes / Mphs), being cells - are predecessors of all cells of epithelioid type, 2) ECs are differentiated only from population of pre-ECs, committed to a direction of epithelioid-cell-like cytomorphogenesis, 3) at long-lived cultivation and stimulation of PCs of mice the part "mature" ECs is capable to transfer in a phase of a proliferation, forming colonies, consisting of fitting closely to each other cells - epithelioid-cell-like clusters and epithelioid-cell-like layers. Actually obtained data have allowed to conclude that the process of epithelioid-cell-like formating in vitro limited by a separate single-cell epithelioid cell line, that is histogenetic non-macrophage cell line.

7. The rating of a contents of cells-precursor of epithelioid cells in a blood and bone marrow (pre-pre-ECs)

Based on already seted criteria of identification of pre-ECs, obtained in the study of cultures of PCs, represented major practical and theoretical interest installation the rating of a contents of cells-precursor of ECs in a blood and bone marrow, conditionally marked as "pre-pre-ECs". By the method of intravital observation in microchambers fixed that within 3 hours after beginning of cultivation of blood monocytes of mice some large-scale "plasmacyte-like" monocytes gain at spreading the shape, characteristic for differentiated pre-ECs - triangular or trapezoidal with characteristic growth cones (to look fig. 5). As against the majority of typical monocytes (being the precursors of Mphs) with characteristic for Mphs "bean-shaped" or "blade-shaped" nuclei, polymorphic type of spreading (distinguished major variety of the shapes) and possessing of phagocytic activity (concerning to microspheres of polystyrene latex, granulas of a zymosan and micobacteria BCG), large-scale "plasmacyte-like" monocytes named “Epitheliocytoblast” did not phagocytosed (Arkhipov, 1997, 1999a, 1999b). With further cultivation (about 1-2 days), "plasmacyte-like" monocytes were polarized and gained a view, characteristic for "juvenile" ECs. The series of experiments for a statistical validity of existence of an epithelioid-cell-like line in a blood - the population of cells, committed to formation of ECs, was conducted. The comparison of estimations of the number of cells – precursors of ECs, obtained on the basis of the analysis of short-term cultures of leucocytes (3 hours), and also analysis of cultures of leucocytes through 2 days after the start of cultivation, was conducted. The study showed that amounts of pre-ECs for mice of line BALB/c (in recalculation on all leucocytes), detected by these methods, and the amounts of the “juvenile” forms of ECs is determined by one quantity - 0,01-0,03 %. It is established that among all cells of a bone marrow there are cells, which on series of morphological indications (sizes of a cells, sizes and shape of nuclei, features of a structure of a chromatin etc.) and on availability of particular colouring in transiting and reflected light, it is possible to refer to cells – precursors of ECs. Their quantity laid within the limits 0,005-0,01 %. For a number of cytomorphological features (after colouring by azure and eosine) unspread cells of this type have likeness to major reticular cells of a bone marrow, which rank as cells of a reticular stroma of a bone marrow. Thus, as a result of al conducted studies was found that quantity of a pool pre-ECs in a peritoneal cavity in norm for mice BALB/c reaches 0,05-0,15%, among leucocytes of a blood - 0,01-0,03%, bone marrow - 0,005-0,01 %.

8. The rating of speed of differentiation of pre-pre-ECs (from blood and bone marrow) and pre-ECs (from peritoneal cavity) at different differential stimulus in vitro

To one of indexs of maturity of pre-ECs it was possible to refer ability them to differentiation. The estimation of potencies of pre-pre-ECs and pre-ECs to differentiation into ECs at adding in medium the stimulants of cell differentiation was conducted. The stimulation of differentiation of pre-pre-ECs and pre-ECs in cultures realized by an incubation in medium RPMI-1640, containing Potassium Orotate (PO; 5 μg/ml) and Dimethyl sulfoxide (DMSO; 0,005 % in the final dilution). The effects estimated at count of in each culture 10^4 PCs, 10^5 leucocytes of a blood and 10^5 cells of a bone marrow (5 hours after the start of cultivation). At cultivation of PCs within 5 hours the greatest amounts of

the "juvenile" forms of ECs is detected only in those cultures, to which imported PO and DMSO. It is exhibited that the acceleration of processes of differentiation of pre-ECs into cells of an epithelioid type is higher in cultures of PCs received from animals stimulated by Freund's complete adjuvant or BCG. The similar patterns of cytomorphogenesis of ECs are detected at examination of effect of a stimulation of differentiation of pre-ECs (or "pre-pre-ECs") of a blood and cells of a bone marrow (Arkhipov, 2001b). It was found that under selected requirements of cultivation, potency of cells of a bone marrow, leucocytes of a blood and PCs, referred by cytomorphological measure to pre-ECs, to differentiation into ECs are various. They have minimal for pre-ECs of bone marrow, but are high enough for pre-ECs of a blood. It is exhibited that the number of active peritoneal pre-ECs, capable of rapid starting of the mechanisms of differentiation of pre-ECs, defining ECs-forming, exceeds the number of active pre-ECs in the blood. Apparently, that at usage of any other conditions or methods of cultivation of cells of bone marrow (at usage of the padding growth factors, any more effective stimulators of cell differentiation) more optimal requirements, promoting for pre-pre-ECs of bone marrow to differentiation into ECs in vitro can be found.

9. The rating changes of a pool of pre-pre-ECs in a blood in the development of an experimental chronic inflammation in mice

Based on the obtained data it it was possible to assume that the number of pre-pre-ECs in the blood may vary depending on the "stage" or "outcome" of the pathological process, in some way definitely reflecting the dynamics of development or involution of epithelioid-cell-like granulomas in an organism. To answer this question has been used a model of granulomatous inflammation in the liver granulomas with involution, and the developed on its base model of chronic granulomatous inflammation in the liver with different outcomes (Arkhipov, Shkurupy, 1996). It was shown that changing the number of inputs pure zymosan granules (GZ) and the number of grain boundaries associated with acid fuchsin (GZF), you can change the dynamics of granuloma Mphs update, their differentiation and, consequently, to model different versions of morphogenesis of ECs granulomatous inflammation. It is shown that 7 days after intravenous injection of pellets of GZ and zymosan, chemically modified acidic fuchsin (GZF), develop in the liver macrophage granulomas, containing a small amount of ECs and lymphocytes. When using GZF the number of ECs-granulomas was slightly higher (by 25 %), than with using GZ. By 21-th days the results in the indicated experimental groups differed significantly. The number of granulomas induced by GZ was decreased to 4.0 in 10 fields of view, and induced by GZF practically no change, reaching a value of 63,7%. It is established that a pool of pre-ECs in blood changes in the development of granulomatous inflammation in some way reflect the dynamics of the development of granulomas and their involution. It was important to evaluate changes of pool progenitor cells of ECs in a typical epithelioid-cell granulomatous inflammation. To meet this challenge the model of chronic disseminated tuberculous inflammation was used. Granulomatous inflammation in various organs of mice of BALB/c induced by intraperitoneal injection of Mycobacterium Bovis BCG (1 mg/ animal). Estimate of the number of granulomas in liver was carried out on histological preparations, stained with hematoxylin and eosin. The number of pre-ECs in the blood was assessed in short-term cultures of leukocytes. It is shown that 30 days after intraperitoneal injection of BCG in the

liver developed typical epithelioid-cell granulomas. These granulomas are stored in the liver for several months. It was assessed the changes in the blood pool of pre-ECs at 1 and 2 months after BCG injection. It is shown that in mice at 1 month after injection of BCG was an increase in the circulating pool of progenitor cells of ECs in the blood of almost 3,5 times, compared with the control (intact animals). The number of granulomas in histological preparations in 10 fields of view was 7,2. In 2 months after the induction of granulomatous process the amounts of granulomas in the liver decreased by 1,7 times. In the proportional reduction of pool pre-ECs in blood is noted (1,8-fold, compared to that at 1 month after the intraperitoneal injection of BCG). Correlation analysis of the data showed that between the number of epithelioid-cell granulomas in the liver of mice and the number of epithelioid-cell precursors (pre-pre-EC) in the blood is a correlation ($r = 0,96$, $P < 0,05$). Thus it is shown that the processes of morphogenesis of epithelioid-cell granulomas may greatly determine by inflow to the site of granulomatous inflammation progenitor cells of ECs.

10. The rating of a circulating pool of pre-ECs in mice of different genetic lines of different predisposition to development of a tubercular infection

It is known that immunological failure of those or other links of immunity, contributing to some inflammatory and infectious diseases, including tuberculosis, are genetically stipulated and are interlinked to failure of the particular immunological factors (Cardona, 2004; Shkurupy, 2007). It was expressed the guess that the quantity of pre-ECs also can be genetically determined. For study of a genetic condition of the control of epithelioid-cell-like cytomorphogenesis the following lines of mice were selected: BALB/c, C57BL/6, CBA, DBA. According to the data of the scientific literature these lines of mice at pairwise comparison can be referred to "opposite reacting lines", concerning formation of epithelioid-cell-like granulomas by infection of the same agents - micobacteria of a vaccine BCG or virulent forms of micobacteria of a tuberculosis. For all lines of mice estimated amounts of cells-precursors of ECs in abdominal cavity, committed in an epithelioid-cell-like direction of differentiation - pre-ECs. The indicated parameters estimated for intact animal, and also after an induction in mice an inflammation in abdominal cavity by introduction of an emulsion of a Freund's complete adjuvant. It is exhibited that per quantity of a starting pool of pre-ECs the explored lines of mice can be arranged on incremental in following series: DBA (0,02-0,09 %; 0,07±0,01 %), BALB/c (0,05-0,15 %; 0,11±0,01 %), CBA (0,07-0,19 %; 0,15±0,02 %), C57BL/6 (0,11-0,27 %; 0,24±0,03 %). At an induction of a chronic inflammation in abdominal cavity by introduction of emulsion of a Freund's complete adjuvant this tendency is maintained. However, thus the interlinear differences, estimated by quantity of induced cells of an epithelioid type, essentially will increase. If for mice of a line DBA, the amounts of ECs-precursors of ECs type at induction of an inflammation in abdominal cavity will increase only in 1,5 times that for mice BALB/c, CBA, C57BL/6 - accordingly in 3,9, 4,0 and 6,7 times. The obtained results allow to make a deduction about existence of a genetic determinancy of a datum level of an epithelioid-cell-like reactivity. They specify also that the morphogenesis of epithelioid-cell-like granulomas in tuberculosis can be determined by the different starting genetically determined level of a pool pre-ECs, inflow pre-ECs in the center of an inflammation, and also intensity of processes them differentiation into ECs.

11. The conclusion about necessity of revising of the old concept of a origin of epithelioid cells of cells of system of mononuclear phagocytes

The analysis of the scientific literature touching problems of a nature and cytomorphogenesis of ECs, and also analysis of the experimental data, introduced in the present chapter, allows to raise the question about revising the resisted representations about a parentage ECs from Mphs. Tuberculosis - very dangerous, serious and artful disease. And we can not neglect even slightest chance in improving comprehension of its etiology, pathogeny and morphogenesis of epithelioid - cell-like granulomas (or tuberculomas), which are surveyed by the majority of research peoples as structures, which function is isolation of an organism from the centers of an infection that is "function" of protection of an organism from a further dissimination of micobacteria of a tuberculosis in an organism. From positions of the concept of a parentage of ECs from Mphs, to ECs the role of enough “passive” cells, namely modified Mphs with the “truncated” functions (decay the phagocytic activity etc.) is tapped. Thus it is supposed that early or late in tubercular process Mphs in formatived granulomas are transformed into ECs, which function till now is not spotted. We attempt to present a histogenesis of ECs and morphogenesis of tubercular granulomas process completely on new, from positions of a parentage from specialized cells precursors of ECs. In a particular stage of forming of granulomas from Mphs, the germ of pre-pre-ECs in bone marrow is stimulated, the pool of pre-pre-ECs in a blood will increase, they arrive in the center of granulomatous inflammation, which structural basis are Mphs, as well as the different subpopulations of lymphocytes, dendritic cells, and also coming monocytes (precursors Mphs). Pre-ECs are differentiated into ECs, and the process of augmentation of a pool ECs in granulomas happens both for the score of differentiation of pre-ECs, and at the expense of proliferative activity of ECs (division of cells as a result of an endomitosis or amitosis). The genetically determined reduce content of pre-ECs in an organism will give that in tubercular granulomas will dominate Mphs, formative macrophage granulomas (with huge multinuclear cells of foreign bodies), possessing to reduce "abjoint" potential. In the complete absence in the circulation system pre-ECs will be formed granulomas that do not contain ECs. If to go in our reasonings is farther it is possible to assume that ECs have any high special-purpose functions, which are not solved yet, and which allow these cells at prompt enough ascending of their pool in the center of contamination by micobacteria of a tuberculosis and prompt organization in granulomatous epithelioid-cell-like frames, to handicap with a dissimination not only tubercular micobacteria from the centers of a tubercular lesions, but to fulfill «functions of abjoint» in other granulomatous diseases of a infectious and noninfectious etiology. The comparative analysis of cytomorphologic features of different types ECs, formatived in cultures in vitro, in a context of modern representations about differentiation of mesenchymal stem cells in different histogenetic directions cell-like differentiation, allow in a new fashion to survey the concept of a histogenesis of ECs from cells-precursors, committed in an epithelioid-cell-like direction of differentiation. It is possible that some epithelioid-like cells of the forms of cells, formatived in the centers of tubercular granulomatous inflammation (for example, " fibroblast-like" shape of ECs) can be offsprings of mesenchymal stem cells. The clearing up of the earliest stages of a histogenesis of ECs will allow to answer a problem not only about biological substance of ECs, formatived at different chronic granulomatous processes, but also more

precisely to spot their functionality in an organism in tubercular pathological process. The original positions of the new concept of a parentage and differentiation of ECs in chronic inflammatory granulomatous processes, including in a tuberculosis, schematically represented in fig. 8. For matching in the same scheme the conventional concept of a histogenesis of ECs represented.

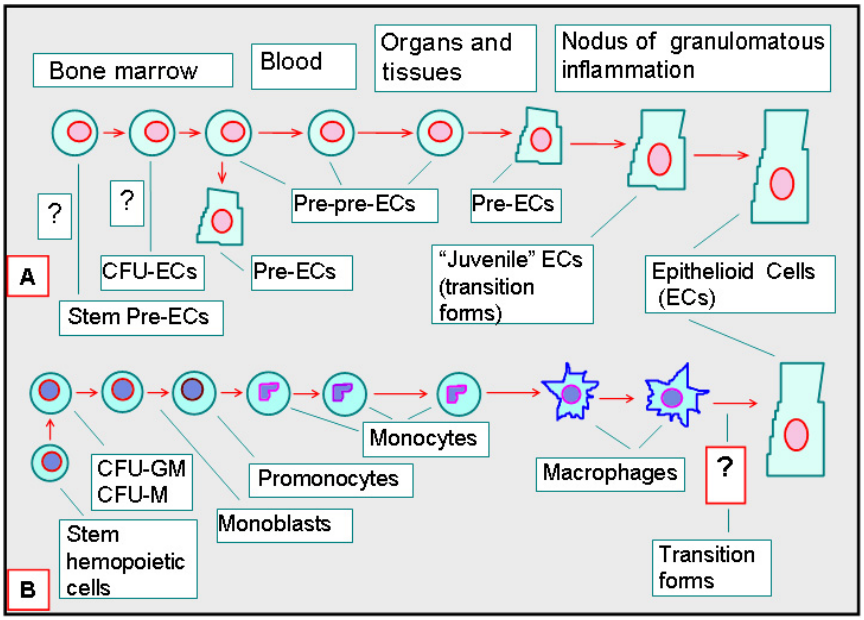

Fig. 8. The scheme of a histogenesis of ECs, constructed on the basis of the results of own examinations (A), in matching with the conventional scheme of epithelioid-cell-like cytomorphogenesis in chronic inflammatory granulomatous processes (B).

12. The algorithm of further researches of epithelioid cells and the rating that can give new knowledge of epithelioid-cell histogenesis in comprehension of a morphogenesis of the tubercular process, in improvement of diagnostics of a tuberculosis, development of methods of its treatment and prognosis of development

Now, unfortunately, we yet do not allocate precise enough immunophenotypic or cytochemical markers of ECs, permitting to correlate data, received in examinations of ECs with the help of morphological methods and on cultures of cells. Therefore, to one of the proximate tasks in examinations of ECs it is necessary to refer searching high-specific markers (clusters of differentiation, cytoskeleton proteins, receptors, enzymes), characteristic only for ECs and pre-ECs. Thus during these examinations any would be clarified «specific or specialized» functions of ECs, which they fulfill in the center of tubercular granulomatous inflammation. In my opinion, it is very complicated to calculate for major progress in the struggle against tuberculosis and in developments of new agents for treatment of tuberculosis, not having faithful representations about a histogenesis of ECs

and about functions, which they fulfill in tubercular granulomas. The new knowledge of a histogenesis of ECs from committed pre-ECs gives new comprehension of mechanisms underlying a morphogenesis of epithelioid-cell-like granulomas in tubercular process, and in long-range researching in this direction, can promote improving of diagnostics of a tuberculosis, help in development of new methods of treatment and prognosis of development of this disease. For example, the estimation of quantity of pre-ECs in a blood in the begining of disease can specify on what "trajectories" there can be a process of formation of tubercular granulomas: macrophage, epithelioid-cell-like or mixed. The lack of pre-ECs in a blood can specify probable unfavorable development of granulomatous process with a probable prompt dissimination of micobacteria of a tuberculosis in an organism. On the contrary, relatively large value of a pool of pre-ECs in blood and its ascending after infection can testify to probable more effective cupping of tubercular process as a result of prompt formation of epithelioid-cell-like granulomas. The same measure can be surveyed as an index of efficiency of a conducted immunotherapy and medicinal therapy of a tuberculosis as a whole. In conclusion follows to answer the question which will undoubtedly be raised. What does the new conception of origin and differentiation of epithelioid cells give? The new conception of epithelioid cells origin and differentiation - first of all, is a key to new understanding of concrete cell mechanisms that participate in forming of epithelioid cells granulomas and epithelioid cells-clusters in a tuberculosis, it is a key to correct understanding of pathogenesis and morphogenesis of the tuberculosis disease; probably it is the basis for elaborations of new diagnostic methods of the prognosis and new medical treatment methods of a tuberculosis. It would be desirable to hope that the results of experimental researches on study of histogenesis, cytomorphogenesis, morphofunctional potency of ECs, their role in pathogenesis and morphogenesis of the tubercular process, introduced in the present chapter, will not stay without attention and become a basis for further examinations in this direction.

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8

How *Mycobacterium tuberculosis* Manipulates Innate and Adaptive Immunity – New Views of an Old Topic

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1. Introduction

Tuberculosis (TB), once regarded an historical disease due to the discovery of antibiotics, is one of the most wide-spread human infections today, and a major cause of death from bacterial infections. The causative agent, *Mycobacterium tuberculosis,* has evolved over ages along with the human species, the oldest human finding being 9 000 year-old skeletons with tuberculosis lesions (Hershkovitz, Donoghue et al. 2008). The most widely known form of tuberculosis, pulmonary TB, affects the lungs and is characterized by cough with bloody expectorations associated with fever, night sweats and weight loss. Extrapulmonary TB can be found almost anywhere in the body, either localized in organs such as the lymph nodes, pleura, abdomen, bones, joints and the central nervous system or appearing in a more disseminated form known as miliary TB. Active TB disease develops in 5-10% of infected individuals, whereas most exposed individuals contain the infection in the form of latent disease or more rarely eradicate the bacteria. To prevent progression of latent TB to active disease, an equilibrium between the host and the microbe has to be maintained. Today, about one third of the world population are carriers of TB infection, which constitutes an enormous reservoir for potential spread of disease.

1.1 Preface

Despite decades of research on TB, studies of the cellular mechanisms involved in TB pathogenesis have lagged behind, particularly in human systems. In addition, TB immunology is a competitive area of research and available data is still controversial and often based on interpretations of results obtained from artificial experimental systems. The events associated with the induction and maintenance of *M. tuberculosis*-specific immune responses are complex and there are still many questions to be answered. This chapter describes the mechanisms by which *M. tuberculosis* manipulates host cell functions and the specific immune responses that are induced during mycobacterial infection. The balance between the bacteria and the host is delicate and a large number of research studies have

been devoted to better understanding of the critical factors involved in the cellular and molecular regulation of this balance. As will be discussed, the interplay between innate and adaptive immune responses as well as different cells of the immune system is closely interconnected. A complex network of cells at different locations and stages of differentiation contribute to the induction of the *M.tuberculosis*-specific immune responses and the regulation of TB disease. In order to understand the pathogenesis of TB, it is therefore necessary to study and relate host-specific immune responses to the microbiological and biochemical events initiated by the bacteria that result in the establishment and progression of *M. tuberculosis* infection. This chapter describes experiments ranging from macrophage infections to immunological analysis of pathogenesis in clinical tissue and cell samples obtained from the site of infection of TB infected patients. The presented methods include molecular analysis of mycobacterial cell wall components, a method for rapid determination of bacterial numbers and *in situ* quantitative image analysis. The chapter is based on current literature where animal models and patient materials is integrated with comprehensive cellular and tissue model systems, which are used to explore *M. tuberculosis*-specific host-pathogen interactions in human TB. Basic knowledge and the recent advances in the field of host-mycobacterial interactions and how pathogenic mycobacteria can manipulate and escape the immune system will be discussed with a special focus on the knowledge obtained with experimental models in relation to human TB.

1.2 Mycobacterial structure

M. tuberculosis is a rod-shaped bacterium belonging to the family of Mycobacteria (*Mycobacteriaceae)*, which are gram-positive bacteria. The mycobacterial cell wall is rich in waxes and lipids, which contribute to the virulence of the bacteria in different ways (Shui, Petzold et al. 2011). Mycobacterial waxes are composed of diverse mycolic acids, which form a layer reminiscent of the outer membrane of Gram-negative bacteria (Bhamidi, Scherman et al.; Sani, Houben et al.). The waxy cell wall together with the recently described capsular layer, the major component of which is α-glucan, contribute to the extraordinary resistance of mycobacteria to stresses such as drought, low pH and antibiotics (Sani, Houben et al.; Liu, Barry et al. 1996). Pathogenic mycobacteria can regulate their cell wall thickness in response to stresses posed by host immunity (Cunningham and Spreadbury 1998) and there is recent evidence that the altered cell wall in stressed bacteria is refractory to acid-fast staining (Seiler, Ulrichs et al. 2003; Deb, Lee et al. 2009), a finding which may have important implications for diagnosis (Garton, Waddell et al. 2008).

1.3 The immunological checkpoints *M. tuberculosis* needs to pass

M. tuberculosis is a highly successful intracellular pathogen that has developed strategies to survive even in the presence of high immune pressure. The usual site of entry into the human body is through the airways, beginning with the inhalation of infected droplets expelled from another infected individual through coughing. The bacilli are transported into the respiratory tract to be engulfed by alveolar macrophages, cells that are designed to kill bacteria. Although being caused by a quite simple microorganism, TB is a multifaceted disease with a spectrum of antimicrobial effector pathways at play during different stages of infection, ranging from early innate to late adaptive immune responses during acute and

chronic infection. The infectious dose (the number of microorganisms required to cause infection) is very low, but nevertheless, most exposed individuals maintain the infection in a latent state. As the ability to control *M. tuberculosis* infection is strongly correlated with intact immune functions of the infected human host, individual differences in the ability to mount a proper immune response delivers an explanation for the low percentage of disease progressors. In order to cause active TB, *M. tuberculosis* has to pass several host immunity checkpoints (Barry, Boshoff et al. 2009). These checkpoints, illustrated in figure 1, include the initial attacks posed by innate immune mechanisms and the following adaptive immune response.

1.3.1 Checkpoint one: Avoiding being killed by the macrophage

The first checkpoint that *M. tuberculosis* has to overcome is to prevent itself from being killed by the antimicrobial effector mechanisms harboured by macrophages. Macrophage effector mechanisms include acidification of the phagosome, exposure to proteases and antimicrobial peptides and the generation of reactive oxygen and nitrogen species. The pathogen has evolved strategies to evade and/or tolerate these stresses, and manages to survive in cells that are otherwise effective killers of most microorganisms. However, there may be circumstances under which the bacteria are actually eradicated inside the human host. For example, it is well established that many subjects, who are continuously exposed to *M. tuberculosis* (*e.g.* household contacts of TB patients), do not display any immunological memory of the pathogen, as evidenced by the lack of reaction to interferon-γ release assays (IGRA) and tuberculin skin test (TST). These individuals possibly exert a massive innate immune pressure on the inhaled pathogens, making any adaptive immune reaction unnecessary. A clue to the mechanism behind this phenomenon comes from the observation that healthy household contacts of TB-patients produce high amounts of bactericidal compound nitric oxide (NO) (Idh, Westman et al. 2008). Although this observation needs further confirmation in human *in vitro* systems, it is possible that the ability of macrophages to produce sufficient amounts of NO actually allows the innate immune system to eradicate *M. tuberculosis* infection.

1.3.2 Checkpoint two: Defeating innate immunity

Failure of innate immune mechanisms to control the growth of the bacteria, possibly related to insufficient production of NO and other immune mediators, admits *M. tuberculosis* through the second checkpoint, after which adaptive immunity becomes important. The increasing immune pressure mounted by the adaptive immunity restores the immunological control. Latent TB is characterized by immune reactivity towards TB antigens (*e.g.* IGRA or TST) along with absence of any clinical symptoms. In the lungs tissues, healed or active granulomatous lesions at different stages may be present. Individuals with latent TB include non-progressors, in whom the infection is kept at equilibrium through host immune mechanisms and progressors, in whom the infection progresses towards active disease.

1.3.3 Checkpoint three: Defeating adaptive immunity

As long as immune control is maintained, *M. tuberculosis* infection is kept latent; however, sooner or later, the bacterium may take advantage of a declining immunocompetence of the

host, *e.g.* due to ageing, malnutrition, drug abuse, HIV infection or other immunosuppressive diseases or drug treatments. Thus, by passing the last checkpoint of adaptive immunity *M. tuberculosis* will inevitably cause its host to transmit the infection and eventually succumb, if left untreated. On the following pages, we will describe the protective immune defence mechanisms and the strategy that *M. tuberculosis* employs to get beyond these checkpoints.

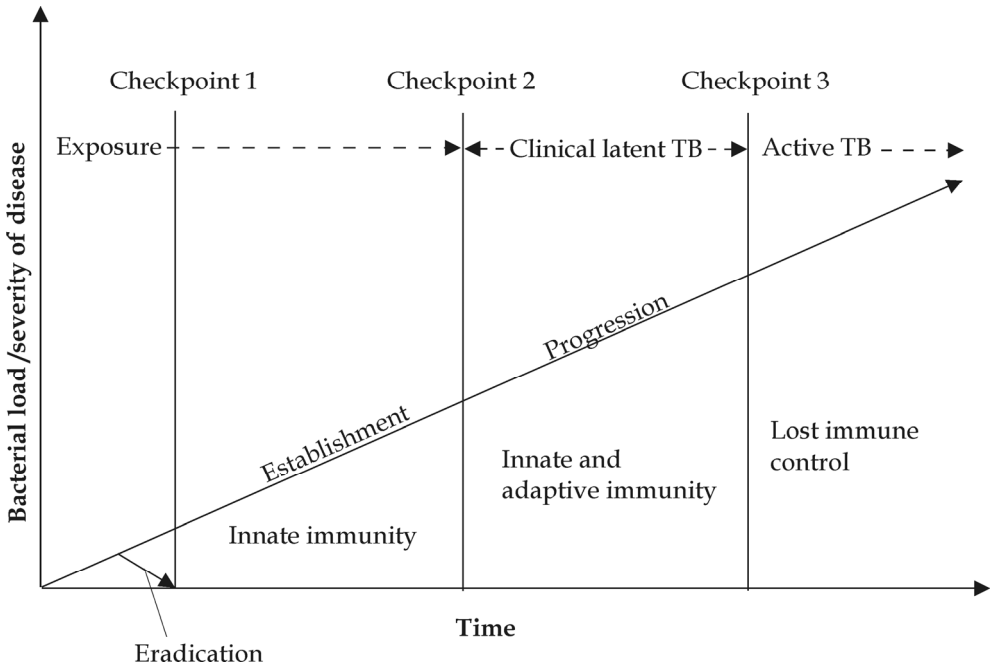

Fig. 1. The checkpoints that *M. tuberculosis* has to pass in order to cause TB. Checkpoint 1 is to avoid being killed by the very early immune mechanisms. Checkpoint 2 is to overcome innate immune control and thereby passing on to presentation to adaptive immunity. Checkpoint 3 is to defeat the effector mechanisms of adaptive immunity. The lost immune control admits effective replication of bacteria, which leads to necrosis and spread to other individuals.

2. Beyond checkpoint one: Establishment of *M. tuberculosis* infection

As mentioned above, the macrophage is the major host cell for *M. tuberculosis* infection, and the alveolar macrophage is described as the first cell that encounters *M. tuberculosis* on its journey within the host. As it is difficult to investigate the very initial events of mycobacterial infection in a living host, a major part of the studies on initial events is performed with macrophages monocultures, most often cell lines of human or murine origin or with macrophages derived from human or murine monocytes. It is well established that production of NO is strongly induced in murine macrophages during mycobacterial infection, but that isolated human macrophages fail to do the same upon *in vitro* infection.

Thus, there are important differences between mouse and human macrophages, which suggests that different macrophage immune mechanisms may exist in the two species. It is also important to realise that macrophages are heterogeneous even if obtained from the same individual and thus can have substantially different phenotypes depending on the protocol used for differentiation. In the body, the circulating precursor cells, the monocytes, are already heterogeneous when they enter the blood stream from the bone marrow. Subsequent differentiation in the various tissues further adds to the complexity. Nevertheless, experiments performed with pure macrophage cultures have substantially contributed to the understanding of *M. tuberculosis.*

2.1 Manipulation of phagosomal maturation

The alveolar macrophage is able to take up microorganisms into a vacuole that subsequently undergoes a process known as phagosomal maturation, where a series of fusion and fission events takes place to localize microbicidal activity to the vacuole. *M. tuberculosis* has evolved mechanisms to inhibit this process, and these mechanisms have been studied in detail (for review, see Vergne, Chua et al. 2004). The process of phagosomal maturation is traditionally viewed as a gradual process of complete fusion of lysosomes, which contain microbicidal effector molecules, with phagosomes. However, it turns out that this process is much more complex and selective than previously thought. Desjardins suggested that phagosomal maturation is a continuous process of fusion and fission of cellular organelles, the transfer of membranes and materials from these being a rather transient and highly specific event (Desjardins, Houde et al. 2005). There is evidence in the literature for separate sources of lysosomal proteins such as LAMPs and the proton pumps that are required for lowering the phagolysosomal pH. Whereas LAMPs can be found on both lysosomes and endosomes, the proton pumps can be recruited to the phagosome from the ER. Therefore, studies solely based on traditional markers for phagolysosomal fusion such as LAMPs may not reflect the actual milieu inside the phagolysosome. Instead, assays addressing more functional aspects of the phagosome must be employed. Fully matured phagolysosomes are known to have a pH below 5.0 (Russell, Vanderven et al. 2009), and the low pH is a prerequisite for functionality of the proteolytic, degradative enzymes delivered by lysosomes. Thus, phagosomal acidification, which can be studied using pH-reactive, fluorescent dyes, is a correlate of phagosomal activity.

2.1.1 Inhibition of phagosomal maturation through lipoarabinomannan

Mycobacterial lipids are known to be important for the bacterium to resist the host immune functions, the waxes being more like passive protectors, whereas other lipids exert more specific effects. The most studied glycolipid, lipoarabinomannan (LAM), is composed of glycophosphatidyl inositol (GPI), which functions as a lipid anchor, and a branched arabinomannan residue that extends through the mycobacterial cell wall to be exposed on the outside of the bacterium (Shui, Petzold et al. 2011). LAM differs between virulent, slow growing mycobacteria and their non-virulent, fast-growing relatives in that the virulent species carry mannosylated LAM (ManLAM) (Dao, Kremer et al. 2004). ManLAM has been shown to inhibit phagosomal maturation in both murine and human macrophages (Fratti, Chua et al. 2003; Hmama, Sendide et al. 2004; Kang, Azad et al. 2005), thus playing a central role in manipulation of an important host cell function. LAM can interact with different

receptors on the host cell surface, including the mannose receptor and the complement receptor (Le Cabec, Carreno et al. 2002; Kang, Azad et al. 2005) and during prolonged infection of cell cultures, the glycolipid is trafficked throughout the membrane compartments of the host cell (Xu, Cooper et al. 1994). In fact, LAM can be detected in the urine of TB-patients, illustrating the extensive distribution of this molecule in *M. tuberculosis*-infected hosts (Hamasur, Bruchfeld et al. 2001). Although the ability of LAM to inhibit phagosomal maturation is well established, the mechanism by which it causes this effect has not been addressed by many groups. To date, there is no mycobacterial strain, which is specifically deficient in the production of ManLAM, making mechanistic studies difficult. A frequently used model to study LAM function is based on LAM-coated latex beads (Hmama, Sendide et al. 2004; Kang, Azad et al. 2005), and these studies have provided evidence for the inhibitory effect of ManLAM on phagosomal maturation but failed to provide a mechanism by which this occurs. In order to allow mechanistic studies of LAM functions, we created a unique model based on the previously established fact that LAM can be intercalated into the host cell membrane via its GPI anchor (Ilangumaran, Arni et al. 1995). Assuming that at least initially, the membrane surrounding the phagosome is more or less derived from the plasma membrane, we allowed cells that had incorporated LAM in their plasma membrane to phagocytose opsonised zymosan particles. Resulting phagosomes displayed a reduced ability to attract markers of phagosomal maturation in comparison to control conditions (Welin, Winberg et al. 2008), a phenomenon which was dependent on the ability of LAM to incorporate into the lipid rafts. There are ongoing studies investigating this phenomenon in more detail (Torrelles and Schlesinger 2011). Albeit a totally unrelated pathogen, the protozoan parasite *Leishmania donovani* carries a glycolipid lipophosphoglycan (LPG), which is structurally related to LAM, in its cell membrane. In analogy with LAM, LPG is shed into the lipid rafts of host macrophages during infection with *L. donovani*, also causing inhibition of phagosomal maturation, thereby providing the parasite with a suitable niche inside the host cell (Winberg, Holm et al. 2009).

2.1.2 The abilities of non-virulent *vs.* virulent strains to manipulate host cell functions

Many studies on the ability of *M.tuberculosis* to inhibit phagosomal maturation are based on the attenuated vaccine strain of *M. bovis*, better known as Bacillus Calmette-Guérin (BCG). Although this strain is not able to grow inside macrophages or to cause disease (at least not in immunocompetent individuals), it seems to be as capable as virulent strains of its relative, *M. tuberculosis* to manipulate phagosome functions (Jayachandran, Gatfield et al. 2008; Sun, Wang et al. 2010). Indeed, LAM from the BCG strain is of the ManLAM type, showing no major structural difference to ManLAM from virulent strains (Prinzis, Chatterjee et al. 1993). Thus, the BCG strain and other attenuated strains of *M. tuberculosis* can be exploited as a relevant model for initial events during mycobacterial infection (Rhoades, Hsu et al. 2003; Jayachandran, Sundaramurthy et al. 2007). The reason for the lacking virulence of BCG is attributed to the deletion of the Region of Difference-1 (RD1) in its genome (Lewis, Liao et al. 2003). This region encodes a Type VII secretion system and proteins that are secreted via this system. These proteins, termed Early Secreted Antigenic Target-6 (ESAT-6) and Culture Filtrate Protein-10 (CFP-10) have both been identified as important for virulence (Smith, Manoranjan et al. 2008). Interestingly, they seem to be secreted as a one-to-one complex, the CFP-10 protein being a putative chaperone to assist ESAT-6 folding (de Jonge, Pehau-Arnaudet et al. 2007). There is no evidence of a role for ESAT-6 for the inhibition of

phagosomal maturation, but instead it is involved in the events that takes place after the initial uptake. Therefore, extended infection experiments are required to obtain a more holistic picture of *M. tuberculosis* infection.

2.2 The interaction between *M. tuberculosis* and the macrophage during prolonged infection

Most of the earlier studies on the interaction of *M. tuberculosis* with the host macrophage are based on short-term experiments of a few hours up to one day. Until recent years, researchers have underestimated the importance of extended infection experiments to study a chronic infection with a slow-growing bacterium such as *M. tuberculosis*. Thus, later studies performed with human macrophages and virulent bacteria have provided insight into the broader strategy of *M. tuberculosis*, which is certainly not limited to a life within a vacuole. Instead, it seems that the pathogen, after adapting to the intracellular environment by interfering with phagosomal maturation, will disrupt the phagosome, enhance replication and eventually kill the host cell.

2.2.1 New experimental models for studies of *M. tuberculosis* interaction with the host macrophage

As outlined above, the fate of mycobacteria inside macrophages is more difficult to predict than previously assumed and it becomes crucial to study mycobacterial replication inside macrophages under varying conditions. The available methods for assessment of intracellular bacterial growth have long been limited to viable counts or incorporation of radioactive markers into growing bacteria. Therefore, alternative methods for these studies of *M. tuberculosis* that could be performed more effectively are convenient. Such methods, run on a medium-throughput scale, allow more thorough analysis of intracellular fate of *M.tuberculosis* in terms of extended time of infection, bacterial factors contributing to the ability of the bacterium to establish infection and varying conditions that support the host cell to control the infection. To this end, we validated the use of *M. tuberculosis* bacteria, engineered to express luciferase, against the gold standard determination of colony forming units (CFU). We found that luciferase-based determination of bacterial numbers was actually superior to the CFU-method in accuracy as determined by intraassay variation (Eklund, Welin et al. 2010) when run in a 96-well plate format. The use of luciferase, which requires a co-factor, $FMNH_2$ that is present only in living bacteria, allows inclusion of viable bacteria only. Fluorescent markers such as GFP are also used to enumerate bacteria; however, a loss of viability does not necessarily correlate with loss of fluorescence. Many studies that measure replication of mycobacteria inside cells do not include parallel measurements of extracellular and intracellular bacteria. The easy handling of the 96-well plates and the efficient readings with the plate reader with luminometry function admit measurements of both extracellular and intracellular growth, without the need for additional manipulation of the system (*e.g.* addition of extracellular antibiotics). Another shortcoming of many studies on intracellular growth of mycobacteria is that cell viability is not addressed. If the number of bacteria is determined in a lysate of adherent cells, it is very important to know how many cells were left, as loss of cells would result in loss of measurable bacteria. Thus, many studies that conclude that mycobacteria were killed by the macrophages cannot rule

out cell loss as cause of reduced numbers of CFUs. The use of luminometry for determination of bacterial numbers as in our system allows fluorescence-based measurement of cell viability in the same wells, which gives reliable data on the host-pathogen balance in the individual experiments.

2.2.2 Phagosomal acidification and control of mycobacterial growth

Using the described method, we have established that depending on the initial bacterial load, non-activated human macrophage cultures will experience different fates when infected with virulent *M. tuberculosis*. Whereas a higher bacterial load results in effective bacterial replication and rapid cell death (further discussed below); a lower bacterial load results in a state where the macrophage controls the pathogen. The latter situation provides us with a tool to study the immune mechanisms that the macrophage employs to control the infection ("balanced infection").

By assessing the macrophage functions that were at play during the balanced infection, we narrowed down acidic pH of the phagosome and functional cathepsin D as essential for controlled infection, whereas the generation of reactive oxygen species did not appear to play a role in this context (Welin, Raffetseder et al. 2011). We found that the restricted intracellular growth of *M.tuberculosis* the ability of the macrophage to acidify phagosomes, but not to translocate LAMPs. This finding is supported by earlier studies, where the proton pumps (v-ATPase) but not LAMP-1, were excluded from mycobacterial phagosomes (Sturgill-Koszycki, Schlesinger et al. 1994). We were not able to delineate whether the acidic pH itself was harmful to the bacteria, but given the relative resistance of mycobacteria to low pH, it was more likely the activation of hydrolytic enzymes (such as cathepsin D) inside the acidic compartments that conveyed the killing activity.

2.2.3 Killing of *M. tuberculosis* or just growth restriction

The reduction of the net growth of the bacteria observed with the balanced infection could be explained by either absence of growth or by a steady-state between growth and killing. Importantly, we have never been able to see a reduction in bacterial numbers using the human macrophages, which would point to a net killing of bacteria. Other groups have reported killing of mycobacteria by macrophages (Thoma-Uszynski, Stenger et al. 2001; Fortune, Solache et al. 2004), however, many reports interpret reduced growth of mycobacteria as killing. Ehrt and colleagues showed that even in IFN-γ-activated mouse macrophages, virulent *M. tuberculosis* were not killed but rather subject to growth restriction (Vandal, Pierini et al. 2008). Only deletion of a specific membrane protein of *M. tuberculosis*, Rv3671c, resulted in loss of bacterial viability. The authors further showed that this virulence factor is crucial for the maintenance of intrabacterial pH, as assessed by a pH sensitive variant of GFP. Together, these studies suggest that replication and induction of host cell death is only one strategy employed by *M. tuberculosis* when it enters the cell. The other strategy, which occurs if the macrophage is able to mount a significant level of phagolysosomal activity, is to switch the phenotype to a slower growth rate associated with enhanced ability to resist the harsh environment inside the host cell, allowing it to persist for extended time. Thus, the inhibition of phagosomal maturation may be rather a means to buy time to induce the optimal phenotype. Ageing of the

macrophage eventually unleashes the growth, but as long as immunosurveillance is strong, uptake by neighbouring cells will not result in significant net growth. This hypothesis is summarized in figure 2.

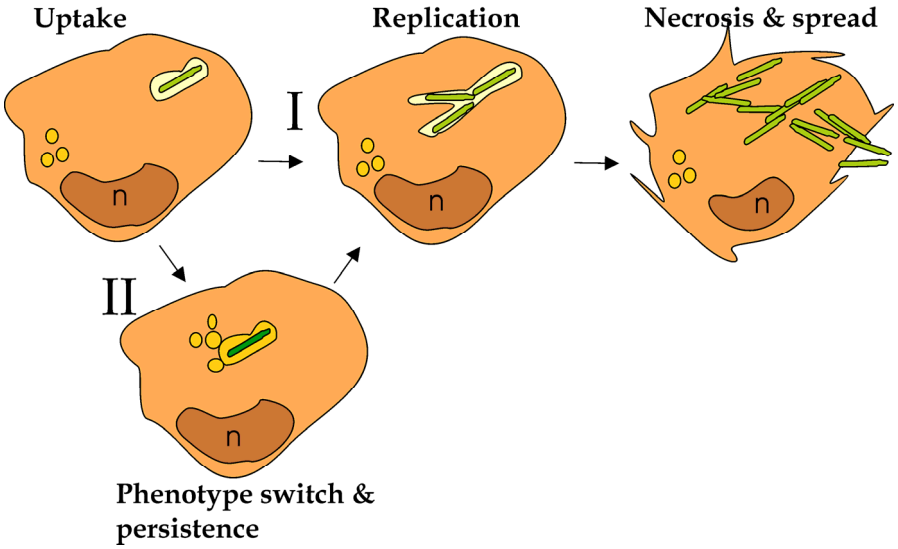

Fig. 2. Schematic view of two strategies that *M. tuberculosis* employs during infection of the macrophage. The first one (I) is to replicate after successfully inhibiting phagosomal maturation. The second (II), which can be used if the macrophage manages to acidify the phagosome, is to switch to a phenotype that tolerates the stresses posed by the macrophage. Ageing of the macrophage eventually allows the bacterium to resume growth.

2.2.4 Host-protective apoptosis *vs.* detrimental necrosis

In 2007, Peter Peters and colleagues demonstrated that *M. tuberculosis* can, like the close relative *M. marinum* (Stamm, Morisaki et al. 2003), escape from the phagosome and replicate inside the macrophage cytoplasm (van der Wel, Hava et al. 2007). The issue has been a matter of contradiction during the past few years and many studies have emphasized the absence of bacilli void of phagosomal membranes in many experimental settings and cells obtained from infected humans. Given the facts that host cell death follows the mycobacterial escape from the phagosomes and that remnants of dead cells are rapidly removed in tissues by residual macrophages, it is logical to assume that any such event is difficult to capture in alveolar macrophages and tissues. Regardless, the protocols used for preparation of and the origin of macrophages (mouse *vs.* man) may be crucial determinants of the outcome for *M. tuberculosis* during experimental infection, and this may cause different results in the hands of different research groups. The mechanisms by which the phagosomal escape occurs are currently being investigated. A favoured candidate is ESAT-6, which plays an important role for the phagosomal escape in *M. marinum*, probably through its membrane-damaging properties (Smith, Manoranjan et al. 2008). In any case, phagosomal escape is likely to precede host cell death. There is conflicting evidence for the

mode of cell death induced by *M. tuberculosis.* In fact, there is convincing evidence that *M. tuberculosis* actively prevents apoptosis by the action of the protein nuoG (Velmurugan, Chen et al. 2007). The mechanism, by which this occurs, remains elusive, but a microbial rationale for apoptosis prevention is that this mode of cell death seems to be lethal to intracellular pathogens (Keane, Remold et al. 2000). In contrast to apoptotic cell death, which proceeds in a programmed, quiescent manner to avoid excessive inflammation, necrosis is an uncontrolled loss of cell integrity caused by different kinds of stresses such as mechanical forces, heat or infections. Typically, cellular contents, such as ATP, High Mobility Group Box (HMGB)-1 protein and DNA are released in the extracellular matrix. These endogenous danger signals attract the attention of innate immunity by interacting with purinergic and pattern recognition receptors, causing release of inflammatory cytokines and chemokines. Active tuberculosis lesions are associated with excessive inflammation and necrosis. It is a matter of debate whether inflammation is good or bad for *M.tuberculosis*. An illustrative example comes from the observations with IL-1β. This pro-inflammatory cytokine is crucial for the control of mycobacterial infection in mice as deficiencies in the production of the cytokine or its receptor causes more severe symptoms (Sugawara, Yamada et al. 2001; Mayer-Barber, Barber et al. 2010). On the other hand, *M. tuberculosis* has been shown to activate the NLRP3 inflammasome, which is a protein complex responsible for IL-1β production in macrophages (Petrilli, Dostert et al. 2007). Mycobacteria-induced production of IL-1β causes destructive inflammation in the mouse, the conclusion being that IL-1β is deleterious for the host (Carlsson, Kim et al.). Thus, IL-1β can act like a double-edged sword, in analogy to the pro-inflammatory cytokine TNF-α, which is required for TB control, but is also a component of accelerated inflammation during disease progression (Mootoo, Stylianou et al. 2009).

The recently described modes of cell death termed pyroptosis and pyronecrosis due to the association with the pyrogenic (fever-causing) cytokine IL-1β, has been described to occur during *Salmonella* and *Neisseria* infection, respectively (Fink and Cookson 2007; Duncan, Gao et al. 2009). As we found that a higher bacterial load resulted in cell death and a massive release of IL-1β, we sought to determine whether either of these novel modes of cell death was at play. Thorough analysis of the pattern of cell permeability, nuclear fragmentation and mitochondrial damage pointed to a more disorganized form of cell death of human macrophages infected with virulent *M. tuberculosis*. Any attempt to inhibit apoptosis (Caspase-dependent), pyroptosis (Caspase-1-dependent) and pyronecrosis (Cathepsin-B-dependent) failed, suggesting that the fate of the cells was necrosis. We turned to investigate the microbial factors that were causing necrosis, selecting ESAT-6 as a possible candidate. Deletion of this well-known but poorly understood mycobacterial virulence factor showed a clear relationship with the necrosis-causing ability of *M. tuberculosis.* Another report arrived at the same conclusion that macrophages are killed by *M. tuberculosis* in a necrosis-like fashion. However, the authors correlated the ability of the bacterium to induce cell death to functional PhoP two-component signalling instead of an intact RD-1 locus, in which ESAT-6 and the ESX-1 machinery is localized. The reason for this discrepancy remains elusive, but again, the different cell types used (human monocyte-derived macrophages in our study *vs.* bone-marrow-derived mouse macrophages in the other study) are possible reasons for the differing results.

3. Beyond checkpoint two: Interplay between innate and adaptive immunity and *M. tuberculosis*

As described above, *M. tuberculosis* is able to survive within the host even during a strong inflammatory response. The ability of the bacterium to enter and persist in the phagosomal system (Clemens and Horwitz 1995) is essential for establishment of infection, but also to avoid and/or manipulate protective host immune responses (Flynn and Chan 2003). Elucidation of the immune mechanisms that control the initial infection and prevent reactivation of latent TB is ongoing. Certainly genetic and environmental variations exist within the human population that may significantly affect an individual's susceptibility to develop active TB disease. However, pathogenic mycobacteria and their virulence factors can also modulate specific host immune responses, delay the onset of crucial anti-TB immunity and evade antimicrobial effector functions. Pathogenic mycobacteria are well adapted to the human host and have a range of complementary evasion mechanisms that contribute to the ability to avoid elimination by the immune system and establish a persistent infection (Tufariello, Chan et al. 2003; Gupta, Sharma et al. 2010). A close interplay between both innate (Jordao and Vieira 2011) and adaptive (Urdahl, Shafiani et al. 2011) antimicrobial effector pathways are required to control the progression of *M. tuberculosis* infection. Since the induction of the adaptive immune response including clonal expansion of antigen-specific T cells takes time, the innate immune system provides a first line of defence upon infection. In this section, we describe and discuss different antimicrobial effector pathways that are vital to maintain a balance between the mycobacteria and its host.

3.1 Induction and regulation of innate immune responses in *M. tuberculosis* infection

While macrophages are the primary host cell to be infected with *M. tuberculosis*, providing a shelter for growing bacteria, macrophages are also key effector cells with the ability to eliminate intracellular bacteria. Another innate immune cell with important antigen presenting and T cell activating functions is the dendritic cell (DC). Both macrophages and DCs are major players in the induction of proinflammatory responses in the early stages of TB infection and later on bridge innate immunity to the adaptive immunity.

3.1.1 Nitric oxide

In the initial phase of *M. tuberculosis* infection, infected host macrophages can be activated to become bactericidal by producing compounds such as oxygen and nitrogen radicals, primarily NO, which is important in order to restrict intracellular growth of bacteria. NO has been shown to be critical to control *M. tuberculosis* infection in mice (Chan, Xing et al. 1992; MacMicking, North et al. 1997; Scanga, Mohan et al. 2001; Reece, Loddenkemper et al. 2011) and it was recently shown that NO-mediated apoptosis of *M. tuberculosis*-infected murine macrophages was required for efficient mycobacterial killing (Herbst, Schaible et al. 2011). Moreover, inhibition of phagolysosomal maturation mediated by the *M. tuberculosis*-specific lipid trehalose dimycolate (TDM) is fully abrogated in IFN-γ/LPS-activated murine macrophages and involves NO synthase (NOS2) and reactive nitrogen intermediates (RNI), which suggests that macrophage-specific NO can promote inactivation of a mycobacterial molecule with an essential virulence function (Axelrod, Oschkinat et al. 2008). These results also support the assumption that NO-mediated mycobacterial clearance is dependent on acidification of *M. tuberculosis*-containing phagosomes (MacMicking, Taylor et al. 2003).

Despite substantial evidence of a protective role of NO in the mouse, the importance of NO-mediated elimination of *M. tuberculosis* in humans is controversial. Our group as well as others have used immunohistochemistry or mRNA analysis to show that NO expression can be detected in human *M. tuberculosis*-infected macrophages *in vivo* (Nicholson, Bonecini-Almeida Mda et al. 1996; Nathan 2002; Schon, Elmberger et al. 2004; Andersson, Samarina et al. 2007). There is also a synergistic effect of vitamin D and IFN-γ treatment to enhance *M. tuberculosis*-induced NO synthesis and bacterial killing in human monocyte-derived macrophages *in vitro* (Lee, Yang et al. 2009). Inhibition of NO-production *in vitro* using compounds such as L-NMMA or NMA promote intracellular *M. tuberculosis* growth, which suggests that NO indeed has a significant function in the human defence against *M. tuberculosis* (Jagannath, Actor et al. 1998). However, some strains of *M. tuberculosis* have developed strategies to neutralize the actions of NO and RNI by expressing a detoxifying protein encoded by the AhpC gene (Chen, Xie et al. 1998; Master, Springer et al. 2002). Interestingly, apart from its bactericidal activity, NO may possess regulatory effects on T cell activation leading to impaired effector functions (Bingisser, Tilbrook et al. 1998; Angulo, de las Heras et al. 2000; Hoffman, Mahidhara et al. 2002). As local production of oxidative stress and NO may severely impair the immune system, these effectors can mediate both beneficial and detrimental effects during mycobacterial infection.

Whereas NOS2 gene-disrupted mice have been used to show that NO and RNI are required to control murine TB (Scanga, Mohan et al. 2001; Reece, Loddenkemper et al. 2011), most methods to evaluate the relevance of NO in humans are indirect and dependent on detection of different NO synthases or specific NO-metabolites such as nitrate. Thus, reliable methods for direct assessment of NO are missing. The detection of NO radicals in tissues is particularly difficult due to the short lifetime and relatively low concentration of these compounds. Hence, better techniques are required to be able to test if the expression of NO synthases corresponds to the induction of an active enzyme. Simultaneous assessment of the NO synthase and metabolites can be used to overcome part of this problem. Furthermore, an important difference between mouse and human macrophages is their ability to induce either NO or the NO synthase *in vitro*. It is widely accepted that LPS and IFN-γ can be used to activate robust NO production in murine macrophages, while very few publications show a similar induction of NO in human macrophages *in vitro*. The failure to induce NO in human macrophages under the same conditions that works for mouse macrophages (Arias, Zabaleta et al. 1997), support the hypothesis that NO possibly plays a more profound role in murine TB compared to human TB. It will be a future challenge to develop better model systems to continue to study and evaluate the bactericidal function of NO in human TB and also in relation to the potential suppressive effects on T cell responses at the site of TB infection. In this regard, we are currently establishing TB infection in a lung tissue model that we use to mimic human TB infection in the context of a more relevant physiological environment. The model consists of 3-dimensional organotypic cultures of stromal cells as well as primary human macrophages infected with virulent *M. tuberculosis*, which provides us with an experimental tool that can be used for mechanistic studies on human TB.

3.1.2 Antimicrobial peptides

Innate immune effector mechanisms in TB also include the induction and action of antimicrobial peptides (AMPs) such as human cathelicidin, LL-37 (Liu, Stenger et al. 2007;

Martineau, Newton et al. 2007). These are small cationic molecules, with the potential to kill microbes at mucosal surfaces. In addition to its antimicrobial properties, LL-37 is pro-inflammatory and could stimulate migration of various cell types (Coffelt, Marini et al. 2009) and also affect the inflammatory functions of neutrophils. Importantly, AMPs may be crucial to prevent initial uptake of mycobacteria in the respiratory tract. It has also been shown that LL-37 confers protection against *M. tuberculosis* infection by the induction of autophagy in human monocytes (Yuk, Shin et al. 2009). Autophagy is an important physiological process that involves degradation of intracellular components after fusion of autophagosomes and lytic lysosomes. Among other functions, autophagy is an important defence mechanism to inhibit intracellular survival of mycobacteria since it can overcome the phagosomal maturation block induced by *M.tuberculosis* (Gutierrez, Master et al. 2004). Although activated by different signalling pathways, the induction of both LL-37 and autophagy in human macrophages is dependent on the presence of active vitamin D. It is well known that individuals with vitamin D deficiencies have an increased susceptibility to TB, which may be associated with reduced production of LL-37 as well as reduced autophagy (Liu, Stenger et al. 2006; Liu, Stenger et al. 2007). Our unpublished observations suggest that vitamin D deficiency in a cohort of Russian TB patients with chronic pulmonary TB correlates with reduced levels of LL-37 in pulmonary TB lesions. More studies are needed to investigate the regulation of LL-37 expression *in vivo* and *in vitro* and whether mycobacteria have developed strategies to circumvent the function of this antimicrobial peptide.

3.1.3 Toll-like receptors

The capacity of the innate immune system to recognize foreign antigens is restricted to a set of conserved molecular patterns called pathogen-associated molecular patterns (PAMPs). The receptors for PAMPs are called pattern-recognition receptors (PRR), to which the Toll-like receptors (TLRs) belong. TLRs are PRRs that specifically recognize certain microbial antigens, including mycobacterial proteins and lipids. Antigen presenting cells (APCs), such as macrophages and DCs, express several different TLRs, which control the activation and induction of antimicrobial functions in the cells. Furthermore, TLR-stimulated APCs will interact with *M. tuberculosis*-specific T cells and induce clonal differentiation and expansion of these T cells. During mycobacterial infection, recognition by TLR 1, 2, 4, 8 and 9 are important for maturation of APCs including up-regulation of antigen-presenting molecules, production of inflammatory cytokines and chemokines and also the induction of antimicrobial effector functions (Means, Wang et al. 1999). TLRs mainly recognize lipid-rich mycobacterial molecules such as LAM (van Crevel, Ottenhoff et al. 2002) and TDM (Bowdish, Sakamoto et al. 2009). TLR2/1 activation, which triggers LL-37 production in a Vitamin D-dependent manner, has been shown to enhance the killing of *M. tuberculosis* bacilli in human macrophages (Liu, Stenger et al. 2006). In addition, mycobacterial cell wall products can regulate CD1 antigen presentation through TLR2 signalling (Roura-Mir, Wang et al. 2005). On the other hand, pathogenic mycobacteria have developed mechanisms to manipulate TLR-induced signalling pathways and may thus suppress important antimycobacterial immune responses (van Crevel, Ottenhoff et al. 2002; Bowdish, Sakamoto et al. 2009). In this regard, *M. tuberculosis* ManLAM can bind to the C-type lectin DC-SIGN on the surface of DCs and actively interfere with TLR-mediated DC maturation (Geijtenbeek, Van Vliet et al. 2003). Prolonged exposure to *M. tuberculosis* 19kD lipoprotein

inhibits MHCII expression as well as alternative MHC-1 antigen processing and presentation by IFN-γ activated macrophages via TLR2, which may allow the bacteria to decrease recognition by CD4+ and CD8+ T cells and maintain chronic infection (Noss, Pai et al. 2001; Tobian, Potter et al. 2003; Pecora, Gehring et al. 2006). The ESAT-6 protein of *M. tuberculosis* can also interact with TLR2 and inhibit TLR signalling in macrophages in order to restrict innate immune responses (Pathak, Basu et al. 2007). Moreover, an elegant study recently provided strong evidence that active release of mycobacterial vesicles contain TLR2 lipoprotein agonists, which contribute to an enhanced pulmonary inflammation and progression of TB in infected mice (Prados-Rosales, Baena et al.). Cytokine analysis indicated the induction of atypical proinflammation since large amounts of immunosuppressive IL-10 was also produced by macrophages treated with mycobacterial vesicles. Altogether, these studies show that TLR interactions with *M. tuberculosis* ligands can activate antimicrobial and antigen presenting functions of macrophages and DCs but can also be exploited by the bacteria to escape the host immune response.

3.2 Induction and regulation of adaptive immune response in *M. tuberculosis* infection

Induction of cell-mediated immunity and the formation of a granulomatous response are mandatory in human TB. Many immune cells are involved in this process that is guided by CD4+ helper T cells and CD8+ cytolytic T cells (CTLs). These T cell subsets possess complementary and interacting functions, which are therefore hot targets for mycobacterial manipulation, as improper activation of this arm of immunity facilitates bacterial invasion of the host.

3.2.1 Th1 effector cells

CD4+ T cells are so-called helper T cells (Th cells) with a specialized function in cytokine production in order to activate CTLs as well as B cells and other professional APCs. However, CD4+ T cells are also important for the activation of immune cells through specific receptor-ligand interactions. Differently polarized subsets of CD4+ T cells including Th1, Th2 and Th17 cells secrete distinct patterns of cytokines, which will control the fate of the CD4+ mediated immune response in TB. Therefore CD4+ T cells play a central role in the induction and maintenance of immune regulation during TB infection. Th1 effector cells mainly produce IL-12, IFN-γ, TNF-α and IL-2 and induce cell-mediated immunity which is fundamental for immune protection in TB infection. Accordingly, patients with mutations in the IL-12 or IFN-γ receptor genes have a strongly enhanced susceptibility to develop active TB (Newport, Huxley et al. 1996; de Jong, Altare et al. 1998). Particularly, IFN-γ activation renders the macrophages capable of killing intracellular mycobacteria by overcoming the block in phagosomal maturation and also stimulates production of microbicidal effectors including NO. In addition, patients with latent TB who are treated with anti-TNF-α antibodies rapidly develop active TB (Keane, Gershon et al. 2001; Bruns, Meinken et al. 2009). A newly discovered subset called Th17 cells produce IL-17, IL-22 and IL-23, which has been shown to regulate the production of antimicrobial peptides (Liang, Tan et al. 2006) as well as the activation and recruitment of IFN-γ expressing T cells at mucosal sites including the lung (Khader, Bell et al. 2007). Th1 and Th17 effector cells cooperate to induce protective immunity in TB.

3.2.2 Th2 effector cells

In contrast, Th2 effector cells producing IL-4, IL-5, IL-9 and IL-13, regulate the differentiation of antibody secreting plasma cells that has been shown to enhance intracellular persistence of *M. tuberculosis* (Potian, Rafi et al.). Thus, a dominant Th2 response can undermine Th1-mediated immunity and drive inappropriate alternative activation of macrophages (Rook 2007). The traditional view is that IFN-γ activated or classically activated (M1) *M. tuberculosis*-infected macrophages, produce high amounts of proinflammatory cytokines and NO and become highly bactericidal. However, recent evidence suggests that the initial M1 activation is followed by alternative activation (M2) of *M. tuberculosis*-infected macrophages in the lung, which would support a switch in macrophage polarization upon progression of TB disease (Redente, Higgins et al. 2011). IL-4 and IL-13 could promote alternative macrophage activation characterized by collagen deposition and formation of fibrosis in the inflamed tissue, which are typical traits of advanced TB disease. IL-4 and IL-13 have also been shown to block autophagy-mediated killing of mycobacteria in both murine and human macrophages (Harris, De Haro et al. 2007). Thus, *M. tuberculosis* virulence factors may interfere with M1 polarization and instead promote polarization of alternatively activated M2 macrophages or deactivated macrophages that are immunosuppressive and poorly microbicidal (Benoit, Desnues et al. 2008).

3.2.3 Regulatory T cells

CD4+ T cells may also be induced to acquire regulatory functions and to secrete immunosuppressive cytokines like TGF-β and IL-10 rather than the classical Th1 or Th2 cytokines. There is plenty of evidence that *M. tuberculosis* can induce specific regulatory T (Treg) cells that potently restrict protective immune responses during human TB. Increased levels of FoxP3+ Treg cells have been discovered in blood (Ribeiro-Rodrigues, Resende Co et al. 2006), lung (Guyot-Revol, Innes et al. 2006), lymph nodes (Rahman, Gudetta et al. 2009) and pleura (Chen, Zhou et al. 2007) of patients with active progressive TB. Induction of FoxP3+ Treg cells is also evident in murine TB (Shafiani, Tucker-Heard et al.). *In vitro* experiments demonstrate that ManLAM induces expansion of human Treg cells through mechanisms that depend on and prostaglandin E2 (PGE2) (Garg, Barnes et al. 2008) or PD1 (Periasamy, Dhiman et al.). These Treg cells produced significant amounts of TGF-β and IL-10 and inhibited IFN-γ expression by autologous CD4+ and CD8+ T cells. Several studies provide evidence that Treg cells could suppress antigen-specific IFN-γ production by human T cells, by which mechanism they would limit immunopathology but also down-regulate cellular immunity in TB (Hougardy, Place et al. 2007; Li, Lao et al. 2007; Li and Wu 2008). Thus, local CD4+ T cell responses could be inhibited, which may result in a failure to recruit CD8+ effector T cells to the granulomatous lesions in TB. Premature induction of an immunosuppressive Treg response may blunt important CTL activity and instead enhance pathological alterations in TB infected tissue.

3.2.4 Polyfunctional T cells

A popular concept that has been intensively studied is the induction of polyfunctional T cells that has been demonstrated to correlate with immune protection in several chronic

intracellular infections including TB (Darrah, Patel et al. 2007; Forbes, Sander et al. 2008). Polyfunctional T cells are characterized by the simultaneous production of effector molecules such as several Th1 cytokine co-expressed with inflammatory chemokines and markers for CTL degranulation. Even if several studies show that polyfunctional T cells have an impact on TB immunity, we still lack specific immune signatures or correlates of immune protection that could be used as specific biomarkers in novel vaccine- and drug development. Expansion and activation of Treg cells and Th2 (IL-4, IL-13) cells in chronic TB infection may prevent important polyfunctional Th1 responses and the development of fully functional CTLs. Our research group applies multicolor flowcytometry, multiplex mRNA and luminex analysis to characterize polyfunctionality and unravel the complex network and functional relationship between cytokine and chemokine profiles in lung vs systemic circulation of patients with active TB compared to uninfected controls. We also plan to use a systems biology approach to address immunological defects induced by mycobacteria and associated with progression of clinical disease.

3.2.5 Cytolytic T cells

CD4+ Th1 cells activate CD8+ CTLs which act in concert with natural killer (NK) cells as professional killers of *M. tuberculosis*-infected target cells. Activation mediated by CD4+ T cells or provision of other co-stimulatory signals provided by professional APCs are necessary to trigger the differentiation and maturation of CTLs. CTL-induced destruction of *M. tuberculosis*-infected cells is characterized by lysis of the cell membrane as well as disruption of the cell nucleus and characteristic fragmentation of target cell DNA. CTL-mediated lysis is primarily executed by one of two different mechanisms, involving either perforin- or Fas/Fas-ligand(L)-based killing. However, whereas perforin-mediated killing seems to be crucial for the elimination of infected target cells (Canaday, Wilkinson et al. 2001), Fas-mediated killing seems to play a major role in lymphocyte homeostasis (Kremer, Estaquier et al. 2000; Watson, Hill et al. 2000). Interestingly, *M. tuberculosis* can induce an increased expression of FasL on infected macrophages which may induce apoptosis among Fas expressing cells like CTLs and Thl cells (Mustafa, Phyu et al. 1999). Death receptor ligand-mediated apoptosis could also lyse *M. tuberculosis*-infected cells in the absence of bacterial killing and thus contribute to activation-induced cell death of CTLs. Here, it has been suggested that disease progression is associated with Fas/FasL mediated apoptosis of *M. tuberculosis*-reactive T cells (Li, Bassiri et al. 1998; Rios-Barrera, Campos-Pena et al. 2006).

Hence, only CTLs using the perforin-dependent, granule-mediated pathway of target cell killing are effective in decreasing the viability of intracellular M. *tuberculosis* and properly control the infection (Stenger, Mazzaccaro et al. 1997; Lewinsohn, Bement et al. 1998; Silva and Lowrie 2000; Samten, Wizel et al. 2003). Perforin is a pore-forming protein that is stored in the cytolytic cell within cytoplasmic granules together with other lytic and antimicrobial molecules called granzymes and granulysin (Stenger, Hanson et al. 1998; Okada, Li et al. 2003). Similar to the actions of human cathelicidin, the antimicrobial peptide granulysin can interact with the negative cell surface of the bacteria and induce osmotic lysis (Ernst, Thoma-Uszynski et al. 2000). Importantly, mice lack a known homologue of granulysin, which again indicates that mouse TB is regulated differently from human TB. The capacity

of granulysin to kill *M. tuberculosis* bacilli located inside infected cells is dependent on the pore-forming properties of perforin. Upon binding to a specific target cell, the CTL reorients these lytic granules to the site of cell-cell contact and releases its contents in the intercellular space between itself and the infected target cell. Perforin will integrate into the target cell membrane in a Ca^{2+}-dependent manner and create polyperforin pores, which result in permeabilization and osmotic failure of the target cell. It is also believed that perforin forms pores in cellular membranes to facilitate entry and endosome-mediated transportation of granzymes and/or granulysin to the intracellular compartments via a newly identified membrane-repair mechanism (Keefe, Shi et al. 2005). Interestingly, we have found that short peptides of granulysin are particularly effective in killing of slow-growing multidrug resistant TB (MDR-TB) strains with a reduced fitness phenotype (Toro, Hoffner et al. 2006). These results suggest that a cost of resistance, measured as reduced growth among MDR-TB strains, could be associated with increased susceptibility to natural immune defence mechanisms, such as antimicrobial peptides of granulysin. However, a robust cell wall as well as the membrane of host cells still provides physical shelter for the bacteria that may spare them from being killed.

Individuals with active, progressive TB disease most likely have an inadequate up-regulation of *M. tuberculosis*-specific CD8+ CTLs and cytolytic effector molecules, resulting in insufficient killing of infected cells and bacilli at local sites of TB-infection. Importantly, impaired CTL function has been associated with the progression of clinical TB disease, particularly in anti-TNF treated patients with autoimmune diseases, who demonstrate selective depletion of perforin and granulysin expressing CD8+ CTLs (Bruns, Meinken et al. 2009). We have previously discovered that an impaired expression of both perforin and granulysin at the site of TB infection in pulmonary (Andersson, Samarina et al. 2007) and lymph node (Rahman, Gudetta et al. 2009) lesions from TB patients correlated with the progression of active TB disease. The cause and mechanism behind this impaired expression of antimicrobial effector molecules is unknown, but an important subject for future studies on human TB.

3.2.6 Mycobacterial manipulation of T cell effector functions

Bacterial factors are critical determinants in the decision between Th1 or Th2 polarization, and although *M. tuberculosis* does induce a strong inflammatory host response, Th1 responses may be delayed and insufficient to eradicate the infection. Mycobacteria may skew immune activation toward an improper regulatory or Th2 profile and simultaneously inhibit a Th1/Th17 response and a subsequent CTL response. Recent results demonstrate that the ESAT-6 protein from *M. tuberculosis* directly inhibits human T cell proliferation and IFN-γ production in a p38 MAPK-dependent manner (Peng, Wang et al. 2011). It has also been shown that apart from IFN-γ, ESAT-6 can inhibit IL-17 and TNF-α production as well as expression of early activation markers on human T cells (Wang, Barnes et al. 2009). Similarly, the Th1 cell surface molecule Tim3 can stimulate antimicrobial immunity in *M. tuberculosis*-infected macrophages but simultaneously inhibit the expansion of Th1 cells to prevent excess tissue inflammation (Jayaraman, Sada-Ovalle et al. 2011). It has also been shown that PD1 and its ligands, can inhibit CTL function in human TB (Jurado, Alvarez et al. 2008), suggesting that PD1 interferes with T cell effector functions against *M. tuberculosis*.

Thus, the pathogen may have evolved mechanisms to modulate the expression of negative regulators including Tim-3 and PD1 to favour bacterial persistence. On the other hand, PD1-deficient mice are highly susceptible to progressive TB infection and show abnormal immune activation including a dramatic increase in proinflammatory cytokines but low numbers of lymphocytes infiltrating the lung (Lazar-Molnar, Chen et al. 2011). Importantly, a shift of the immune response towards excessive inflammation characterized by extensive production of proinflammatiory cytokines such as IL-1, TNF-α, IL-6 and IL-17 may promote pathology and result in severe tissue damage instead of immune control (Lazar-Molnar, Chen et al. 2011; Torrado and Cooper 2011). Hence, a balance between Th1 and Th17 responses needs to be achieved to control bacterial growth and limit immunopathology in the chronic phase of TB infection.

3.3 Passing checkpoint three – loss of immune control and progression to TB

Intracellular replication of *M. tuberculosis* is followed by the release of inflammatory mediators and the recruitment of additional immune cells including monocytes, macrophages, neutrophils and lymphocyte to the site of infection. The resulting clusters of immune cells are known as granuloma, which are immunopathological hallmarks of tuberculosis. The formation and organization of the granulomatous inflammatory response is believed to contain the infection and to generate an immunological balance between the pathogen and the host. Thus, the function of the granuloma is generally described to be host-protective in its nature; however, recent research has also provided evidence that *M. tuberculosis* bacteria may use the granuloma as a vehicle to seed the infection in the local environment of the lung. It is noteworthy that the major route of spread of the infection to other individuals is through the rupture of fully matured caseating granuloma, which results in drainage of viable bacteria into the airways. This implies that *M. tuberculosis* would not be a successful pathogen if it would not be able to cause necrotic granulomas.

3.3.1 The granuloma –good or bad for the *M. tuberculosis*-infected host?

Formation of *M.tuberculosis* granulomas typically requires the initiation of a delayed-type hypersensitivity reaction and chronic inflammation. Granulomas are composed of clusters of infected macrophages surrounded by sheets of lymphocytes and fibroblasts that are recruited to the site of infection with the aim to contain the infection (Flynn, Chan et al. 2011). The specific host and bacterial factors that orchester granuloma development are still poorly defined. Whereas it is well established that the granuloma is formed as part of the protective immune response to *M. tuberculosis*, there is evidence that mycobacteria can also utilize early granuloma formation to seed the infection to uninfected macrophages that are recruited to the site of granuloma (Davis and Ramakrishnan 2009). This ability of pathogenic mycobacteria to exploit granuloma function is dependent on the ESX-1 secretion system that promotes cell death of infected macrophages, which further enhances uptake and expansion of bacteria in the local environment. Granulomas are highly dynamic structures with multiple appearances in infected organs during active TB disease, including solid non-necrotizing and caseous necrotic granulomas (Kaplan, Post et al. 2003; Rahman, Gudetta et al. 2009). Rupture of mature granulomas drains the caseous necrotic fluid, which is full of

viable bacteria, into the airways of patients with TB, followed by expectoration in the form of infectious aerosols. This is the only known mechanism for *M. tuberculosis* to spread to other individuals. Therefore, although facing the risk of being restricted or killed by host immunity, *M. tuberculosis* is dependent on mature granulomas and host immunity. Interestingly, a recent analysis showed that many T cell antigens are actually conserved in virulent *M. tuberculosis* strains, implying that the bacterium actually wants to be presented to the host immune system (Comas, Chakravartti et al. 2011). Taken together, at least in the initial and late stages of *M. tuberculosis* infection, the bacterium takes advantage of the host immune response, and host immunity is therefore both protective and detrimental for the host during mycobacterial infection, which adds a level of complexity to the understanding of TB.

3.3.2 Manipulation of T cell functions in the granuloma

Our research group has built a technological platform based on quantitative cell- and tissue analysis of clinical materials from TB patients and controls. We have obtained patient samples from the site of TB infection and used immunohistology and *in situ* computerized image analysis to assess expression and distribution of CD4+ T cells, CD8+ CTLs and antimicrobial effector molecules in *M. tuberculosis*-infected tissue. Importantly, immune cells are recruited and collected at sites where the bacilli reside, *i.e.* in infected macrophages in the lung, lymph nodes, pleura or other organs (Barnes, Mistry et al. 1989; Schwander, Torres et al. 1998; Wilkinson, Wilkinson et al. 2005; Jafari, Ernst et al. 2008; Nemeth, Winkler et al. 2009). Hence, organ-specific cell-cell interactions and the functional expression of different proteins can be studied in a physiological environment. Protein expression can be quantified at the single-cell level (Bjork, Andersson et al. 1994) using microscopy and a highly sensitive digital image analysis system with the ability to detect and separate many million different colours. In our studies, we have found that the abundance of CD8+ CTLs expressing the important anti-TB effectors perforin and granulysin, are very low in the granulomatous lesions (Andersson, Samarina et al. 2007; Rahman, Gudetta et al. 2009), indicating bacterial manipulation of host immune functions (Fig. 3). Quantitative real-time mRNA analysis of tissue from *M.tuberculosis* infected lymph nodes revealed that IFN-γ, TNF-α and IL-17 were not up-regulated while there was a significant induction of TGF-β and IL-13. Whereas CD8+ CTLs were absent from the TB lesions, NO-producing macrophages were abundant and the expression of the *M. tuberculosis*-specific antigen MPT64 was high (Fig. 3). These results suggest that CTL activation is impaired and that the few effector CTLs that are present in the tissue cannot make contact with and kill *M. tuberculosis*-infected cells, which accumulate in the granuloma. Instead, these lesions may provide a protective niche for the bacteria, especially at later stages of *M. tuberculosis* infection after induction of adaptive immunity. Whereas compartmentalization of immune responses involved few CD8+ CTLs, we found that the levels of FoxP3+ Treg cells and also TGF-β were significantly elevated in the tuberculous granulomas, suggesting that active immunosuppression takes place at this site (Rahman, Gudetta et al. 2009). This methodology provides important information about the regulation of immune responses in the microenvironment of the human TB granuloma and generates a valuable contribution and complement to the knowledge gained from animal and *in vitro* experimental systems.

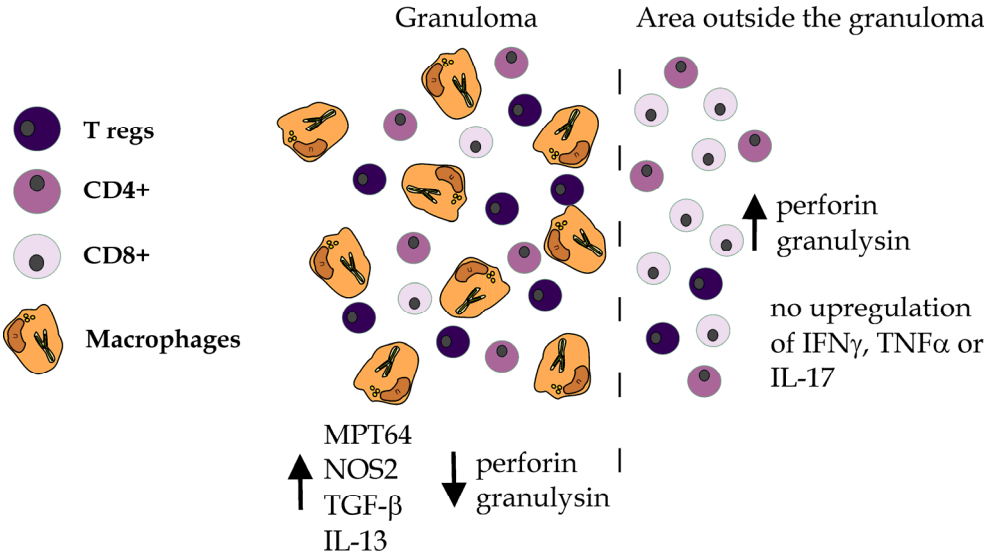

Fig. 3. Schematic illustration of the expression and distribution of *M.tuberculosis* infected macrophages, T cell subsets and cytolytic and antimicrobial effector molecules inside and outside the human granuloma. While macrophages expressing high amounts of the *M.tuberculosis*-specific antigen MPT64 as well as NO synthase are accumulated at the site of infection in the granuloma, the ratio of CD8+ CTLs and Treg cells are significantly higher outside the lesions. Expression of the important anti-TB effectors perforin and granulysin, is low in the infected tissue, and almost absent in the *M.tuberculosis* granuloma. Impaired CTL activation correlates with a low induction of Th1 cytokines, IFN-γ, TNF-α and IL-17, but an increased expression of the immunosuppressive cytokines TGF-βand IL-13. This type of immune response will provide a protective niche for the bacteria inside the granuloma and favour bacterial persistence in the host.

4. Conclusion

The complex mechanisms that are at play during the development of chronic and acute TB involve host immunity and mycobacterial manipulation of both innate and adaptive immunity. The tug-of-war between the bacteria and host immune cells has been illustrated in this chapter as a spectrum between early infection and active disease. The described checkpoints, which *M. tuberculosis* has to pass in order to cause acute infection, are represented by immune mechanisms of increasing complexity, ranging from early innate defence to the multifaceted antibacterial effects posed by adaptive immunity. If immunological control fails, the pathogen takes advantage of immune activities such as excessive release of inflammatory mediators, thus creating the necrotic tissue through which it conveys its spread to other individuals. Innovative thinking and new models that can be used to address questions on the interaction between host immunity and *M. tuberculosis* are required to move the research field forward towards new knowledge. Thus, by questioning old dogmas, many research groups are now approaching the pathogenicity mechanisms of *M. tuberculosis* from new angles, and this is already paying off as improved understanding of the disease it causes, which is a promising step towards new therapies.

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9

Role of TNF in Host Resistance to Tuberculosis Infection: Membrane TNF Is Sufficient to Control Acute Infection

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1. Introduction

Tuberculosis (TB) infection is a major public health problem caused by *Mycobacterium tuberculosis* (*M.tb*). The present estimate is that one third of the world population harbors *M.tb* in a latent form (http://www.who.int), which may be reactivated when the host immune response is suppressed such as in HIV infection (Dye et al., 2005). Only 10% of the population which has been in contact with the pathogen develop overt clinical symptoms while roughly 90% of the infected persons contain the infection. A recent quantification of bacterial growth and death rates showed that *M.tb* replicates throughout the course of chronic TB infection in mice and is restrained by the host immune system (Gill et al., 2009). Unraveling the host immune response during primary and chronic/latent infection is therefore a major challenge. Prominent mechanisms of the host leading to protective immunity controlling tuberculosis and reactivation of infection are associated with T cells, macrophages, interferon-γ (IFN-γ), TNF, interleukin-12 (IL-12), nitric oxide (NO), reactive oxygen and reactive nitrogen intermediates (RNI), as reviewed (Cooper, 2009; Flynn, 2004; Flynn and Chan, 2001a; North and Jung, 2004). While IL-23 and IL-17 contribute to host resistance (Umemura et al., 2007), they do not seem essential to control acute TB infection (Khader et al., 2007).

2. The TNF family

TNF is the founder member of cytokine TNF-like superfamily (for review see (Locksley et al., 2001; Ware, 2005; Watts, 2005). TNF is expressed by many different cell types including macrophages, dendritic cells, CD4+ and CD8+ T cells, B cells, but also by other cells such as

adipocytes, keratinocytes, mammary and colon epithelium, osteoblasts, or mast cells. TNF is first synthesized as a homotrimeric 26 kDa membrane bound protein or transmembrane TNF (tmTNF). After proteolytic cleavage by TNF-alpha converting enzyme (TACE), 17 kDa soluble TNF is released. Levels of circulating TNF in healthy individuals are nearly undetectable however they increase substantially in pathological situations. Lymphotoxin alpha is a member of TNF superfamily and structurally the closest TNF relative. It exists as a soluble homotrimer (LTα3) or forms a membrane-bound heterotrimeric complex with the anchor LTβ.

TNF, LTα and LTβ genes are tightly clustered within 12kb inside the major histocompatibility complex locus on murine chromosome 17 (human 6), while the receptors (R), TNF-R1 and LTβR genes are clustered on mouse chromosome 6 and human chromosome 13 (Nedospasov et al., 1986; Spies et al., 1986). Membrane-bound as well as soluble TNF interact with two receptors, TNFR1 (p55 in mouse, p60 in humans, CD120a) and TNFR2 (p75/p80, CD120b). TNFR1, the high affinity receptor for soluble TNF, is constitutively expressed in nearly all tissues and cell types. TNFR1 contains a protein module called "death-domain" which is essential for induction of apoptosis, as well as for other non-apoptotic functions (Locksley et al., 2001). The expression of TNFR2 is more restricted to lymphoid tissues (Chan et al., 2000). Soluble LTα3 also binds and activates both TNFR1 and TNFR2, whereas membrane bound LTαβ exerts its unique functions through the engagement of LTβR (for review see Ware, 2005).

Receptor ligation initiates signals through a complex cascade to activate the nuclear factor NFκB, JNK-AP1 and p38 signaling axis resulting in activation of TNF-dependent program of gene expression (for review see (Grivennikov et al., 2006). Both TNR1 and TNFR2 are constitutively shed in substantial amounts *in vivo* and soluble TNF receptor shedding is likely to play an important role in regulating TNF activity under physiologic conditions (Pinckard et al., 1997). Macrophage infection by *M.tb* was shown to induce release of soluble TNFR2 that formed inactive TNF-TNFR2 complexes and reduced TNF bioactivity (Balcewicz-Sablinska et al., 1998). *M. bovis* BCG in vivo infection upregulates soluble TNFR1 and TNFR2 release in the circulation following release of TNF (Garcia et al., 2000).

Thus, tmTNF, soluble TNF and soluble LTα3 appear to mediate both overlapping and distinct physiological responses *in vivo*. Their relative roles in inflammatory models and in host defense have not been fully unraveled, in large part due to the limitations in physiologically relevant *in vivo* models. Membrane-bound TNF mediates cellular responses such as apoptosis, proliferation, B cell activation, and some inflammatory responses. To date, the main evidence for an *in vivo* role for tmTNF has come from genetically modified mice expressing uncleavable membrane-bound TNF {Ruuls, 2001, Alexopoulou, 2006). While the role of TNF in controlling tuberculosis has been extensively studied using a panel of available mouse models (Bean et al., 1999; Kaneko et al., 1999; Roach et al., 2002; Zganiacz et al., 2004), the role of LTα3 had to be implicated indirectly from the comparative phenotypes of mice deficient for LTα versus LTβ or TNFR1/TNFR2 versus TNF and therefore remained much less defined.

3. Non-redundant role of TNF to control mycobacterial infection

Macrophages, DC and epithelial cells are among the first cells encountering *M.tb* bacilli in the airway. Phagocytosis induces the transcriptional machinery resulting in the secretion of

several proinflammatory cytokines, chemokine, expression of costimulatory molecules and effector molecules including nitric oxide which has mycobactericidal activity (**Figure 1)**. Mycobacterial proteins are degraded and presented by class II proteins to the T cell receptor inducing clonal activation of CD4 T cells. IFNγ derived from T cells and NK or NKT cells is a potent activator of APCs, enhancing the killing of *M.tb* and presentation of mycobacterial peptide to T cells. The concerted action of cytokines and chemokines leads to accumulation of activated macrophages containing a few surviving bacilli surrounded by activated T cells, which constitutes the typical mycobacterial granuloma (**Figure 1)**. Other cell types may participate in this process and include neutrophils, eosinophils, NK, NKT and mast cells and possibly γδ-T cells (Cooper, 2009; Flynn and Chan, 2001a; North and Jung, 2004; Umemura et al., 2007).

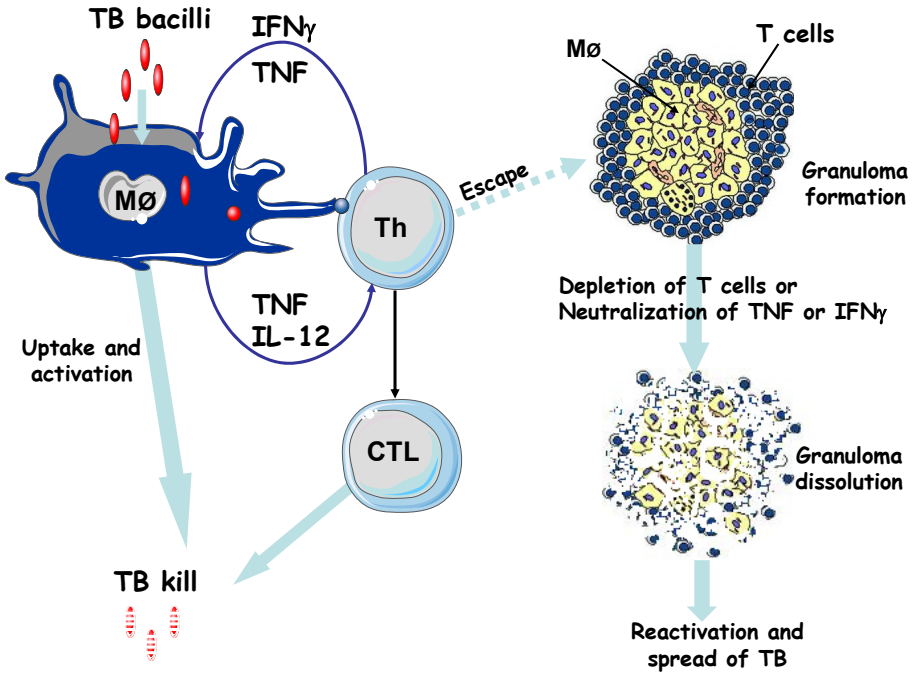

Fig. 1. Macrophage and T cell activation, killing of TB bacilli and granuloma formation. Macrophages are activated by TB bacilli and produce cytokines and T cell activation. Activated macrophages are mycobactericidal, but a few bacilli escape. The cell activation induces lymphocyte recruitment orchestrated by chemokines leading to the formation of granulomas which contain the bacilli. Antibody neutralization of TNF or IFNγ or T cell depletion result in dissolution of the granuloma structure, rescue of surviving bacilli with dissemination of infection.

Infection with the vaccine strain *M. bovis* BCG is well controlled in normal C57Bl/6 mice. However, the control of *M. bovis* BCG infection is TNF dependent as mice treated with anti-TNF antibodies showed impaired granuloma formation and increased bacillus content (Kindler et al., 1989). Transgenic mice expressing soluble TNFR1-Fc fusion protein

neutralizing TNF and LTα succumbed to *M. bovis* BCG infection (Garcia et al., 1997; Guler et al., 2005). Using the originally available TNF-LTα double deficient mice (Eugster et al., 1996), we showed that TNF and/or LTα signaling is required to activate cells of the immune system (Jacobs et al., 2000). TNF-LTα double deficient mice display high susceptibility and succumb to BCG infection between 8 and 10 weeks. The granuloma response was severely impaired with reduced T cell recruitment and macrophages expressed reduced inducible nitric oxide synthase (NOS2), a key mediator of antibacterial defense (Jacobs et al., 2000). We and others further compared the susceptibility of single TNF and LTα deficient mice, and showed that both single gene deficient mice succumbed to *M. bovis* BCG infection, suggesting that both TNF and LTα are necessary and non-redundant to control *M. bovis* BCG infection (Bopst et al., 2001). Reintroduction of LTα as a transgene into TNF-LTα double deficient mice prolonged survival but failed to restore resistance to *M. bovis* BCG (Bopst et al., 2001).

Although *M. bovis* BCG is an attenuated strain, the absence of TNF or TNF signaling induced a phenotype essentially similar to an infection with virulent *M.tb*. Indeed, mice deficient for TNF (Bean et al., 1999; Kaneko et al., 1999; Roach et al., 2002; Zganiacz et al., 2004), or TNF-R1 (Flynn et al., 1995), or mice treated with soluble TNFR1 or TNFR2 to neutralize TNF (Adams et al., 1995; Garcia et al., 1997; Smith et al., 2002) have poorly formed granulomas with extensive regions of necrosis and neutrophilic infiltration of the alveoli, and an inability to control mycobacterial replication upon infection with virulent *M.tb* strains. Bean et al. found comparable MHC class II and inducible nitric oxide synthase expression, serum nitrite levels, and normal activation of T cells and macrophages, while the organization of granulomas was clearly defective and not compensated by LTα (Bean et al., 1999). TNF was not required for granuloma formation, but rather for maintaining granuloma integrity indirectly by restricting mycobacterial growth within macrophages and preventing their necrosis in *M. marinum*-infected zebrafish (Clay et al., 2008). Similarly, in a murine model of *M. bovis* BCG infection, established hepatic granuloma showed a profound decrease in size and in their population of non-infected macrophages within 2-4 days of anti TNF treatment (Egen et al., 2008).

As observed in *M. bovis* BCG infection studies, both TNF and LTα seemed necessary to control infection with virulent H37Rv strain of *M.tb* (Roach et al., 2002; Roach et al., 2001). However, the very close mutual proximity of genes coding for TNF, LTα and LTβ on mouse chromosome 17 raises the issue of collateral gene damage in mouse models employing targeted modifications of TNF/LT genomic locus. For example, independently generated mouse strains with TNF deficiency behave identically in a number of infection and stress models but demonstrate discrepant phenotypes with regard to the development of Peyer's patches, apparently due to differences in the configuration of the targeted locus (Kuprash et al., 2005). Based on published reports, both removal of a regulatory element controlling transcription of the LT genes and their compensatory upregulation by the actively transcribed neo resistance cassette can be envisioned. Since LT expression essential for the development of Peyer's patches has to be cell type specific and may be subject to autoregulatory feedback loops, concluding resolution of these discrepancies proved to be a technically challenging task.

Another example of collateral gene damage, probably more relevant to TB research, is dysregulation of TNF expression in LTα knockout mice. Recently generated $LT\alpha^{\Delta\square\Delta}$ mice were fully capable of producing TNF at normal levels, whereas "conventional" LTα KO animals displayed significant decrease in TNF synthesis in several critical types of leukocytes both *in vitro* and *in vivo* (Liepinsh et al., 2006). In conventional LTα KO mice, TNF deficiency could be corrected by transgenic TNF expression (Alexopoulou et al., 1998). In agreement with the results of TNF promoter studies, the deficiency appears to be restricted to macrophages and neutrophils (Liepinsh et al., 2006). Defective TNF production has been noted, to various extent, by several published reports utilizing conventional LTα KO mice (Bopst et al., 2001; Schluter et al., 2003). Once again, cell type-specific collateral damage to transcriptional initiation may be difficult to unambiguously discriminate from physiological mutual regulation of two closely related cytokines sharing some of their receptors. Nevertheless, any conclusions indicating an independent protective role of soluble LTα in intracellular infections based on experiments with conventional LTα KO mice should be taken with certain caution. Our recent study comparing "conventional" LTα KO mice and $LT\alpha^{\Delta/\Delta}$ KO mice during *M.tb* and *M. bovis* BCG infections demonstrated that LTα might have a less essential role than anticipated for the control of acute infection, and the phenotype previously observed might indeed result at least in part from additional defects such as reduced TNF expression (Allie et al. 2010).

4. Molecular mechanisms of mycobacterial killing/resistance

Activation of macrophages and dendritic cells by *M.tb* induces several proinflammatory cytokines including TNF, LTα and IL-12, and expression of costimulatory molecules that enhance antigen presentation and activation of T cells. Activated T cells produce TNF, IFNγ and LTα inducing further activation of macrophages and likely other cells including stroma cells. Activated macrophages express NOS2, producing nitric oxide and reactive nitrogen intermediates (RNI), which are critical for killing and inhibiting growth of virulent *M.tb* and BCG (Chan et al., 1992; Garcia et al., 2000; MacMicking et al., 1995).

Mycobacteria may inhibit phagosomes maturation and fusion with lysosomes, thereby escaping killing (Mwandumba et al., 2004; Russell et al., 1996; Sturgill-Koszycki et al., 1996; Xu et al., 1994). Activated macrophages recruit T cells to form granulomas, which contain bacterial growth. The granuloma is a dynamic structure, which requires a permanent signal from activated T cells and macrophages (Ehlers et al., 1999). Any perturbation of this signaling such as neutralization of TNF causes dissolution of granulomas (Kindler et al., 1989) and allows reactivation and spread of infection (**Figure 1**). Activated T cells not only provide help, but acquire cytotoxic functions, which eradicate bacilli, although the relative contribution of CD4 versus CD8 cells to control TB infection is not fully established.

In order to better understand the effect of TNF on intracellular replication of mycobacteria, we investigated the growth of the vaccine strain BCG in TNF deficient macrophages. *M. bovis* BCG infection resulted in logarithmic growth of the intracellular bacilli, while recombinant BCG-expressing TNF (BCG-TNF) led to bacillary killing associated with production of NO. Therefore, TNF contributes to the expression of NOS2 and to bacterial growth inhibition indirectly (Bekker et al., 2001).

IFNγ has been shown to be an essential component of immunity to tuberculosis. It activates infected host macrophages to directly inhibit the replication of *M.tb* (Flynn and Chan, 2001a). Although IFNγ-inducible NOS2 is considered the principal effector mechanism, other pathways exist. *M.tb* has developed several mechanisms to escape eradication including inhibition of phagosome maturation (Flynn and Chan, 2003). Mycobacteria, blocking Ca^{2+} signaling and phagosome maturation in human macrophages or inhibiting sphingosine kinase, may allow the escape from eradication in the phagocyte (Malik et al., 2000; Malik et al., 2001; Malik et al., 2003). The role of autophagy and ensuing inhibition of phagolysosome formation (Deretic, 2008) may be considered, as well as Coronin-1 inhibition as an alternative pathway to prevent phagosome maturation (Jayachandran et al., 2007).

Defensins such as cathelicidin (LL37) have an important anti-mycobacterium activity in human macrophages. Liu and colleagues have reported that activation of Toll-like receptors (TLRs) up-regulates the expression of the vitamin D receptor and the vitamin D-1-hydrolase generating $1,25(OD)_2D3$, the active form of vitamin D, leading to induction of the microbicidal peptide cathelicidin and killing of intracellular *M.tb* in human macrophages (Liu et al 2006).

Mycobacteria induce apoptosis of macrophages and cause the release of apoptotic vesicles that carry mycobacterial antigens to uninfected antigen-presenting cells, including dendritic cells which are indispensable for subsequent antigen cross-presentation through MHC-I and CD1b. This new pathway for presentation of antigens from a phagosome-contained pathogen illustrated the functional significance of infection-induced apoptosis in the activation of CD8 T cells specific for both protein and glycolipid antigens in tuberculosis (Schaible et al., 2003).

Induction of TNF and other proinflammatory cytokines is mediated through several mycobacterial motives triggering different pattern recognition receptors, including TLR2, TLR4 or TLR9. However, while the control of acute TB infection was severely compromised in the absence of MyD88 (Feng et al., 2003; Fremond et al., 2004), TLR2, TLR4 and/or TLR9 do not seem essential for the control of acute TB infection but may interfere in the control of chronic infection (Bafica et al., 2005; Drennan et al., 2004; Holscher et al., 2008). The MyD88 pathway may thus contribute rather through IL-1R signaling to control acute TB (Fremond et al., 2007). TLR/MyD88 dependent signaling is also required for phagosome maturation (Blander and Medzhitov, 2004).

In summary, TNF participates in resistance to mycobacteria in the following ways: (1) activation of macrophages, (2) induction of chemokines and cell recruitment, (3) activation of T cells, (4) killing by macrophages, T and other cells and (5) regulation of apoptosis and signals from TLR/MyD88/IL-1R pathway that contribute to the host response. Since separating the effects of these different TNF functions *in vivo* is presently difficult or impossible, a computational model was applied to understand specific roles of TNF in control of tuberculosis in a single granuloma. The model predicted that macrophage activation is a key effector mechanism for controlling bacterial growth within the granuloma, TNF and bacterial numbers represent strong contributing factors to granuloma structure, and TNF-dependent apoptosis may reduce inflammation at the cost of impaired mycobacterial clearance (Ray et al., 2009).

5. Membrane TNF biological activity controls acute M.tb infection

Although a key role of TNF in controlling intracellular bacterial infections is uncontested, it is only recently that the specific function of membrane TNF has been appreciated. Membrane TNF is cleaved by the metalloproteinase-disintegrin TACE (TNF alpha converting enzyme) (Black et al., 1997) into the secreted, soluble trimeric TNF. Several functions of membrane TNF have been described, such as cytotoxicity, polyclonal activation of B cells, induction of IL-10 by monocytes, ICAM-1 expression on endothelial cells and liver toxicity (Decker et al., 1987; Grell et al., 1995; Kriegler et al., 1988; Ruuls et al., 2001). The transgenic expression of membrane TNF suggested an *in vivo* role of membrane TNF (Akassoglou et al., 1997). Olleros et al. investigated the resistance to mycobacterial infection in transgenic mice expressing a membrane TNF (Δ12-10; -1; one substitution, K11E) under the control of proximal TNF promoter and on a TNF-LTα deficient background. In this model membrane TNF had a fully protective effect against *M. bovis* BCG but only partial protective effect against *M.tb* infection (Olleros et al., 2002; Olleros et al., 2005). Mice with functional, normally regulated and expressed membrane-bound TNF represents a major advance and allowed interesting insights in the role of membrane TNF in lymphoid structure development and inflammation. Knock-in mice expressing the uncleavable Δ1-9,K11E TNF (Ruuls et al., 2001) and TNF-deficient mice (Marino et al., 1997) were compared in their resistance to mycobacterial infection. As previously reported for membrane TNF transgenic mice, we and others demonstrated that membrane TNF has important biological functions and substitutes soluble TNF to a large extent (Dambuza et al., 2008; Fremond et al., 2005; Saunders et al., 2005; Torres et al., 2005). Membrane TNF knock-in mice survived a *M.tb* aerosol infection for three months, were able to recruit and activate macrophages and T cells, generate granuloma and partially control mycobacterial infection in the early stage, unlike complete TNF deficient mice (Fremond et al., 2005; Saunders et al., 2005). However, during the chronic phase of infection membrane TNF knock-in mice demonstrated reduced bacterial clearance and succumbed to infection (Fremond et al., 2005). In another model of targeted mutagenesis in mice, the shedding of membrane TNF was prevented by deleting its cleavage site (Alexopoulou et al., 2006). Mice expressing non-cleavable and regulated Delta1-12 TNF allele partially controlled *M. bovis* BCG infection, with recruitment of activated T cells and macrophages and granuloma formation, while mice with complete TNF deficiency succumbed (Allie et al., 2008). It was confirmed that membrane TNF conferred partial protection against virulent *M.tb* infection and inter-crossing these mice with TNF-R1 or TNF-R2 KO mice showed that tmTNFxTNFR2 KO mice were very sensitive, essentially as much as TNF KO mice, while tmTNFxTNFR1 KO mice behaved more like tmTNF mice, suggesting that the protective effect of membrane TNF against acute *M.tb* infection is mediated through TNF-R2 signalling (Allie et al., 2008).

Therefore data from the genetic mouse models suggest that membrane expressed TNF is sufficient and soluble TNF dispensable to control the first phase of acute TB infection. However, during the chronic phase membrane TNF alone is not sufficient and soluble TNF seems to be required to control chronic TB infection. The reason for the progressive loss of infectious control is unclear. As previously discussed, soluble TNF may be required to negatively control the Th1 type cytokines. This TNF function may become important during the chronic phase of infection by regulating excess production of IL-12 and IFN-γ by DC and T cells.

6. TNF in reactivation of TB infection

Clinical tuberculosis in humans may be due to a primary infection or reactivation of latent controlled infection. Secondary immunosuppression due to HIV/AIDS is the most common cause of *M.tb* reactivation. In recent years, over a million patients received TNF neutralizing therapy for the treatment of severe rheumatoid arthritis, Crohn's disease or severe psoriasis. The most common complication of TNF blockade has been the emergence of opportunistic infection and tuberculosis. Both reactivation of latent tuberculosis and increased susceptibility to new tuberculosis in patients without a clinical history of active TB infection was observed (Wallis, 2008). In some patients, neutralizing TNF antibody, infliximab, or soluble TNFR2-IgG1 Fc fusion protein, etanercept, yielded reactivation of latent TB within 12 weeks and overt clinical disease (Keane, 2005; Keane et al., 2001; Mohan et al., 2004), often with extrapulmonary disease manifestations (disseminated infection in lymph node, peritoneum and pleura). The frequency of tuberculosis in association with infliximab therapy was higher than the reported frequency of other opportunistic infections associated with this drug (Keane, 2005). Reactivation of latent tuberculosis and primary infection in patients treated with TNF inhibitors are still difficult to be clearly defined in many cases. Anti-TNF antibody may be more associated with latent TB reactivation than etanercept. The majority of etanercept-associated cases of TB appears late (90% after 90 days of treatment) suggesting that these cases may have occurred as a result of the inability to control new *M.tb* infection while 43% of infliximab associated cases of TB occurred during the first 90 days of treatment, indicating that they likely represent reactivation of latent infection (Wallis, 2008; Wallis et al., 2005). The reactivation of latent TB under TNF blocking therapy indicates that the normal immune system is able to control, but not able to eradicate a primary infection, and that TNF plays a role in the long term containment of residual *M.tb* in tissues.

In order to study the factors leading to reactivation of chronic or chemotherapy controlled latent infection, several experimental models have been developed (Flynn, 2006). In the Cornell model, after an intravenous administration of *M.tb* H37Rv and treatment with pyrazinamide and isoniazide (INH) for 12 weeks, mice appear to have cleared the bacilli from organs, but a substantial proportion of animals spontaneously reactivate with acute disease upon cessation of chemotherapy. Since the original publication of the Cornell model a few variations have been reported (Botha and Ryffel, 2002; Flynn and Chan, 2001b). In the low-dose model, infection is exclusively controlled by the host in the absence of chemotherapy (Flynn and Chan, 2001b). Although considered to better reflect the human host response, bacterial numbers in the organs of these mice remain high during the chronic persistent phase of infection. To date, these models have yielded significant information on the immune effector mechanisms participating in latent or chronic persistent and reactivated tuberculosis.

We established the first aerosol infection model of drug-induced latent and reactivated murine tuberculosis using rifampicin and isoniazide (Botha and Ryffel, 2002, 2003). In this model, latency was defined as almost undetectable levels of bacilli in mouse organs for a prolonged period of time. Reactivation of infection could be achieved by inhibiting nitric oxide synthase activity by aminoguanidine (Botha and Ryffel, 2002). Using this model, we showed that a 4 weeks rifampicin and isoniazide administration cleared infection as assessed by viable bacterial accounts in the organs in both wild-type and TNF deficient

mice. Upon cessation of therapy massive spontaneous reactivation of *M.tb* infection occurred within several weeks in TNF deficient mice with necrotic pneumonia and death, while wild-type mice displayed mild subclinical reactivation (Botha and Ryffel, 2003). This model allows studying the role of TNF neutralization in a reactivating infection in the presence of an established specific adaptive immune response.

The role of soluble vs membrane TNF was then studied in this model (**Figure 2** and unpublished data). Although TNF KO mice rapidly lost weight and had to be terminated within 6 weeks after the end of the antibiotic treatment with uncontrolled infection, tmTNF KI mice survived as wild-type mice. Therefore, membrane TNF suffices to provide some control of the *M.tb* infection after reduction of the bacterial burden by an antibiotic treatment, while complete absence of TNF results in rapid progression of the infection.

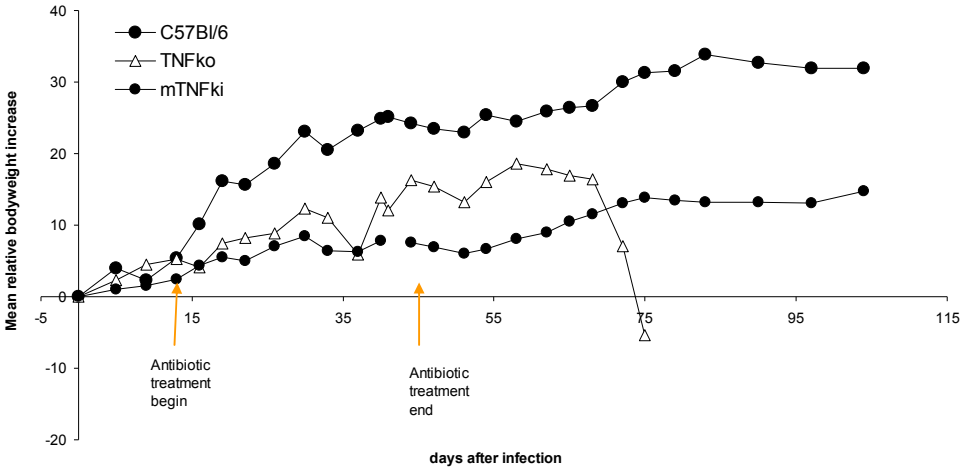

Fig. 2. Comparison of susceptibility of membrane TNF KI and conventional TNF KO reactivating, chronic *M.tb* infection.
Wild-type, TNF KO and mTNF KI mice were infected with *M.tb* (ca 100 CFU i.n.) and treated for 4 weeks with rifampicin and isoniazid (day 14-42) to control the infection. TNF KO mice started to die 6 weeks after the end of the antibiotic treatment while all mTNF KI mice survived as wild-type mice. In parallel groups infected with *M.tb* but not treated with antibiotics TNF KO had to be killed at 3 wk, while 6 out of 8 mTNF KI survived with no marked body weight loss.

7. Pharmacological TNF neutralization and TB control

The experimental models of TB reactivation described above allow to test the potential risk of diverse TNF neutralizing therapies to induce reactivation of TB. Administration of neutralizing TNF antibody but not of soluble TNF receptor was able to reactivate experimental latent infection (Plessner et al., 2007). TNF neutralization resulted in marked disorganization of the tuberculous granuloma and to the enhanced expression of specific proinflammatory molecules. (Chakravarty et al., 2008). A computational approach suggested that TNF bioavailability following anti-TNF therapy is the primary factor for

causing reactivation of latent infection and that even very low level of soluble TNF is essential for infection control (Marino et al., 2007).

Novel approaches to experimentally block soluble TNF are being tested in murine models of TB. One approach is to compete for natural TNF by the use of dominant negative mutant TNF (DN-TNF; see **Figure 3**) reported to block soluble TNF while sparing membrane TNF (Steed et al., 2003). *In vivo*, DN-TNF attenuated arthritis without suppressing innate immunity to *Listeria monocytogenes* (Zalevsky et al., 2007). Similarly, DN-TNF protected mice from acute liver inflammation, without compromising host control of *M. bovis* BCG and *M.tb* infections (Olleros et al., 2009). This was in contrast to TNFR2-IgG1 etanercept that inhibits murine soluble and membrane TNF as well as LTα, which severely compromised the host response to *M.tb* infection (Olleros et al., 2009). Another novel approach is an active immunization selectively targeting soluble TNF. Vaccination with a virus-like particle linked to a TNF N-terminal peptide resulted in high titers of autoantibodies against soluble TNF. It protected mice from arthritis without inducing reactivation of latent tuberculosis (Spohn et al., 2007), while immunization against the entire TNF molecule yielded enhanced reactivation of latent TB. This difference was attributed to recognition of only soluble TNF vs recognition of both transmembrane and soluble TNF by the elicited antibodies. Thus,

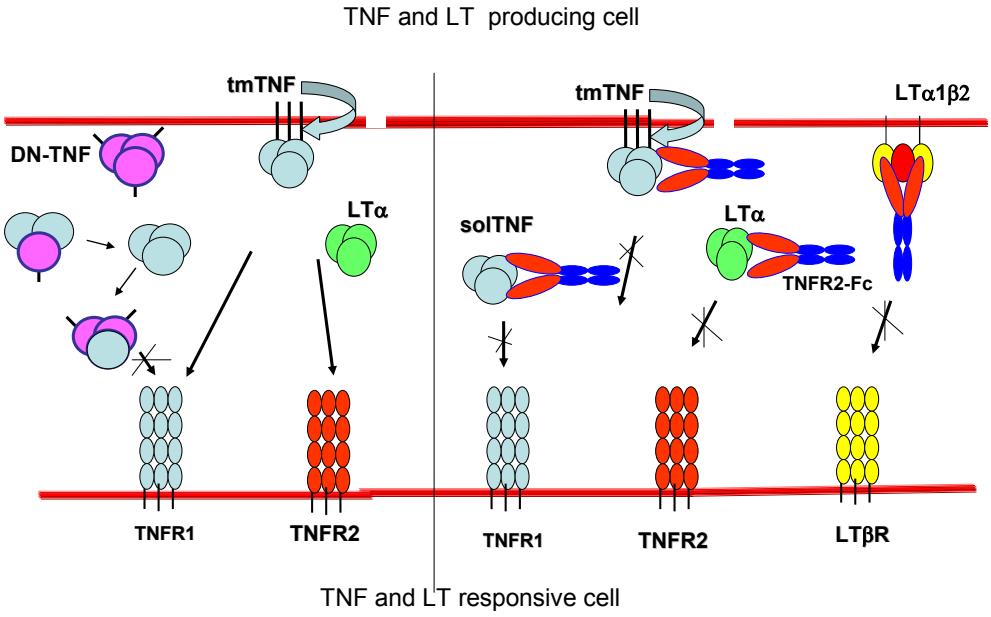

Fig. 3. Mechanisms of action of dominant-negative-TNF (DN-TNF) biologics and soluble TNFR2-Fc (Etanercept). *Left*, DN-TNF, a mutated form of human solTNF with disrupted receptor binding interfaces eliminates solTNF by a subunit exchange mechanism, but is unable to interact with tmTNF and LTα. *Right*, solTNF, tmTNF, LTα and LTαβ can be neutralized by Etanercept, inhibiting interaction with corresponding receptors. Thus, DN-TNF (XENP1595) inhibits solTNF receptor signaling without suppressing tmTNF- or LTα responses to TNFR1 and TNFR2, mediating inflammatory and immune responses

specifically targeting soluble TNF has the potential to be effective against inflammatory disorders while overcoming the risk of opportunistic infections associated with the currently available TNF antagonists.

8. Conclusions and perspectives

In conclusion, TNF is an essential mediator for the integrity of microbiocidal granulomas and the control of *M.tb* infection. Experimental tuberculosis infection of gene deficient mice has demonstrated the non-redundant contribution of several pro-inflammatory cytokines such as TNF, IL-12, IFNγ or IL-1 to the host response to M.tb infection (Flynn, 2006; Fremond et al., 2007). An important notion is the fact that latent mycobacterial infection can be reactivated by TNF neutralization. Sparing membrane TNF in neutralizing TNF therapy used in rheumatic arthritis or Crohn's disease may diminish the infectious complications and reactivation of latent TB infection.

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Immunoregulatory Role of GM-CSF in Pulmonary Tuberculosis

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1. Introduction

1.1 Tuberculosis

Tuberculosis is mainly an infectious disease of the lung (1, 2). Pulmonary tuberculosis continues to devastate the lives of millions of people worldwide, despite the availability of potent antibiotics against the causative pathogen *Mycobacterium tuberculosis* (3, 4). Eight to ten million new cases of clinical *M. tuberculosis* infection are diagnosed yearly, and the death toll due to tuberculosis has remained above 1.7 million people annually over the last two decades. The vast majority of *M. tuberculosis* infections occur in immuno-competent individuals (4, 5). Populations in Southeast Asia and sub-Saharan Africa are particularly vulnerable to the disease (3, 6). HIV co-infection and the recent emergence of drug resistant strains of *M. tuberculosis* have contributed to the escalation of tuberculosis in recent years (4, 7-9). *M. tuberculosis* infection exhibits a complex life cycle driving the development of primary, latent, and post-primary tuberculosis in the human host (2, 5, 10-12).

Primary tuberculosis is a granulomatous immune process that effectively contains *M. tuberculosis* within a few weeks after initial infection (1, 10, 13-15). Primary infection begins when previously uninfected individuals inhale aerosol droplets containing a few *M. tuberculosis* organisms that invade alveolar macrophages, epithelial cells, and dendritic cells in the distal airway mucosa. Mycobacteria arrest phagolysosome fusion and proliferate intracellularly in alveolar macrophages (16). The fate of mycobacteria in lung epithelial cells is not understood, but these cells are thought to participate in granuloma formation (17, 18), mycobacterial dissemination (19) and local antigen presentation to T lymphocytes (20). Infected dendritic cells migrate to regional lymph nodes resulting in activation and

[*] Original work presented here represents ten years of collaborative research between Zissis C. Chroneos in his previous position at the University of Texas Health Science Center at Tyler and Chinnaswamy Jagannath at the University of Texas Health Science Center at Houston.

migration of Th1 T lymphocytes back to the lung halting mycobacterial proliferation through intercellular contact with infected alveolar macrophages (21, 22). Dissemination of live mycobacteria or transport of mycobacterial antigens to peripheral immune organs by macrophages and dendritic cells establishes potent anti-mycobacterial immunity at the periphery (23-26). Elaboration of key cytokines such as TNFα, IL-12, and IFNγ and chemokines drive formation of granulomas at sites of infection (27). Granulomas evolve into caseating lesions containing mycobacteria within a central core of lipid and activated foamy macrophages enclosed by layers of epithelioid cells and lymphocytes. Foamy macrophages undergo necrosis and calcification killing most *M. tuberculosis* organisms. The source of lipid causing foam cell formation is not established but may involve uptake of alveolar type II epithelial cell-derived surfactant lipids or adjoining adipose tissue (17). Healing of caseous lesions represents resolution of the primary infection which occurs asymptomatically in most immuno-competent individuals (2, 28).

M. tuberculosis is not completely eradicated by primary cell-mediated immunity causing latent tuberculosis infection (LTBI) (10-12, 29). LTBI is thought to result from mycobacterial adaptation to the hypoxic lipid environment of caseous granulomas (14, 30, 31). A small number of bacteria survive presumably in macrophage phago-lysosomes in a non-replicative or dormant state. Cell-mediated immunity established during primary infection exerts life-long control of LTBI in the host. Immunosuppressive therapy aimed at treating inflammatory and autoimmune diseases by targeting TNFα, a crucial host factor in control of LTBI, results in reactivation of tuberculosis in latently infected individuals (29). LTBI is diagnosed using the purified protein derivative (PPD) skin test or interferon gamma release assays that measure mycobacterial antigen-specific recall responses of cell-mediated immunity. LTBI is present in over 3 billion people worldwide. Recent imaging observations in humans and non-human primates indicate that LTBI represents active rather than dormant subclinical infection contained within heterogeneous granulomatous lesions in the same host (11).

Reinfection or reactivation of LTBI results in development of post-primary also called secondary tuberculosis in the lungs of individuals that have already developed robust immunity to the primary infection (2, 5). For reasons that are not understood, over-activation or perhaps unanticipated activation of the pulmonary immune system may steer the development of post-primary tuberculosis in the face of strong anti-tuberculosis immunity at the periphery. Individuals with LTBI have a 10% life-time risk of developing post-primary tuberculosis. Interestingly, post-primary tuberculosis occurs mainly in young adults aged 15-40 years old (4, 5). Post-primary tuberculosis is responsible for 80% of clinical cases of tuberculosis and is the source of most transmission of the infection in the community. The more widely reported account of secondary tuberculosis pathology in the post-antibiotic era is that liquefaction of caseous granulomas results in formation of cavities. According to Hunter (5), however, post-primary tuberculosis manifests mainly as a lipid broncho-pneumonia, rather than a granulomatous disease process, that resolves spontaneously in most cases without further medical intervention. The view of post-primary tuberculosis as a lipid pneumonia agrees with the description of the disease reported by pathologists in the pre-antibiotic era when tuberculosis was rampant in the Western World. Hunter's observations are based on lung autopsies from patients who succumbed to tuberculosis but did not receive antibiotic therapy, reevaluation of specimen from the John Hopkins tuberculosis historical repository, and post-mortem lung biopsies of tuberculosis patients from Russia. Unresolved

post-primary tuberculosis lipid pneumonia progresses into caseous necrotic lesions in which mycobacteria reach high numbers. Caseous necrosis results in formation of cavities that release necrotic material along with large numbers of *Mycobacterium tuberculosis* organisms into main stem bronchi. Coughing then expels *M. tuberculosis* into the atmosphere completing the cycle of airborne transmission of the infection to other members of the community. Individuals with productive cavities but with minimal clinical symptoms can transmit the disease for many years before becoming seriously ill (5).

Understanding geographical differences in immune responses to tuberculosis is essential for the rational design of effective vaccines against tuberculosis (4, 6, 32). Exposure to higher infection loads in countries where tuberculosis is endemic may contribute to immune exacerbations that increase the risk for development of active tuberculosis infection. Infection with helminths and environmental mycobacteria in developing countries is thought to cause immunological predisposition to active tuberculosis driven by a mixed Th1/Th2 cell-mediated immune response that also renders BCG vaccination ineffective (6, 32-35). Helminth infections are prevalent in areas of the world were tuberculosis is also endemic. Helminths were shown to diminish the efficacy of the BCG vaccine. Co-infection of mice with the helminth *Nippostrongylus brasiliensis* and *Mycobacterium tuberculosis* increased mycobacterial burden in the lungs due to accumulation of alternatively activated macrophages. The effect of helminth infection was abrogated in mice lacking the IL-4 receptor IL-4Rα indicating that IL-4 drives accumulation of alternatively activated macrophages with reduced capacity to control *M. tuberculosis* infection even in the presence of robust Th1 cell-mediated immunity (35).

The role of GM-CSF in the broader context of vaccine development in the developing world where tuberculosis is an endemic disease is not yet understood. Monitoring of GM-CSF levels as a product of poly-functional T lymphocytes following BCG vaccination was adopted only recently (36). BCG vaccination of infants and children is effective but does not provide protection beyond adolescence and exhibits variable efficacy when administered in adults (37, 38). The failure of BCG vaccines to protect against tuberculosis in developing countries has confounded clinical and basic researchers for many years (38, 39). By contrast, BCG vaccination provides lasting protection in 80% of individuals in developed countries (33, 37). It is possible that primary infection is the predominant form of tuberculosis in developed countries where BCG vaccination works, whereas post-primary tuberculosis is the major form of infection in endemic countries were BCG vaccination does not work. GM-CSF has been used in a variety of formulations and boosting strategies to enhance BCG priming of anti-tuberculosis cell-mediated immunity with promising results (40-45). So far, GM-CSF has been shown to enhance T cell responses and provide temporary benefit against primary infection with *M. tuberculosis* in pre-clinical models (41).

2. GM-CSF functions in the lung

GM-CSF is crucial for pulmonary homeostasis as a modulator of alveolar macrophage differentiation (46, 47). GM-CSF signals through a bi-component receptor of α and βc subunits mediating concentration-dependent functions of GM-CSF in macrophages and other cell types. Assembly of the GM-CSF receptor heterodimer into hexameric or dodecameric forms underlies pleiotropic effector functions of GM-CSF (48). Secreted

constitutively at low levels by alveolar type II epithelial cells, GM-CSF is required for terminal alveolar macrophage differentiation (47, 49). Disruption of GM-CSF in both humans and mice results in development of alveolar proteinosis, a disease characterized by excessive accumulation of surfactant proteins and lipids in the alveolar space (50, 51). In the absence of GM-CSF, alveolar macrophages are arrested at an immature state with reduced capacity to internalize and catabolize surfactant (47, 52, 53). Normally, alveolar macrophages contribute 20-50% of surfactant catabolic activity in alveoli (54). In addition to surfactant catabolism, GM-CSF regulates phagocytosis and innate immune responses in alveolar macrophages (47, 55). GM-CSF modulates expression of the transcription factors PU.1 and PPARγ. Optimal levels of PU.1 as maintained by GM-CSF are crucial for differentiation and multiple immune functions of alveolar macrophages (46, 47, 55-57) while in addition to PU.1, expression of PPARγ is necessary for the catabolism of surfactant lipids by alveolar macrophages (58-60).

GM-CSF coordinates regulatory functions of alveolar macrophages that modulate activation of pulmonary mucosal immunity and protection of the distal airway epithelium from inflammatory stimuli (61-63). GM-CSF regulates, recruitment, activation, and expansion of alveolar macrophages, alveolar type II epithelial cells, and dendritic cells (47, 64-66). Under normal conditions alveolar macrophages maintain immune homeostasis by inhibiting activation of T lymphocytes by innocuous antigens. Anti-inflammatory mediators such TGFβ, IL-10, nitric oxide, and constituents of pulmonary surfactant, SP-A, SP-D, and lipids contribute to the suppressive activities of alveolar macrophages (67, 68). In particular, the SP-A receptor SP-R210 moderates proliferation of lymphocytes in peripheral blood mononuclear cells of PPD+ individuals (69). High levels of GM-CSF inhibit the ability of alveolar macrophages to suppress mucosal immunity (63), representing an important switch mechanism between steady-state conditions and activation of cell-mediated immunity in the lung. On the other hand, optimal alveolar macrophage differentiation as normally maintained by epithelial derived GM-CSF in the lung, is necessary for appropriate innate immune responsiveness of alveolar macrophages. For example, induction of TNFα secretion by alveolar macrophages in response to LPS results in paracrine stimulation of high levels of GM-CSF by alveolar type II epithelial cells. High levels of GM-CSF stimulate proliferation of alveolar type II epithelial cells protecting the integrity of the alveolar epithelial barrier during acute inflammation (70).

3. GM-CSF in treatment of tuberculosis

Pre-clinical studies in animal models have shown that GM-CSF enhances the effectiveness of current vaccines against *Mycobacterium tuberculosis* (42). Studies in GM-CSF-deficient ($GM^{-/-}$) mice showed that GM-CSF is necessary for the development of cell-mediated immunity and formation of granulomas against primary *M. tuberculosis* infection in the lung (71, 72). Prior vaccination of mice with GM-CSF-secreting Bacillus Calmette Guerin (BCG) reduced pulmonary *M. tuberculosis* numbers up to 100-fold when administered locally (41, 42). The best protection was achieved when BCG-GM-CSF was delivered directly to the lung.

Systemic administration of high levels of GM-CSF, however, reduced anti-tuberculosis immunity in mice (73). Furthermore, GM-CSF-deficient mice in which GM-CSF is constantly secreted at high levels by lung epithelial cells (SP-C-$GM^{+/+}$) are resistant to the infection initially but do not sustain long-term protective immunity against tuberculosis (71, 72),

suggesting that high levels of GM-CSF impact cell-mediated immunity in the chronic phase of the disease. On the other hand, lack of GM-CSF in mice was detrimental for survival against virulent *M. tuberculosis* Rv (74) and Erdman strains (72). These findings indicate that GM-CSF expression impacts host resistance to tuberculosis at different stages of the infection.

Remarkably, neither GM-CSF deficiency nor constitutive over-secretion of GM-CSF influence clearance of the vaccine strain *M. bovis* BCG (71). Similarly, clearance of BCG and BCG-GM-CSF expressing strains were not different in WT mice (41). In a related study, pulmonary clearance of *Mycobacterium avium* was similar between WT and GM$^{-/-}$ mice, despite decreased activation of macrophages in the absence of GM-CSF (75). Both absence or high levels of GM-CSF disrupted normal granuloma formation in GM$^{-/-}$ and SP-C-GM$^{+/+}$ but did not impact clearance of pulmonary BCG infection (71). Furthermore, abnormal levels of GM-CSF did not impair secretion of IL-12p40 in response to BCG infection in GM$^{-/-}$ and SP-C-GM$^{+/+}$ mice (71). IL-12p40 is secreted by dendritic cells and is crucial for Th1 cell-mediated immunity against tuberculosis (27, 76, 77). To this extend, lack of GM-CSF did not alter the ability of BCG to induce IL-12p40 (71). BCG infection in the presence of high levels of GM-CSF, however, generated an early IFNγ burst in SP-C-GM$^{+/+}$ mice compared to WT and GM$^{-/-}$ mice 9 days after infection (Figure 1), and remained at the same high levels over the course of the experiment. In contrast, IFNγ increased gradually in WT mice reaching similar levels as SP-C-GM$^{+/+}$ mice by 29 days after BCG infection. IFNγ levels in GM$^{-/-}$ lungs also increased gradually but were significantly lower than both WT and SP-C-GM$^{+/+}$ mice. The kinetics of IFNγ secretion in WT mice (Figure 1) correlates with the development of granulomatous inflammation following BCG infection (71). These studies indicate that GM-CSF modifies the initial IFNγ response to BCG vaccination.

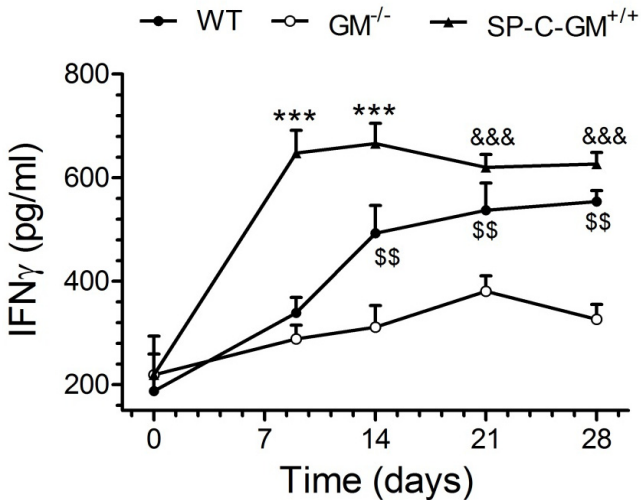

Fig. 1. GM-CSF modulates IFNγ levels in response to *M. bovis* BCG infection.
WT, GM$^{-/-}$, and SP-C-GM$^{+/+}$ mice were infected with 1.5 x 10^7 cfu of *M. bovis* BCG via the intranasal route. The concentration of IFNγ was measured by ELISA in lung homogenates at indicated times after infection. ***$p<0.001$ compared to WT, GM$^{-/-}$; &&&$p<0.001$ compared to GM$^{-/-}$; $$$p<0.01$ compared to GM$^{-/-}$. Data shown are means±S.E.M. n=9 for WT, n=10 for GM$^{-/-}$, and n=6 for SP-C-GM$^{+/+}$ mice.

Differences in GM-CSF levels alter long-term immune responses to BCG vaccination. ELISA assays compared levels of inflammatory mediators in the lungs of GM$^{-/-}$ and SP-C-GM$^{+/+}$ mice at 53 and 75 days after infection with BCG (Figure 2). Figure 2A shows that GM$^{-/-}$ and SP-C-GM$^{+/+}$ mice maintained similar levels of IFNγ 53 and 75 days after infection with BCG.

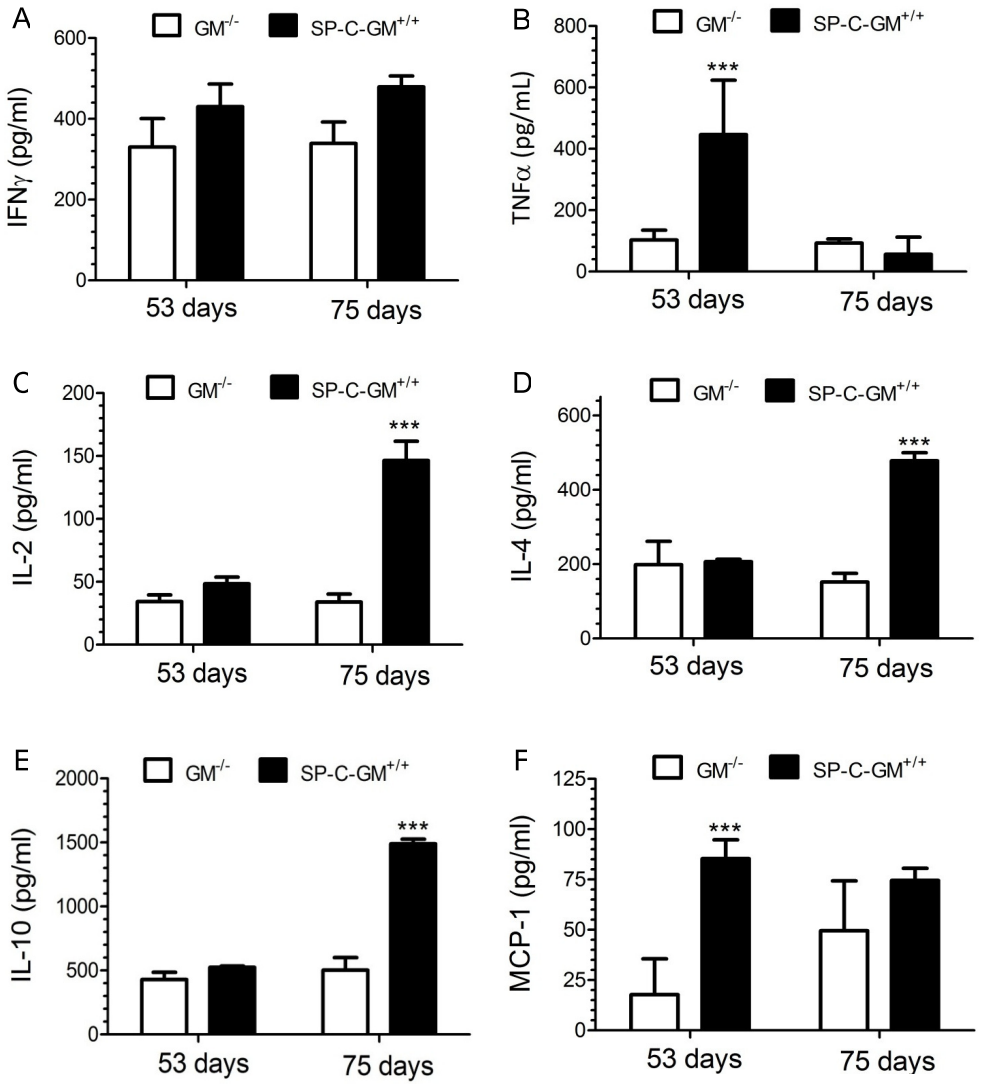

Fig. 2. High levels of GM-CSF promote Th2 polirization after *M. bovis* BCG infection. WT, GM$^{-/-}$, and SP-C-GM$^{+/+}$ mice were infected with 1.5 x 10^7 cfu of *M. bovis* BCG via the intranasal route. The concentrations of IFNγ, TNFα, Il-2, IL-4, IL-10, and MCP-1 were measured by ELISA in lung homogenates at 53 and 75 days after infection. ***$p<0.001$ compared to GM$^{-/-}$. Data shown are means±S.E.M. n=4 for all mice.

The levels of TNFα, however, were significantly higher in the lungs of SP-C-GM$^{+/+}$ mice compared to GM$^{-/-}$ mice 53 days after infection, consistent with increased macrophage activation in the GM-CSF over-secreting mice (Figure 2B). The level of TNFα in SP-C-GM$^{+/+}$ mice, however, decreased 75 days after intranasal immunization with BCG and was similar to GM$^{-/-}$ mice (Figure 2B), indicating suppression of macrophage activation in SP-C-GM$^{+/+}$ mice at the later time point. Correspondingly, the levels of IL-2 (Figure 2C), IL-4 (Figure 2D), and IL-10 (Figure 2E) increased significantly 75 days after BCG immunization of SP-C-GM$^{+/+}$ mice, indicating proliferation of T lymphocytes secreting immunosuppressive cytokines. The increase in IL-4 secretion suggests that high levels of GM-CSF in the context of BCG immunization results in activation of Th2 cell-mediated immunity in the long term. Furthermore, Figure 2F shows significantly increased levels of MCP-1 53 days after immunization with BCG in SP-C-GM$^{+/+}$ mice that remained elevated at 75 days. MCP-1 is considered an important biomarker predictive of active TB infection or increased risk for development of active tuberculosis (78-81). In this regard, the SP-C-GM$^{+/+}$ mice display delayed morbidity compared to GM$^{-/-}$ mice following infection with virulent *M. tuberculosis*; all SP-C-GM$^{+/+}$ mice were alive at 53 days but their survival decreased after 70 days (71, 72). These results indicate that GM-CSF modifies transitional immune responses to mycobacterial infections between primary and chronic phases of the disease.

4. GM-CSF coordinates immunopathology with lung resistance to *M. tuberculosis*

Why does GM-CSF influence susceptibility to pulmonary *M. tuberculosis* but not to other mycobacterial species? Histological analysis, mycobacterial localization, and measurements of immune responses of *M. tuberculosis* infected lungs from WT, GM$^{-/-}$ and SP-C-GM$^{+/+}$ mice provided important insights (71). The previous studies and the findings presented here show that the balance between GM-CSF levels and mycobacterial load underlie the complexity of pathological and host immune responses in tuberculosis.

In WT and SP-C-GM$^{+/+}$ mice, the earliest lung response to *M. tuberculosis* infection was the appearance of macrophage aggregates in the interstitial space underlying alveolar epithelial cells 7 days after infection. This early response did not occur in GM$^{-/-}$ mice whereas interstitial lesions were restored and larger in SP-C-GM$^{+/+}$ mice. Parenchymal interstitial lesions in WT mice had evolved into multicellular structures of epithelioid macrophages interspersed with lymphocytic infiltrates 15 days after infection. The WT lung epithelioid lesions persisted over time in lung parenchyma along with the appearance of highly organized peribronchial and submucosal granulomas containing lymphocyte aggregates and foamy macrophages 15 days onward. Interestingly, formation of epithelioid inflammation in WT mice contrasts the histopathology observed after infection with BCG where only lymphocytic granulomas in peribronchial/submucosal areas were observed (71). Lymphocytic granulomas containing clusters of lymphocytes were small and sparse in BCG infected SP-C-GM$^{+/+}$ lungs. The *M. tuberculosis* infected SP-C-GM$^{+/+}$ however, formed large epithelioid lesions, similar to those observed in WT mice, 15 days after infection but these disintegrated into smaller interstitial lesions over large areas of the lung parenchyma at later time points. Peribronchial and submucosal lymphocytic infiltrates in SP-C-GM$^{+/+}$ mice were smaller than in WT mice but contained foamy and epithelioid macrophages. In contrast, the histology of GM$^{-/-}$ lungs, in

which epithelioid inflammation did not occur at early time points, were characterized by expansion of foamy macrophages consolidating multiple alveoli into necrotic nodules enriched in surfactant lipoprotein 21-28 days after infection (17, 71).

The dynamics of mycobacterial infection was visualized using the highly sensitive auramine-rhodamine stain shown on Figure 3. Interstitial lesions were the site of *M. tuberculosis* proliferation in WT mice and were observed at both 15 and 29 days after

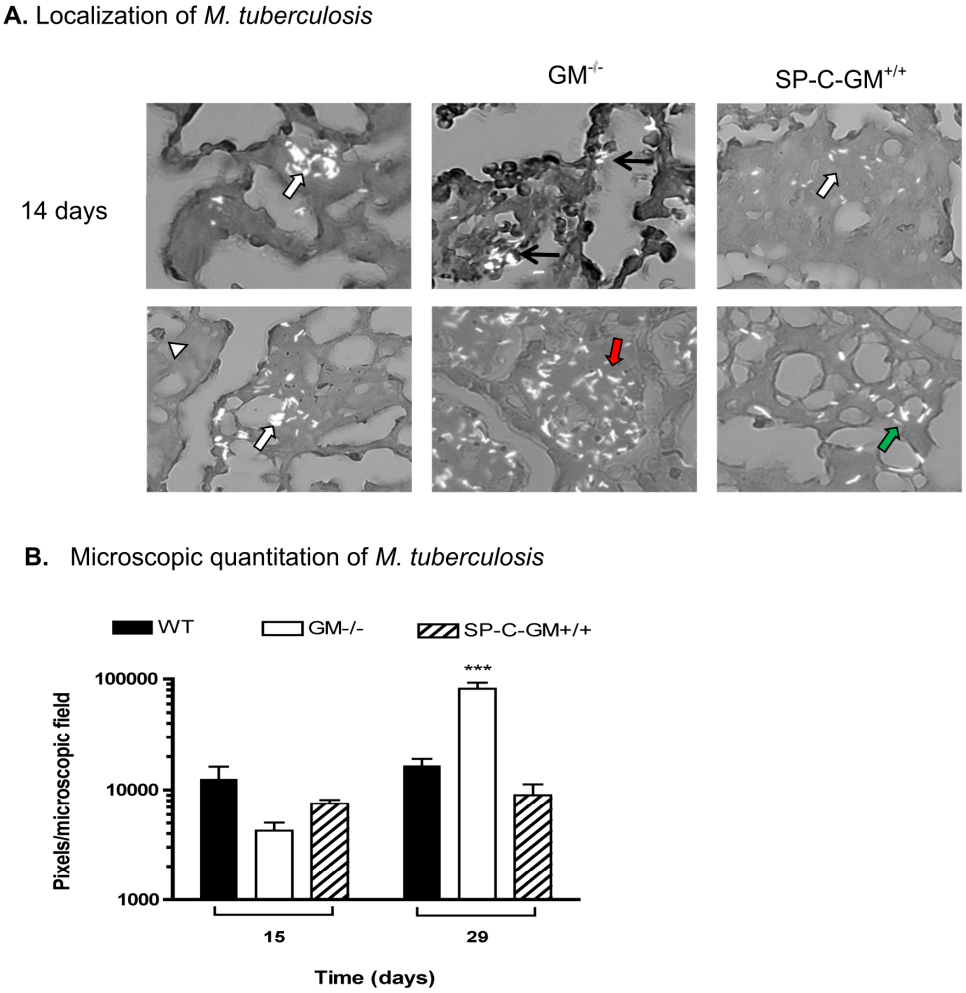

Fig. 3. Effect of GM-CSF on *M. tuberculosis* localization in the lungs.
A) Localization of *M. tuberculosis* infection in lung. WT, GM$^{-/-}$, and SP-C-GM$^{+/+}$ mice were infected with 100 *M. tuberculosis* cfu via aerosol. *M. tuberculosis* organisms were identified in lung tissue section 15 and 29 days after infection using auramine-rhodamine. *M. tuberculosis* were visualized in distal lung interstitial lesions in both WT and SP-C-GM$^{+/+}$ mice (white arrow), but were retained in alveolar macrophages and alveolar lumen (open black arrows)

in GM$^{-/-}$ mice bacilli 15 days after infection. *M. tuberculosis* were found in smaller but more widespread interstitial lesions in both WT (white arrows) and SP-C-GM$^{+/+}$ (green arrows) mice. In contrast, *M. tuberculosis* proliferated in intra-alveolar lesions in GM$^{-/-}$ mice 29 days after infection (red arrows). Magnification x40. B) Microscopic quantitation of *M. tuberculosis* infection in lung. Both WT and SP-C-GM$^{+/+}$ mice form bacteriostatic lesions while GM$^{-/-}$ mice cannot restrict MTB growth on day 29. Data are means ± S.E.M ***p<0.001 in GM-/- vs. all groups.

infection (Fig. 3A, white arrows). Free aggregated *M. tuberculosis* organisms were observed in the alveolar lumen upon the alveolar epithelium. Some interstitial lesions were devoid of mycobacteria while others contained only a few clusters (Fig. 3A, white arrow heads). Previous assessment of mature lymphocytic granulomas in peribronchial and submucosal areas of the lungs localized single organisms in foamy macrophages in WT and SP-C-GM$^{+/+}$ mice (17). The mycobacterial burden remained stable between 15 and 29 days in both WT and SP-C-GM$^{+/+}$ mice (Figure 3B), likely reflecting the balance between poor control in the parenchyma and effective arrest of *M. tuberculosis* in mucosal lesions. In contrast to WT and SP-C-GM$^{+/+}$ mice, *M. tuberculosis* organisms infected alveolar macrophages and were seen on the luminal side of alveolar epithelium 15 days after infection (Fig. 3A, open black arrows), but not in the interstitium of GM$^{-/-}$ mice. The presence of *M. tuberculosis* organisms in parenchymal interstitium of WT and SP-C-GM$^{+/+}$ nut not in GM$^{-/-}$ mice suggests a potential route of local dissemination of the organism between parenchymal and mucosal sites; there were no differences in dissemination of *M. tuberculosis* to the spleen between the different mouse groups (unpublished data). The parenchymal tropism of *M. tuberculosis* was not evident using the more common Ziehls-Nielsen acid fast stain (unpublished results) but was readily detectable using the auramine-rhodamine stain used on Figure 3. The GM$^{-/-}$ mice developed full-blown pulmonary tuberculosis characterized by over a log increase in mycobacterial burden compared to WT and SP-C-GM$^{+/+}$ mice (Figure 3B) (71). Numerous mycobacteria were located in foamy macrophages in intra-alveolar lipid rich lesions undergoing necrosis (Fig. 3A, red arrows) (17). Necrotic lesions also formed in a fraction of BCG-infected GM$^{-/-}$ mice but these were also rich in neutrophils, were enclosed by healing granulation tissue, and were devoid of BCG organisms. Most GM$^{-/-}$ mice eliminated lung BCG infection without formation of granulomas (71). These results support the concept that primary *M. tuberculosis* infection generates a heterogeneous histological response of epithelioid and lymphocytic granulomas, reflecting the ability of *M. tuberculosis* to invade the distal lung interstitium at early stage.

That GM-CSF regulates a critical juncture between early parenchymal inflammation and activation of cell-mediated immunity was evidenced by significant differences in IL-12p40 between WT, GM$^{-/-}$, and SP-C-GM$^{+/+}$ mice on day 14 (Figure 4A). IL-12p40, produced by dendritic cells, is critical for activation of IFNγ-secreting T lymphocytes in the lung. IL-12p40 increased significantly between 7 and 14 days in both WT and SP-C-GM$^{+/+}$ mice but not in GM$^{-/-}$ mice. The levels of IL-12p40 were similar in all mice at later times indicating that, in GM$^{-/-}$ mice IL-12p40 secretion is delayed but not impaired. In GM$^{-/-}$ mice, IL-12p40 increased in a time-dependent manner after day 14. IL-12p40 increased in a time-dependent manner in WT mice that seemed to equilibrate by day 29, but in SP-C-GM$^{+/+}$ mice peak IL-12p40 levels were observed on day 14. The concentration of IFNγ was significantly higher in SP-C-GM$^{+/+}$ mice 7 days after infection compared to WT and GM$^{-/-}$ mice, but not different

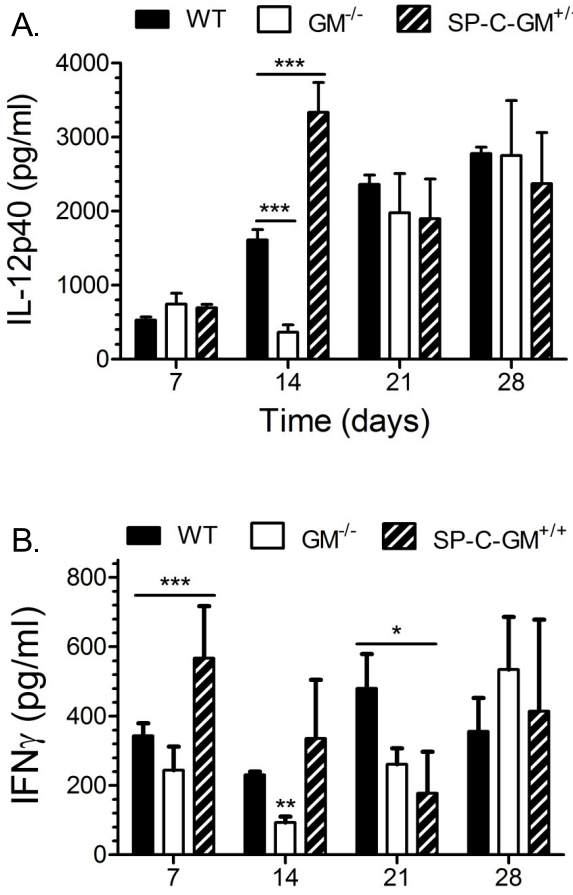

Fig. 4. GM-CSF modulates secretion of IL-12p40 and IFNγ in *M. tuberculosis* infection. WT, GM$^{-/-}$, and SP-C-GM$^{+/+}$ mice were infected with 100 *M. tuberculosis* cfu via aerosol. Lung IL-12p40 (A) and IFNγ (B) were measured in lung homogenates by ELISA. IL-12p40 and IFNγ were attenuated in GM$^{-/-}$ mice on day 14. Significant differences in IL-12p40 were found on day 7 and 21: *$p<0.01$ GM$^{-/-}$ vs WT and SP-C-GM$^{+/+}$ on day 14, **$p<0.02$ SP-C-GM$^{+/+}$ vs WT and GM$^{-/-}$ on day 21. * $p<0.001$ SP-C-GM$^{+/+}$ vs WT and GM$^{-/-}$ on day 7. Data are means ± S.E.M. n=4-6 for all groups.

between WT and GM$^{-/-}$ mice (Figure 4B). The level of IFNγ was lower in GM$^{-/-}$ mice 15 days after infection and significantly below the levels of WT and SP-C-GM$^{+/+}$ mice at this stage of infection. Subsequently, IFNγ plateaued 22 days after infection in WT mice. The highest amount of IFNγ in GM$^{-/-}$ mice was measured 29 days after infection, indicating delayed activation of T lymphocytes consistent with the IL-12p40 results on Figure 4A. Induction of IFNγ in SP-C-GM$^{+/+}$ mice occurred early but subsequently was found to be variable and at overall lower levels (Figure 4B). The effect of different GM-CSF levels in kinetics of lung CD4+ and CD8+ T lymphocytes following *M. tuberculosis* infection are shown on Figure 5. In

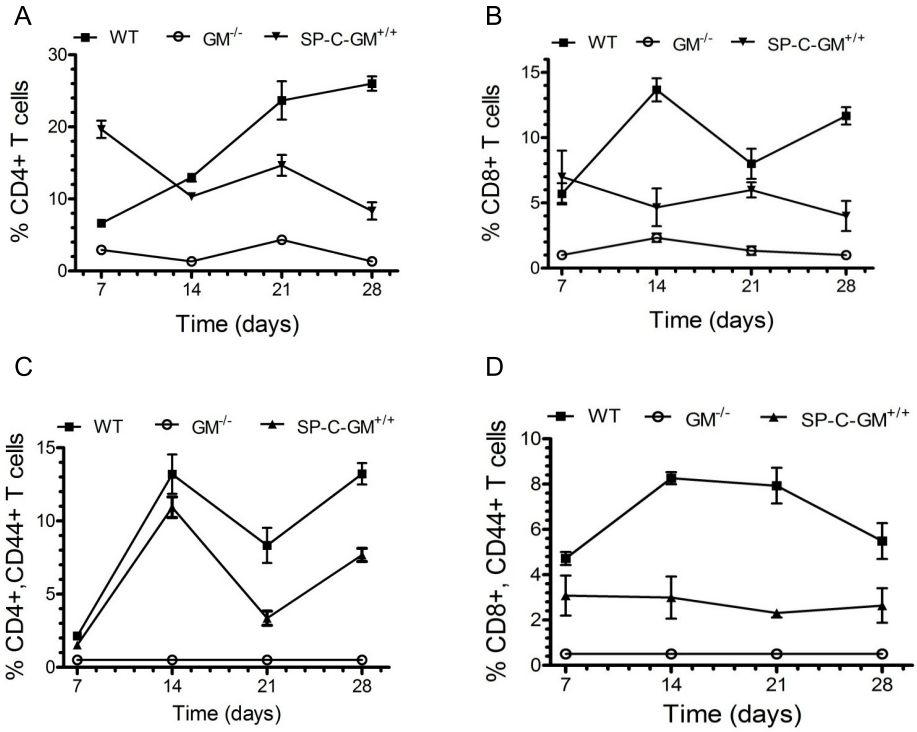

Fig. 5. GM-CSF regulates activation of T lymphocytes in the lung.
WT, GM$^{-/-}$, and SP-C-GM$^{+/+}$ mice were infected with 100 *M. tuberculosis* cfu via aerosol. Lungs were enzymatically dispersed into cell suspensions at indicated times after infection and lymphocytes analyzed by flow cytometry. The content of CD4+ (A), CD8+ (B) T lymphocytes were obtained by gating. Activated CD4+ (C) and CD8+ (D) T lymphocytes were obtained by staining cells with CD44 antibodies. Data are means ± S.E.M. n=4 for all groups.

WT mice, lung CD4+ T lymphocytes increased in a time-dependent manner reaching an apparent plateau 28 days after infection (Figure 5B). In contrast, Figure 5B shows abnormal CD4+ T lymphocyte kinetics in SP-C-GM$^{+/+}$ lungs with early recruitment 7 days after *M. tuberculosis* infection that equilibrated at lower levels 7-29 days after infection. Compared to WT mice, the CD8+ T lymphocyte content did not change over time in SP-C-GM$^{+/+}$ lungs (Figure 5B). Despite the early influx of CD4+ T lymphocytes in SP-C-GM$^{+/+}$ lungs, activation of CD4+ T lymphocytes, as indicated by expression of CD44, was similar in WT and SP-C-GM$^{+/+}$ mice (Figure 5C). In contrast, expression of CD44 was activated in CD8+ T lymphocytes in WT but not in SP-C-GM$^{+/+}$ mice (Figure 5D). Both CD4+ and CD8+ T lymphocytes were found in low numbers and did not express CD44 in lungs of GM$^{-/-}$ mice (Figure 5A-D). Peripheral activation of T lymphocytes occured in the absence of GM-CSF, as shown by similar activation of CD44 in CD4+ and CD8+ T lymphocyte populations after *M. tuberculosis* infection (Figure 6A-D). The content of activated CD8+,CD44+ T lymphocytes, however, was higher in WT mice 28 days after infection compared to both GM$^{-/-}$ and SP-C-GM$^{+/+}$ mice, indicating that peripheral activation of CD8 T cells is not optimal in the absence of GM-CSF.

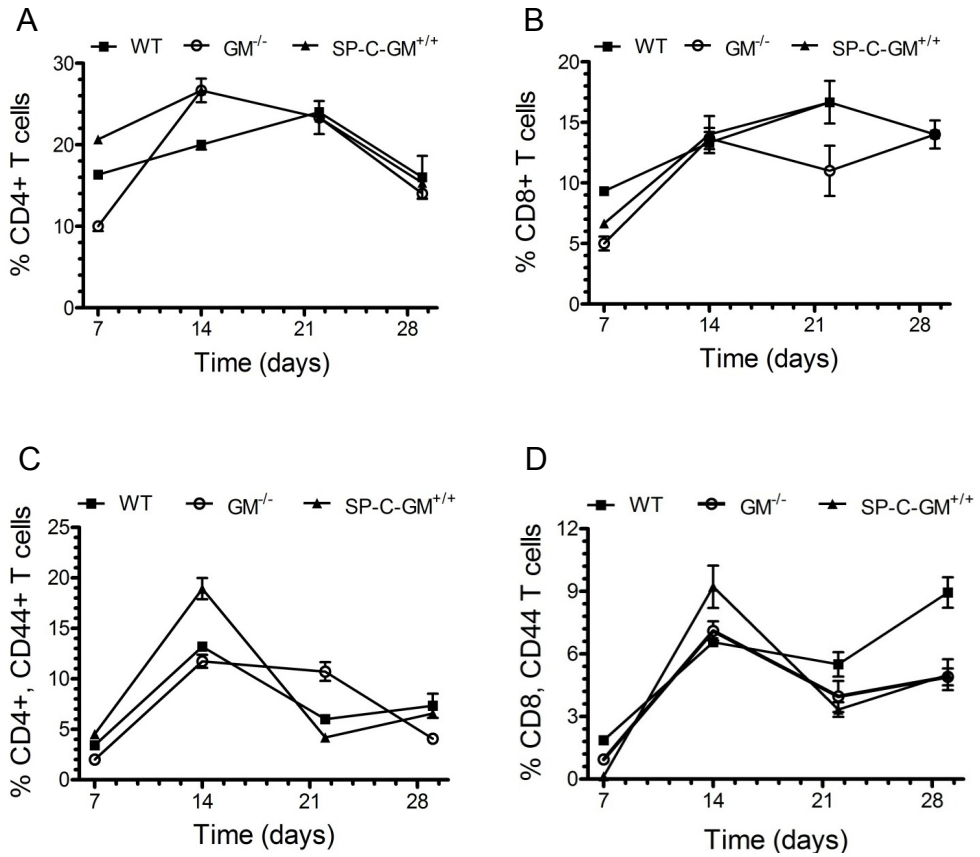

Fig. 6. Effect of lung GM-CSF in activation of T lymphocytes in the spleen. WT, GM$^{-/-}$, and SP-C-GM$^{+/+}$ mice were infected with 100 *M. tuberculosis* cfu via aerosol. Lungs were mechanically dispersed into cell suspensions at indicated times after infection and lymphocytes analyzed by flow cytometry. The content of CD4+ (A) and CD8+ (B) T lymphocytes were obtained by gating. Activated CD4+ (C) and CD8+ (D) T lymphocytes were obtained by staining cells with CD44 antibodies. Data are means ± S.E.M. n=4 for all groups.

Surprisingly, 10-fold reduction in *M. tuberculosis* infection from 100-200 cfu to 10-20 cfu delayed morbidity significantly from 30-40 days weeks to six months in both GM$^{-/-}$ mice and from 70-90 days to 12 months in SP-C-GM$^{+/+}$ mice (17). Histological evaluation of GM$^{-/-}$ lungs 6 months after low dose *M. tuberculosis* infection revealed intra-bronchial obstruction with surfactant rich material and necrotic cellular debris indicating collapse of alveolar spaces into bronchial airways. Interestingly, peribronchial spaces of obstructed airways contained lymphocytic infiltrates, suggesting that cell-mediated immunity against *M. tuberculosis* is abnormal histologically but not impaired in the absence of GM-CSF. On the other hand, the low dose infection in SP-C-GM$^{+/+}$ mice generated large lymphocytic granulomas similar to WT mice and expansion of foamy macrophages in surrounding

alveoli contrasting the results following high dose *M. tuberculosis* infection described above (17, 71). These findings indicate an important relationship between GM-CSF levels and *M. tuberculosis* infection dose modulating histological remodeling in pulmonary tuberculosis.

5. Discussion

Histological studies indicate that GM-CSF facilitates formation of early epithelioid granulomas in lung parenchyma during the innate phase of *M. tuberculosis* infection. GM-CSF expression is required for the development of interstitial granulomas as indicated by the absence of interstitial lesions in GM$^{-/-}$ mice and formation of enlarged epithelioid lesions in SP-C-GM$^{+/+}$ mice in the first 15 days after *M. tuberculosis* infection. This early histological response involves recruitment of macrophages and other immune cells leading the differentiation of interstitial lesions enriched in epithelioid cells. Initial granuloma formation begins with alveolar deposition of *M. tuberculosis* organisms resulting in not only infection of alveolar macrophages but also trafficking and proliferation of *M. tuberculosis* in interstitial granulomas. In contrast to WT and SP-C-GM-CSF$^{+/+}$ mice, alveolar macrophages were the main site of infection in GM$^{-/-}$ mice, indicating that GM-CSF is not required for infection of alveolar macrophages by *M. tuberculosis*, whereas GM-CSF is required for the early dissemination of *M. tuberculosis* into lung interstitium. Differences in GM-CSF levels, however, did not influence or alter distal dissemination to the spleen (unpublished data), indicating that peripheral and local dissemination of *M. tuberculosis* infection involve different mechanisms. Transport of infected macrophages or infection of epithelial cells may contribute to early interstitial dissemination of *M. tuberculosis*. GM-CSF-induced differentiation of alveolar macrophages or expression of mycobacterial receptors on alveolar epithelial cells may be required to relocate *M. tuberculosis* from the alveolar lumen to lung interstitium. Previous studies reported that *M. tuberculosis* uses the cell-surface hemagglutinin HBHA to trancytose across alveolar epithelial cells (82). More recently, it was shown that *M. tuberculosis* and its close relative *M. marinum* spread between cells via the ejectosome (83), an actin-based non-lytic mechanism that requires expression of the ESX-1 secretion system. The ESX-1 secretion system of *M. tuberculosis* mediates export of the highly immunogenic antigen ESAT-6. Mycobacterial organisms such as *M. avium* and *M. bovis* BCG that lack ESX-1 do not disseminate through ejectosomes. The early epithelioid granuloma drives activation of cell-mediated immunity as indicated by marked induction of IL-12p40 in WT and SP-C-GM$^{+/+}$ mice but not in GM$^{-/-}$ mice two weeks after *M. tuberculosis* infection. It is possible that epithelioid macrophages are an intermediate source of *M. tuberculosis* antigen presentation that dendritic cells acquire en route to lymph nodes, a process that takes two weeks to complete before activation of protective immunity against *M. tuberculosis*. Formation of parenchymal epithelioid lesions leading to activation of IL-12p40 secretion preceded the appearance of lymphocytic granulomas in mucosal and peri-bronchial sites establishing cell-mediated immunity and long-term control of the infection in the lung.

GM-CSF suppresses formation of foamy alveolar macrophages in *M. tuberculosis* infected lungs. Macrophage-derived foam cells arise by accumulation of intracellular lipids. Macrophage-derived foam cells are a characteristic feature of primary and post-primary tuberculosis lesions in lungs of both mice and humans (84-86). Macrophage foam cells may facilitate resolution of infection with the vaccine strain *M. tuberculosis* BCG (87), but are thought to have pathogenic roles in *M. tuberculosis* infection. *M. tuberculosis* cell wall lipids contribute to foam cell formation by inducing accumulation of lipid bodies in macrophages

(84, 88). Foam cells may modify the ability of *M. tuberculosis* as a persistent or a resurgent pathogen during latent (89, 90) and post-primary tuberculosis (86), respectively. Lipid bodies that fuse with phago-lysosomes are thought to provide cholesterol and other host lipid nutrients facilitating persistence of *M. tuberculosis* (90-92). Foamy macrophages, in which *M. tuberculosis* organisms were identified at later stages of infection, were a distinguishing feature of lymphocytic granulomas in WT lungs and SP-C-GM$^{+/+}$ mice. In the latter case, large lymphocytic granulomas with expansion of foamy alveolar macrophages was present after low but not high dose infection with *M. tuberculosis.* In contrast to WT and SP-C-GM$^{+/+}$ mice, expansion of foamy macrophages evolving into necrotic granulomas filling alveolar and bronchial spaces was the only lesion that developed three weeks after infection of GM$^{-/-}$ mice with *M. tuberculosis* infection. Induction of IL-12p40 and IFNγ was not abrogated but occurred at late stages of infection apparently providing adequate immunity that prolonged survival at low dose infection with *M. tuberculosis*. Necrotic granulomas obstructing alveolar and bronchial spaces were enriched in pulmonary surfactant. Interestingly, staining surfactant proteins in normal mice indicated that surfactant lipoprotein is actively produced by alveolar type II epithelial within tuberculous granulomas (17). Given the essential and selective role of GM-CSF in degradation of surfactant lipids (46, 58), it is reasonable to speculate that foamy macrophages in tuberculosis represent macrophages that have lost the capacity to respond to GM-CSF forming a suitable environment for *M. tuberculosis* to maintain its life cycle in the long term.

6. Hypothesis

The model on Figure 7 depicts that differences in GM-CSF levels control the balance between protective and escape mechanisms through which *M. tuberculosis* is either controlled or causes infection transmitting infection to other individuals. The histological presentation of primary tuberculosis at early stage of infection is discerned by three morphologically distinct but interacting processes. GM-CSF is necessary for the development of epithelioid granulomas in lung parenchyma 0-15 days after infection, a period known as the innate phase of the infection. Epithelioid granulomas govern the development of cell-mediated immunity in the form of lymphocytic granulomas which curb proliferation of *M. tuberculosis* in the lung. Macrophages that lose the ability to respond to GM-CSF constitute the escape mechanism that transitions primary tuberculosis to chronic infection. Foamy macrophages accumulate lipids that may provide a permissive environment for mycobacteria to survive. Foamy macrophages remain in constant interaction with adaptive immune cells preventing mycobacterial proliferation in the long-term. High levels of GM-CSF generated at the chronic or latent stage of tuberculosis may alter polarization of the immune response resulting in reactivation of the infection in foamy macrophages. As a result, development of post-primary tuberculosis may progress to cavities transmitting the disease to other individuals through coughing. At this stage, depending on the cause of GM-CSF secretion, GM-CSF may work to the host's benefit to resorb lipid through recruitment and differentiation of macrophages facilitating resolution of cavitary tuberculosis, as occurs in most individuals with post-primary tuberculosis (93), or promote disease development through inappropriate polarization of the immune response in the lung. At this point it should be noted that BCG immunization in the context of high levels of GM-CSF was associated with Th2 polarization after clearance of BCG (Figure 2). BCG has been highly effective against tuberculosis in children preventing dissemination of the disease that causes

tuberculous meningitis, but fails to prevent activation of the disease in adults. Millions of children receive BCG vaccination yearly. Differences in lung GM-CSF in endemic populations may contribute to the failure of the BCG vaccine once these individuals become adults and it is

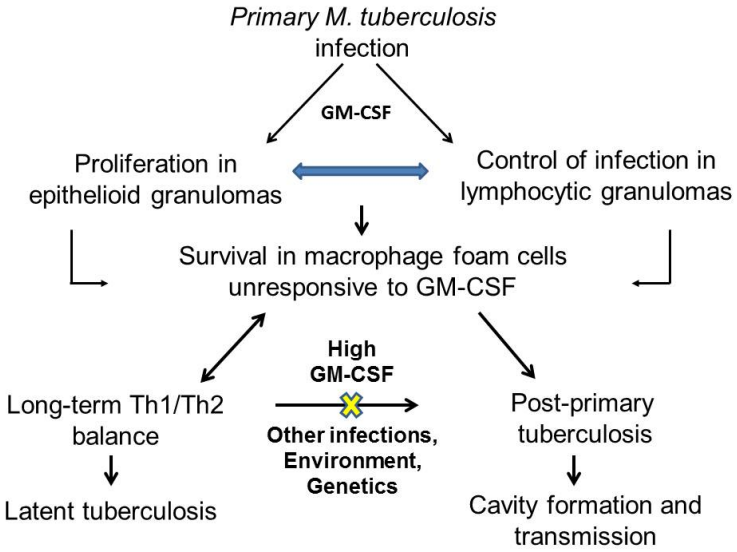

Fig. 7. Hypothesis on the role of GM-CSF in pathogenesis of pulmonary tuberculosis.GM-CSF is required for initiation of protective immunity against primary tuberculosis by inducing optimal formation of epithelioid and lymphocytic granulomas. *M. tuberculosis* survives in foamy macrophages that do not respond to GM-CSF. Long-term adaptive immunity regulates transition to latent tuberculosis by suppressing mycobacterial proliferation in foamy macrophages. Excess production of GM-CSF during latent infection alters the balance of adaptive immunity resulting in reactivation of *M. tuberculosis* infection in foam cells. Reactivation of *M. tuberculosis* causes development of post-primary tuberculosis and formation of cavities that transmit tuberculosis disease.

not unreasonable to suggest that it could contribute to post-primary tuberculosis, the most common form of the disease in the developing world. Given the hypothesis that latent M. tuberculosis infection hides in foamy macrophages under the incomplete surveillance of cell-mediated immunity, the mouse models described here present unique opportunities to test vaccine strategies targeting the infection in its natural host cell.

7. Methods

7.1 Mice

Specific pathogen-free male or female C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). The generation C57BL/6 GM$^{-/-}$ and SP-C-GM$^{+/+}$/GM$^{-/-}$ mice was described previously (50, 94). Mice were housed and bred under specific-pathogen free conditions. Mice were transferred to a BSL-3 vivarium for experiments and were used at between 4-6 weeks of age in accordance with IACUC approved protocols.

7.2 Bacteria

Mycobacterium tuberculosis (MTB) H37Rv (#27294) was from the ATCC repository. The *M. bovis* BCG strain Tokyo strain S-10 was a kind gift of Dr. Malini Rajagopalan, University of Texas Health Science Center at Tyler. Colonies (CFU) grown on 7H11 agar were sub-cultured in 7H9 broth and log phase organisms were harvested 10-12 days later. Bacteria were washed in PBS and sonicated at 5 watts for 15 seconds to disperse organisms and stored in aliquots at –70°C in 70% glycerol-PBS. One thawed aliquot was diluted ten-fold in PBS and plated for CFU counts on 7H11 agar.

7.3 Infections of mice

Pulmonary infections with 1.5 x 10^7 BCG were established intranasaly as previously described. Aerosol infections were performed in a Middlebrook aerosol exposure chamber (Glascol Inc, Terre Haute, IN). A sonically dispersed suspension of MTB at 10^6 CFU/mL in saline was nebulized for 30 min, which implanted around 2.0 $\log_{10}$ CFU/mouse lung. All mouse groups were exposed to the MTB aerosol at the same time. Mice were sacrificed on indicated time points and lungs and spleens were aseptically removed. Organs were homogenized in PBS with 0.05% Tween-80 and ten-fold dilutions plated on 7H11 agar for enumeration of CFU counts.

7.4 Localization of mycobacteria in lungs

Infected organs were fixed in 10% formalin and embedded in paraffin. Mycobacteria were visualized using an auramine-rhodamine staining kit (BD Biosciences, San Diego, CA). Fluorescent images were captured using TE 100 inverted microscope equipped with a Cooke Sensicam digital camera (3I imaging Denver, Co). The MTB burden in infected lesions was estimated as fluorescent pixels/microscopic field from six randomly selected microscopic fields by using morphometry tools embedded in Slidebook software (3I imaging, Denver, Co). Tissues were interrogated at 40x magnification.

7.5 ELISA

Lung homogenates were filtered using 0.2 μ syringe filters prior to ELISA assays that measured the concentration of IFNγ, IL-12, IL-10, IL-4, MCP-1, and TNFα. All kits were obtained from eBiosciences (San Diego, CA).

7.6 Flow cytometry

Single cell suspensions were obtained from lung and spleen. Lungs were perfused with cold sterile phosphate buffered saline (PBS), minced and digested in PBS containing 1 mM EDTA , 100 U /mL collagenase, 50 μg/ml elastase and 1 μg/ml DNAase (Sigma Alidrich, MO) for 45 min at 37°C. The digest was then passed through a 0.45 μ nylon mesh to obtain single cell suspensions. The cells were centrifuged and then suspended and stained in FACS buffer (PBS, supplemented with 2% heat inactivated goat serum, 0.5% fetal calf serum, 10 μg/ml Fc block and 0.02% azide). The T cells were stained with conjugated antibodies against the following antigens (BD PharMingen): CD3 (anti-CD3-PE, clone 145.2C11), CD4 (anti-CD4-FITC, clone H129.19), CD8 (anti-CD8-FITC, clone 53-6.7), and CD44 (anti-CD44-PE, clone IM7). Dual

staining of lymphocytes was accomplished using antibodies to CD4 or CD8 along with antibodies against CD44. Cells were fixed with 2% paraformaldehyde for 1 h and analyzed by flow cytometry using CellQuest software (BD Immunocytometry Systems, San Jose, CA). Cells were gated on the lymphocyte and monocytes by forward and side scatter.

7.7 Statistics

Graphing and statistical analysis was accomplished using Graphpad Prism 4.0 software (www.graphpad.com). Statistical comparisons were assessed by two-way ANOVA, and differences were considered significant at $p<0.05$.

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11

Double Edge Sword: The Role of Neutrophils in Tuberculosis

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1. Introduction

Today, after more than 100 years of tuberculosis (TB) research, which have produced a vaccine and several drugs, TB still remains the most important bacterial infection worldwide. Every year more than 1.5 million people are killed by the infection and around 8 million new cases are reported. The risk of developing the disease is greatly increased by acquired immunodeficiency syndrome (AIDS) and immune-compromising conditions, such as diabetes and malnutrition.

In order to exert a better control of the disease, more effective vaccines and/or chemotherapeutics should be developed. In order to achieve this, better understanding of the immune mechanisms underlying the host-pathogen relationships in TB should be obtained first (Nathan, 2009). Of particular interest is the innate immunity generated by *Mycobacterium tuberculosis* infection, and more precisely the role of neutrophils, since their exact participation in the immunity or pathogenesis of TB is still poorly understood.

Because *M. tuberculosis* is transmitted via aerosols, classically alveolar macrophages, and more recently dendritic cells have been considered to be the first cells to encounter the bacilli in the alveolar sack. This view has just recently started to change dramatically, despite results obtained by Antony *et al.* in 1983, which clearly demonstrated, both *in vitro* and *in vivo*, an active participation of neutrophils in monocyte recruitment, granuloma formation and lung repair (Antony et al., 1983).

Neutrophils are polymorphonuclear cells (PMNs), with abundant granules in their cytoplasm that present large amounts of bactericidal molecules such as antimicrobial peptides and different proteolytic enzymes. Besides this molecular repertoire, PMNs are phagocytic cells and are prone to produce abundant Reactive Oxygen Species (ROS). Usually, PMNs that are part of the tisular inflammatory infiltrate are terminal differentiated

cells and with a short lifespan of 6 to 10 h, a notion that has been challenged recently by Pillay et al. (Pillay et al., 2010).

The goal of this chapter is to describe current knowledge about the role of neutrophils during *M. tuberculosis* infection, and their relationship with other cells. The importance of recent molecular and cellular processes described in neutrophils such as pathogen shuttling, antigen presentation, NET formation and ectosome release will be discussed as well as their contribution to the pathophisiology of TB.

2. Neutrophils

Neutrophils or polymorphonuclear cells (PMN) form the first line of defense of the human innate immune system. They are the most abundant leukocytes in the blood (65% to 75% of all white blood cells) (Nathan, 2006) and are armed with powerful weapons to kill foreign microorganisms. Neutrophils kill by both oxidative (phagocytosis) and non-oxidative (degranulation) mechanisms (Kumar et al., 2010). They are differentiated in the bone marrow from pluripotent hematopoietic progenitor cells into mature neutrophils and have a life span of only a few hours in the bloodstream (Borregaard, 2010). However in a paper published last year, this life span was increase to 5.4 days, ten times longer, forcing to rethink the functions these cells might play in health and disease (Pillay et al., 2010).

Neutrophils possess a multi-lobulated nucleus, abundant storage granules in the cytoplasm (azurophilic or primary, secondary and tertiary granules) (Borregaard et al. 1997), glycogen in the cytosol from which they derive almost all of their energy, and only a few mitochondria. The granules in mature neutrophils contain a variety of proteins that contribute to anti-microbial host defense. Among the proteins in the azurophilic granules are those with direct antimicrobial action (i.e. defensins, bactericidal-permeability-increasing protein, azurocidin), proteases (i.e. elastase, cathepsins), and a peroxidase that is normally expressed only in neutrophils and monocytes (i.e. myeloperoxidase (MPO)) (Borregaard & Cowland, 1997).

The recruitment of neutrophils from the bloodstream to the site of infection is initiated by chemokines and cytokines in a process called extravasation (Kobayashi & DeLeo, 2009). Then, microbes and microbial compounds such as lipopolysaccharide (LPS) activate the neutrophils via transmembrane receptors. Once neutrophils are at the site of infection, they can internalize both opsonized and non-opsonized microbes (Lee et al., 2003). Fc receptors and a subgroup of β2 integrins, which are the principal opsonin receptors of neutrophils, bind to immunoglobulin and to complement-coated particles respectively (Witko-Sarsat et al., 2001). The vesicles containing the pathogens, called phagosomes, fuse with neutrophil granules, and the antimicrobial contents are discharged into the lumen of the phagosome, which is then called the phagolysosome (Segal, 2005).

Upon activation, neutrophils are highly effective at generating reactive oxygen species (ROS) by a process known as respiratory burst. In stimulated neutrophils, ROS are generated almost exclusively by NADPH oxidase (Kobayashi & DeLeo, 2009). If phagocytosis is not to occur following pathogen interaction with the neutrophils, the release of granule contents and ROS formation will be directed to the outside of the cell to eradicate extracellular pathogens. Anti-microbial compounds from granules not only kill the bacteria,

but may also act as chemoattractants for T-cells and immature dendritic cells (iDCs), which in turn recruit more neutrophils to the site of infection and also initiate an adaptive immune response (Burg & Pillinger, 2001).

2.1 Neutrophil Extracellular Traps (NETs) are induced by *M. tuberculosis*

Another recently described microbicidal mechanism of neutrophils is the release of structures called neutrophil extracellular traps (NETs) which can trap and kill microbes. These structures are composed of nuclear chromatin or mitochondrial DNA, associated mainly with nuclear histones and granular antimicrobial proteins (Brinkmann et al., 2004; Yousefi et al., 2009). NETs are formed in response to a variety of pro-inflammatory stimuli such as LPS, IL-8, TNFα and PMA (Brinkmann et. al., 2004), as well as by fungal (Urban et al., 2006; McCormick et al., 2010), bacterial (Beiter et. al., 2006; Brinkmann et al., 2004; Ermert et al., 2009; Ramos-Kichik. et al., 2009), or protozoal (Baker et al., 2008; Guimaraes-Costa et al., 2009) strains and species both *in vivo* and *ex vivo*. The formation of NETs has been demonstrated in many non-infectious pathophysiological conditions in mice, cows and humans (i.e. pre-eclampsia, Crohn's disease, systemic Lupus erithematosus and cystic fibrosis) (Gupta et al., 2005; Hakkim et al., 2010; Marcos et al., 2010; Yousefi et al., 2008).

There is much evidence indicating that NETs are released in the context of a cell death different from apoptosis or necrosis. Other granular cell types, such as eosinophils (Yousefi et al., 2008) and mast cells (Kockritz-Blickwede et al., 2008), but not basophils, also release extracellular traps. Therefore, Wartha et al., introduced the term ETosis as a more generalized term to name the process of extracellular trap release by dying cells (Wartha et al., 2008). During ETosis, the lobulated nuclear morphology of neutrophils is lost. Later, both nuclear and granular membranes disintegrate, but plasma integrity is maintained, allowing the antimicrobial granular proteins to mix with nuclear components. Finally, NETs emerge from the cells as the cytoplasmic membrane breaks (Fuchs et al., 2007). No morphological signs of apoptosis are observed, such as membrane blebbing, nuclear chromatin condensation, phosphatidyl serine (PS) exposure before plasma membrane rupture and internucleosomal DNA cleavage (Fuchs et al., 2007). Caspase activity is only detected during spontaneous neutrophil apoptosis, but not during PMA induced ETosis (Remijsen et al., 2011a). In contrast with necrosis, neutrophils do not stain positive for F-actin after they have undergone ETosis (Marcos et al., 2010; ; Palik et al., 2007; Ramos-Kichik et al., 2009). Although the regulation of subcellular events during ETosis remains unclear, increasing evidence indicates that the collapse of the nuclear envelope during ETosis and concurrent chromatin decondensation are regulated by the interplay between histone citrullination, superoxide production and autophagy (Remijsen, 2011b).

Traditionally, neutrophils are viewed as phagocytes important in the resolution of rapidly growing microorganisms. Thus, they were disregarded in the control of intracellular pathogens responsible for chronic diseases. This is the case of tuberculosis, which today is, after AIDS, the second cause of death from an infectious disease worldwide (Young, 2008). The etiological agent, *M. tuberculosis*, is one of the most successful pathogens at evading the host immune response to establish infection. Its pathology is so complex that it has not been fully understood yet. For several years neutrophils were not believed to have a role in the pathogenesis of tuberculosis due to their short life-span and because their microbicidal

mechanisms, although efficient, were associated with tissue damage and inflammation observed during acute infections. However, evidence has accumulated in the past few years emphasizing the role of neutrophils during *M. tuberculosis* infection. *In vivo* studies have revealed that the earliest immune response during mycobacterial infection is a migration of neutrophils to the site of infection during the acute phase of tuberculosis (Appelberg et al., 1989; Barrios-Payán et al., 2006; Pedroza et al., 2000). Moreover, they are thought to be essential for early granuloma formation during chronic *M. tuberculosis* infection (Seiler et al., 2003).

The question of whether neutrophils play a role in killing *M. tuberculosis* or contribute to the development of the pathology remains controversial, and will be discussed at the end of this chapter.

Recently, Ramos-Kichik et al., reported that two different genotypes of the *M. tuberculosis* complex with different virulence degrees (*M. tuberculosis* H37Rv and *M. canetti*), induce subcellular changes that led to NETs formation in a time dependent manner, causing the death of infected neutrophils. Although the mechanism by which *M. tuberculosis* induces this process has not been elucidated yet, it is possible that a direct recognition through TLR2/TLR4 of mycobacterial cell-wall pathogen associated molecular patterns (PAMPs) such as lipoarabinomannan (LAM), lipomannans (LM), phosphatidylinositol mannosides (PIM2, PIM6) and/or the 19 kDa lipoprotein, may be involved in NETs induction. In Addition, it was shown that NETs can trap mycobacteria (Ramos-Kichik et al.,2009). The outermost layer of the mycobacterial cell wall may be involved in NETs attachment, since this is an electrodense structure exposing negatively charged groups (Paul et al., 1992, Takade et al., 2003). Despite the ability to bind mycobacteria, NETs were unable to kill any of the *M. tuberculosis* genotypes tested, regardless of their virulence. Neither could intact neutrophils kill *M. tuberculosis* genotypes either. Instead, *M. tuberculosis*-induced NETs were able to kill *Listeria monocytogenes* (a rapid-growing intracellular bacteria) confirming their antimicrobial effect, and therefore establishing that *M. tuberculosis* is resistant to the microbicidal activity of NETs. It seems that the molecular composition and structural features of the mycobacterial cell wall confer an effective permeability barrier, thereby evading the host innate immune response. Accordingly, more studies are needed to dilucidate the strategies used by *M. tuberculosis* to resist and escape from the microbicidal effect of neutrophils.

Since NETs trap but do not kill *M. tuberculosis*, the role of NETs *in vivo* could be relevant in maintaining the infectious focus localized, thus preventing mycobacterial spreading and at the same time setting the basis for granuloma formation. On the other hand, it is also conceivable that NETs could act as a barrier avoiding phagocytosis of mycobacteria by macrophages, which are one of the few cells with known microbicidal properties against mycobacteria.

It would be interesting to clarify whether engulfment of mycobacteria could switch-on different cell death pathways or if there are mechanisms behind neutrophil maturation or environmental conditions regulating which neutrophils undergo apoptosis and which ones undergo autophagy and ETosis. The fact that NETs are induced by *M. tuberculosis* opens another perspective about the possible extracellular role that neutrophils might play during tuberculosis infection.

3. Ectosomes released from *M. tuberculosis* infected neutrophils

The release of vesicles from cell membrane of different eukaryotic cells has been observed in response to chemical stimuli (Allan et al., 1980; Scott & Maercklein, 1979; Scott et al., 1979), complement attack (Hess et al., 1999; Morgan & Campbell, 1985; Morgan et al. 1987) or pro inflammatory agents (Hess et al. 1999; Gasser et al. 2003). The process of vesicle release has been named by Stein and Luzio, ectocytosis and the vesicles released from the cell membrane ectosomes (Ects) (Stein & Luzio, 1991). Ects with size ranging from 50 to 200 nm released from PMNs in response to complement attack or fMLP (formyl-methionyl-leucyl-phenylalanine), a bacterial product with chemo-attractant and pro inflammatory properties have been extensively studied. Ects are cholesterol enriched compared with the cell membrane composition (Stein & Luzio, 1991), express CD35 (complement receptor 1, CR1), a marker abundantly expressed on secretory vesicles present in the PMNs cell cytoplasm (Sengelov et al., 1994), myeloperoxidase and human leukocyte elastase (Hess et al., 1999) (both present in the azurophilic granules of PMNs), proteinase 3 and matrix metallopeptidase 9 (Gasser et al., 2003). Due to the presence of these enzymes, a role for Ects as ecto-organelles with anti microbial activity was initially proposed (Hess et al., 1999). Other markers found on the Ects membrane are MHC I, CD11a, CD11b, L-selectin, CD46, CD16, CD32. Ects bind annexin V, suggesting the presence of phosphatidilserine (PS) in the external side their membrane. Interestingly, Ects released by PMNs bind selectively to endothelial and macrophages but not to red cells. These findings suggest that Ects could play a role in the immune response. (Gasser et al., 2003).

Recently anti-inflammatory properties have been attributed to Ects, this effect is exerted on macrophages after they enter in contact with Ects. The anti-inflammatory effect has been attributed to the presence of PS on the Ects and the concomitant production of TGFβ-1 by macrophages. This event could provide a mechanism for the resolution of inflammation (Gasser & Schifferli, 2004). Likewise, it was demonstrated that PS on the Ects membrane can inhibit the maturation of monocyte derived dendritic cells, preventing the expression of co-stimulatory molecules and therefore the proper stimulation of T cells (Eken et al., 2008).

All these interesting findings have been gather from experiments with Ects obtained from human PMNs stimulated with a fMLP. And until recently there were no reports concerning Ects release in an *in vitro* infection model. For this reason we investigated if Ects could be released after the phagocytosis of *M. tuberculosis* by human neutrophils.

Previously Gasser *et al.* noticed that Ects released by human PMNs after fMLP stimulation did not constitute a homogenous population in size, prompting the author to hypothesize that these different sizes Ects could have different properties (Gasser, et al. 2003). In our *in vitro* infection model we observed that after 10 min of infection with *M. tuberculosis* H37Rv, human PMNs produced Ects. The Ects released constituted an heterogeneous population; we observed small Ects, similar in size to the population previously described (50 - 200 nm) and larger Ects (González-Cano *et al*., 2010) (0.5 – 0.75μm). Both populations differed not only in size, but also in the presence of superoxide anion (O_2^-), which was clearly visualized in the lumen of the larger Ects.

These larger Ects were characterized by the presence of CD35, Rab5, Rab7, Ps and gP91^{phox} (a component of the NADPH oxidase) and the presence of O_2^- (figure 1). Human PMNs were infected with *M. tuberculosis* for 10 min, stained with the lypophilic dye CellVue® Jade and

the presence of O^{2-} was demonstrated with diaminobenzidine (Kobayashi et al., 1998). Fluorescence microscopy showed that Ects are membranous compartments (fig. 1A) containing O_2^- (fig 1B). In other experiments we confirmed that the release of these larger Ects was not an exclusive event related to *M. tuberculosis* infection, but a general event induced by Gram positive and negative bacteria, as well as by an intracellular parasite. Therefore Ects release could reflect a mechanism of response of PMNs upon invasion (Gonzalez-Cano et al., 2010). More work needs to be done in order to assess the effect that this Ects may have on the anti-microbial capacity of macrophages.

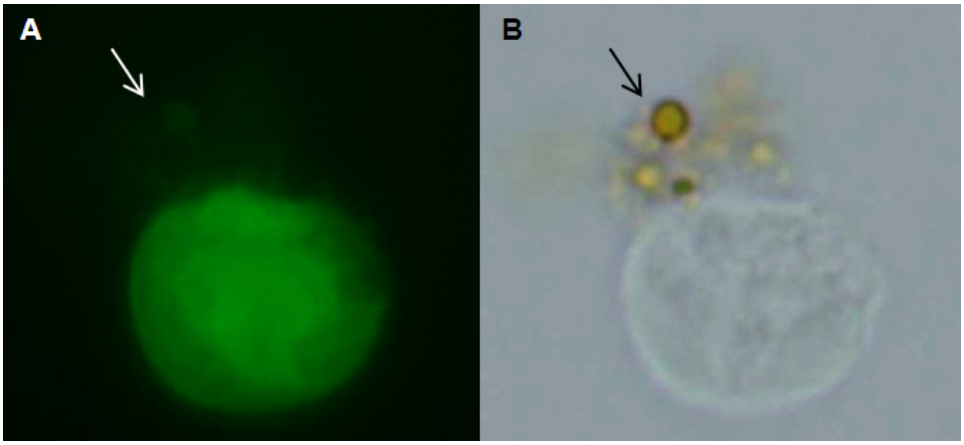

Fig. 1. Ectosomes released by human PMNs infected with *M. tuberculosis* H37Rv showing O_2^- in their lumen. Jade dye staining demonstrates that Ects are membranous compartment (arrow in A), containing O_2^- in their lumen (arrow in B).

Since neutrophils and macrophages "work in a concert" as described by Manuel T. Silva (Silva, 2101a, 2010b) it is essential to analyzed the effect that Ects released by neutrophils may exert on the anti-microbial activity of macrophages.

4. Antigen presentation

PMNs were originally described as short lived and terminally differentiated phagocytes that contribute only to the innate immune response. During the last years they have also been considered to be intimately associated with the establishment of acquired immunity. In resting neutrophils, major histocompatibility complex class (MHC) class I molecules are expressed, while MHC class II and costimulatory molecules are not detected on the cell surface. However, these surface molecules exist intracellularly and some studies indicate that human neutrophils express MHC Class II, CD80 and CD86 molecules on the cell surface, either following *in vitro* activation via CD11b (Sandilands et al., 2005), with IFNγ, IL-3 and GM-CSF (Fanger et al., 1997; Gosselin et al., 1993; Radsak et al., 2000) or IL-4 (Abdel-Salam, 2011).

Potter and Harding demonstrated murine neutrophil Class I restricted antigen presentation and additionally showed that neutrophils processed phagocytosed bacteria via an alternate MHC Class I antigen-processing pathway. Such neutrophils may 'regurgitate' processed

peptide into the extracellular space, this peptide may then bind MHC Class I on neighboring macrophages or dendritic cell for presentation to CD8 cells. Hypothetically, neutrophils may directly present peptide to effector T cells *in vivo* at sites of inflammation, inducing cytokine production, whereas dendritic cells in contact with neutrophil-derived antigenic peptides may migrate to lymphoid organs to initiate T cell responses (Potter & Harding 2001). Additionally, another study demonstrated that murine neutrophils present MHC II-restricted peptides and induced T cell proliferation (Culshaw et al., 2008). These evidences suggested that PMNs may communicate with T cells through direct cell contact.

Since neutrophils have a short life-span and are highly susceptible to apoptosis, their role in antigen presentation has been questioned. However, various pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, produced at the site of inflammation activate neutrophils and suppress apoptosis (Cowburn et al., 2004; McNamee et al., 2005) and as described previously when PMN are cultures in the presence of IFN-γ, GM-CSF or IL-4, these cells show enhanced expression of cell surface molecules and become as competent as dendritic cells or macrophages in their ability to present antigen.

Abi Abdallah *et al.* demonstrated that mouse neutrophils express MHC class II molecules that directly present antigenic peptides, induce T-cell proliferation and promote generation of Th17 effector cells. MHC class II molecules were not constitutively expressed by neutrophils, but instead up-regulation of these proteins required contact with T cells. Most importantly this group showed that ovalbumin-pulsed neutrophils are programmed to induce Th17 differentiation even without addition of exogenous cytokines. This would appear to be an important, and possibly unique, property of PMN, since other antigen presenting cells (APC), such as dendritic cells, typically require addition of recombinant cytokines to mediate optimal T-lymphocyte subset differentiation during cell culture (Abi Abdallah et al., 2011). This is not a recent suggestion, since 1997 PMNs have been demonstrated to act as required accessory cells during T-cell activation with staphylococcal enterotoxin, a superantigen that does not require intracellular processing prior to presentation (Fanger et al., 1997).

Further studies of surface marker expression present additional evidence for the ability of PMNs to differentiate. CD83, a traditional dendritic cell marker, was shown to be expressed on the surface of PMNs stimulated with IFN-γ (Iking-Konert et al., 2001; Iking-Konert et al., 2002; Yamashiro et al., 2000). To assess whether dendritic-like PMN are also generated *in vivo*, cells of patients with acute bacterial infections were tested, the results showed that over half of the patients tested had circulating PMNs expressing CD83. This fact indicates that this phenomenon was not simply the result of unlikely *in vitro* cytokine cocktails, but that the function of CD83 on PMN is still elusive (Iking-Konert et al., 2002). In *ex vivo* experiments Aleman et al. demonstrated that neutrophils in tuberculous pleural effusions, neutrophils expressed CD86, CD83, and major histocompatibility complex class II antigens, acquiring dendritic cell (DC) characteristics. Confirming the fact that the cytokine environment in the pleural space influence the activation of neutrophils allowing them to acquire DC characteristics that in turn influence the immune response against *M. tuberculosis* (Aleman et al., 2005).

PMNs are professional phagocytes that play important roles in many infections, and abundant neutrophils are observed in the bronchoalveolar lavage fluid of patients with

pulmonary tuberculosis (TB) and more intracellular bacilli were found in neutrophils than macrophages in sputum, in bronchoalveolar lavage fluid and in cavities (Eum et al., 2010). The interaction of neutrophils with *M. tuberculosis* induces apoptosis of these cells (Alemán et al., 2002, 2004). Alemán et al. demostrated that *M. tuberculosis* triggers the maturation of DC while it is impaired by the presence of apoptotic PMN, which abrogate Mtb-induced expression of costimulatory and HLA class II molecules, reducing IL-12 and IFNγ release by DC and partially inhibiting Mtb-driven lymphocyte proliferation (Aleman et al., 2007). Other experiments have shown that phagocytosis of apoptotic neutrophils by macrophages results in the decreased viability of intracellular *M. tuberculosis* suggesting a cooperative role of neutrophils in the host's defensive strategy against *M. tuberculosis* infection (Tan et al., 2006). All these experiments provide evidence about the complex interactions between neutrophils and *M. tuberculosis*, supporting the idea that there still much to learn about the immune mechanisms involved in this disease.

5. Interaction of neutrophils with other cells

Neutrophils are viewed as important cellular elements for the control of bacterial infections due to its phagocytic ability and their potential to produce several effector molecules. However, little is known about their role in the regulation of the immune response and the interaction with other cellular elements. One of the first interactions described were between apoptotic neutrophils and macrophages that lead to the removal of apoptotic bodies in a 'silencing' manner because there is not induction of the inflammatory process (Fadok et al., 2000; Newman et al., 1982). So, it is not surprising that neutrophils isolated from patients with active TB are prone to apoptosis either spontaneously or when activated with the bacilli (Aleman et al., 2002). However, apoptotic bodies from Mtb infected neutrophils do not induce an anti-inflammatory state in macrophages, as reflected by the induction of TNFα and IL-1β (Sawant et al., 2010). Moreover, it has been shown that residues of apoptotic neutrophils that are phagocytosed by infected macrophages can co-localize in early endosomes with engulfed mycobateria inducing a decrease in their viability (Tan et al., 2006). Furthermore, the relationship between neutrophils and macrophages has been described *in vivo* through a pleural tuberculosis model. In this model an early recruitment of neutrophils that first ingest bacteria and later undergo apoptosis was observed, a phenomenon that was influenced by the pleural environment (Aleman et al., 2005). The apoptotic bodies, which contained bacteria, were taken by macrophages and these were in turn stimulated to produce suppressor molecules of the inflammatory process such as PGE_2 and TGFβ 1, which could be detrimental for bacilli elimination (D´Avila et al., 2008) and consequently neutrophils would behave as 'Trojan horses" a phenomenon described for other infections caused by intracellular microorganisms (van Zandbergen et al., 2004).

A second cellular element that can interact with neutrophils is the dendritic cell (DCs), which represent an essential element for the induction of T cell responses during mycobacterial infections (Tian et al., 2005). DCs are present as immature cells in different tissues, but when these cells sense a microorganisms or an inflammatory response they fully mature and migrate to draining lymph nodes, where they are responsible for the selection and activation of antigen specific *naïve* T cells. *M. tuberculosis* is capable of inducing this maturation process, however, in the presence of apoptotic bodies derived from neutrophils this process is inhibited. Interestingly cross-presentation is not blocked by this process

allowing antigen presentation to T cells (Aleman et al., 2007). A more recent work showed that *in vivo* neutrophils are important for DCs migration from the lung to mediastinal lymh nodes facilitating the inducton of CD4+ response. The authors suggest that neutrophils deliver *M tuberculosis* to DCs and this process promotes the migration of DC's, making this more efficient and favoring the T cell response (Blomgran & Ernst, 2011).

Although much work has focused on apoptotic neutrophils and their relation with other cell populations, there are other ways by which neutrophil may interact with other cell types. One example of this is TNFα production by *M. tuberculosis* infected neutrophils that is able to activate alveolar macrophages as reflected by an increase in TNFα , IL-1β and hydrogen peroxide production (Sawant & Murray, 2007). Another possible interaction of neutrophils is with elements of the adaptive immune response, for example a recent report described that neutrophils from patients with active TB have shown increased expression of PDL-1 on the cell surface (McNab et al., 2011), a molecule which has been involved in exhaustion of CD8+ T cells during chronic viral infections (Barber et al., 2006) and has been associated with the inhibition of T cell effectors functions during human tuberculosis (Jurado et al., 2008).

A different way of interaction among different cells of the immune system could be through ectosomes (Ect) or neutrophil extracellular traps (NETs), which as mentioned before, are released by *M. tuberculosis* infected neutrophils (González-Cano et al. 2010; Ramos-Kichik et al., 2009). The effect of these (*i.e.* Ect and NETs) *in vivo* in tuberculosis, is still under investigation.

6. Participation of neutrophils in the tissue damage of *M. tuberculosis* infection

Tuberculosis can be considered as the prototype of chronic infectious diseases in which the most important pathogenic factor is the balance between protection and tissue damage mediated by the immune response (Rook & Hernandez-Pando, 1996). Historically the first antecedent of tissue damage mediated by the immune response in tuberculosis was described by Robert Koch in 1891 and was called Koch phenomenon (Anderson, 1891). Koch demonstrated that the intradermal challenge of guinea pigs with whole organisms or culture filtrate, four to six weeks after the establishment of infection, resulted in necrosis at both the inoculation site and the original tuberculous lesion site. A similar phenomenon occurs in persons with active TB, in whom the PPD test site may become necrotic. Koch tried to exploit this phenomenon for the treatment of TB and found that subcutaneous injections of large quantities of *M. tuberculosis* culture filtrate (old tuberculin) into TB patients evoked necrosis in their tuberculous lesions. In fact, this treatment was shown to have extremely severe consequences associated with extensive tissue necrosis and was discontinued (Anderson, 1891). Still today, the task for those working in this field is to understand the differences between protective immunity and progressive disease, including the Koch phenomenon (Rook & Hernandez-Pando, 1996).

It seems that the severity of the Koch phenomenon depends on the dose of antigen, as lower doses induce Th-1 response with high production of IFNγ and macrophage activation which altogether produce the classic delayed type hypersensitivity response. High antigen loads produce local necrosis in which a high Th-2 cytokine production like IL-4 has been founded

(Hernandez-Pando et al., 1997). Interestingly, besides necrotic tissue with macrophage and lymphocytes infiltration there is an increased neutrophils influx (Moreira et al., 2002; Taylor et al., 2003; Turner et al., 2000).

The consistent presence of neutrophils in the necrotic areas could be mediated by IL-17 (Kolls & Linden, 2004; Miyamoto et al., 2003). In fact, IFN-γ is able to regulate the IL-17 response during BCG infection (Cruz et al., 2006), and in IFN-γ absence in TB granuloma there is an increase in neutrophils (Desvignes & Ernst, 2009). Thus, IL-17 could overcome the apparent IFN-γ mediated regulation and participate in immunopathology.

In recent studies, the overexpression of IL-17 and IL23 has been related with neutrophils influx in necrotic pulmonary lesions (Khader & Cooper, 2008). IL-17 induces the production of the chemokine MIP-2 α which is an efficient neutrophils chemoattractant molecule. Indeed, the participation of IL-23, IL-17 and MIP-2 α has been recently demonstrated in a model of repetitive BCG vaccination in mice infected with low dose aereosols (Cruz et al., 2010), implying that IL-17, IL-23 and neutrophils are key molecules in the development of tissue necrosis during advanced pulmonary tuberculosis, and a potential adverse mechanism in specific vaccination schemes such as revaccination with BCG (Cruz et al., 2010). Thus, neutrophils can be protective during early TB infection, but when exposed to excess of IL-23 or IL-17, their function is altered and they become more able to mediate tissue damage (Zelante et al., 2007). Indeed, neutrophils are abundant in the sputum and bronchoalveolar lavage of patients with active TB (Eum et al., 2010), and rapid accumulation of neutrophils that are permissive for bacterial growth is a dominant feature in genetically susceptible mice (Eruslanov et al., 2005; Keller et al., 2006).

The restriction of neutrophil accumulation is dependent on the IFN-γ receptor dependent activity of indoleamine-2, 3-dioxygenase by radio-resistant cells in the lung, which results in increased tryptophan catabolic products that apparently inhibit IL-17 producing cells in situ (Desvignes & Ernst, 2009). These results support the detrimental role of neutrophils in TB pathogenesis.

Increased neutrophil apoptosis is observed in patients with active tuberculosis (Aleman et al., 2002) and mycobacteria is phagocytosed and inactivated by neutrophils, then many of these cells rapidly enter apoptosis via an oxygen-dependent pathway (Brown et al., 1987; Perskvist et al., 2002,). This is a significant process, which prevents the release of toxic compounds from the intracellular compartments. Apoptotic cells are cleared by macrophages which in general induce an anti-inflammatory response by the secretion of TGF-β and other anti-inflammatory cytokines (Fadok et al., 1998; Hernandez-Pando et al., 2006). The production of these anti-inflammatory mediators suppresses the production of significant protective cytokines such as TNF-α and IFN-γ promoting disease progression (Hernandez-Pando, 2006). Interestingly, recent reports have showed that phagocytosis of apoptotic neutrophils by macrophages can result in a pro-inflammatory activation of macrophage including release of TNF-α (Persson, et al., 2008), and high intracellular expression of heat shock proteins 60 and 72 (Hsp60 and Hsp72) in order to protect the cells from damage. HSPs activate immune cells through interaction with several receptors such as CD91, LOX-1, CD14, TLR-2 and TLR-4 (Binder et al., 2004). Thus, mycobacteria induce apoptosis in neutrophils and these cells also release Hsp72 as a consequence of the stress

mediating an early pro-inflammatory stimulation of macrophages during the elimination of the bacilli that induced apoptotic cells. Although this event has been related to early infection bridging innate with acquired immunity, it is possible that this could also happen during advanced infection producing immunopathology, by the combined presence of TNFα and Th-2 type cytokine which can also produce tissue damage (Hernandez-Pando et al., 2004).

In conclusion, neutrophils are significant cells in the initial protective response of the innate immune response against mycobacterial infection, but during the advanced stage of the disease these cells can also contribute to the tissue damage characteristic of this chronic infectious disease.

7. Conclusions

Because neutrophils are the first inflammatory cells to arrive at sites of infection and present a diverse collection of antimicrobial molecules, they are associated as one of the first lines of defense against all microbes; TB is not an exception, in humans this infection elicits apoptosis of neutrophils, ingestion of these by macrophages triggers a pro-inflammatory response, which may or may not control disease progression.

In recent years the whole role of neutrophils in inflammation and bacterial control has been challenged. Remarkable is the work done by Zhang et al., who showed that coactivation of Syk kinase and MyD88 adaptor protein pathways by mycobacteria (BCG or *M. tuberculosis* H37Rv*)* promote previously unsuspected regulatory properties in neutrophils. According with their results, in contrast to monocytes and macrophages, murine neutrophils contribute poorly to inflammatory responses, and secrete high amounts of the anti-inflammatory cytokine IL-10. In a murine model they showed that mycobacteria induced the recruitment of neutrophils secreting IL-10. Interestingly, during the acute mycobacterial infection IL-10 producing neutrophils controlled the inflammatory response of DC, monocytes and macrophages in the lung. However, during the chronic phase of infection (high mycobacteria load), neutrophil depletion promoted inflammation and decreased of the mycobacterial load, these effects could be attributed to a reduce amount of IL-10 and increased TNF-α produced by lung cells in neutrophil depleted animals, and to increased amounts of IL-6 and IL-17, but not IFN-γ. The possible explanation given by the authors for these results is the dual role that neutrophils play, having direct antimicrobial activity (killing) counterbalanced by anti-inflammatory properties (IL-10 production). Neutrophils, at least in mice, are the dominant producers of IL-10 in the lung (Zhang et al., 2009).

Following this line of research, Redford *et al.* found similar results, providing evidence that IL-10$^{-/-}$ mice showed enhanced control of *M. tuberculosis* infection with significant reduced bacterial load in lungs and spleen, which was maintained over the course of the infection. Again, IL-10 seems to regulate the balance of the immune response between pathogen clearance and immunopathology. The reduction of bacterial load in the abscence of IL-10 was preceded by an enhanced cytokine/chemokine production (IFN-γ, CXCL10 (IP-10) and IL-17) and an increased of CD4+ T cells in the lung. Because IL-17 has been shown to promote influx of Th1 cells into the lungs after vaccination against *M. tuberculosis* (Khader et al., 2007), Redford et al. neutralized IL-17, and found a reduction of *M. tuberculosis* load in the spleen, suggesting this cytokine may affect dissemination of mycobacteria, which in turn

may be carried out by neutrophils, since they were also significantly reduced (Redford et al., 2010).

Neutrophils have been shown to be the predominant infected phagocytic cells in the airways of patients with active pulmonary TB (Eum et al., 2010), and in an experimental model neutrophils shuttled live *M. bovis* BCG to draining lymph nodes after intradermal vaccination (Abadie et al, 2005). All together, results from Zhang et al. and Redford et al. (Redford et al., 2010; Zhang et al., 2009) confirm a detrimental role for neutrophils in tuberculosis, and confirms the negative effect that IL-17 have during *M. tuberculosis* infection in humans as shown by Cruz et al. (Cruz et al., 2010).

Neutrophil functions in immunty have been extended thanks to a renewed interest in these neglected cells. Besides having huge amounts of cytokines and effector molecules, they can also produce extracellular traps and ectosomes. In addition, they actively participate in the activation and regulation of both innate and adaptive immune responses (Bratton et al., 2011; Mantovani et al. 2011, Zhang et al., 2009). These newly identified functions might lead to reconsider their role in infectious and non infectious diseases, particularly in tuberculosis, which is a major health concern worlwide.

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12

Role of NK Cells in Tuberculous Pleurisy as Innate Promoters of Local Type 1 Immunity with Potential Application on Differential Diagnosis

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1. Introduction

An efficient host immune response against pathogens encompasses both fast acting innate immunity as well as slower, but more specific, adaptive immunity. The innate immune system is diverse and comprises a variety of cells including natural killer (NK) cells, neutrophils, macrophages, dendritic cells (DCs), as well as soluble factors such as complement. The adaptive immune response is typified by antigen-specific T and B lymphocytes that provide long-lasting protection known as immunological memory. While these two systems are often discussed separately, neither arm of the immune system works in isolation (Medzhitov & Janeway, 1999). The succession of cells interacting with *Mycobacterium tuberculosis* (*Mtb)* comprises tissue macrophages (MΦ) and dendritic cells (DC) followed by chemokine-attracted immigrating neutrophils and monocytes, and then activation and recruitment of natural killer (NK) and γδ T cells, followed by effector T lymphocytes primed in the draining lymph nodes (Ulrichs & Kaufmann, 2006).

Pleuritis is the most frequent clinical manifestation of extrapulmonary tuberculosis (TB) among young adults, and is normally considered a relatively benign form of disease since it may resolve without chemotherapy (Light, 2010). Tuberculous pleurisy is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of *Mtb* infection, but it may also be developed as a complication of primary pulmonary TB infection (Antoniskis et al., 1990). The presence of mycobacterial antigens in the pleural space elicits an intense cellular immune response, initially characterized by abundant neutrophils and macrophages, followed by interferon (IFN)-γ-producing T-helper cell (TH) type 1 lymphocytes, resulting in lymphocyte-predominant exudative effusions (Aleman et al., 2005; Mitra et al., 2005; Porcel, 2009). The cellular trafficking is facilitated by homing surface markers and chemokine gradients. This intense but poorly understood local immune response is synonymous of Koch phenomenon and normally prevents the caseous evolution of lesions. The inflammatory process results in an increased pleural vascular permeability leading to the accumulation of fluid enriched in proteins and the recruitment of specific leukocytes into the pleural space, making this biological sample a physiologically relevant model of human tuberculosis infection (Kroegel & Antony 1997).

Given this effective local resistance, the few bacilli that enter into pleural cavity are rapidly destroyed turning impracticable the rapid diagnosis by direct microscopic observation (Porcel, 2009). This fact often results in the requirement of alternative diagnostics strategies like *Mtb* identification through cultures or PCR amplification of pleural effusions, histopathological examination of pleural biopsies, inflammatory related enzymatic activities and immunological based methods (Liang et al., 2008; Trajman et al., 2008). Among the last ones, detection of mycobacterial antigens, antimycobacterial specific antibodies (Ab), TH1 related biomarkers and *in vitro* evoked T cell responses are matter of active current research (Steingart et al., 2007; Budak et al., 2008; Dheda et al., 2009a; Supriya et al., 2008). Regarding the performance of T and B cell based assays in detection of active forms of disease; the most important trouble is the immunological memory background as result of previous Ag exposition or BCG vaccination (Dheda et al., 2009b; Hooper et al., 2009; Salazar-Lezama et al., 1997). In this line, for high antigen experienced populations, we hypothesized that an innate immune cell based diagnostic assay may circumvent those issues.

Among innate lymphocytes, Natural Killer (NK) cells display an important number of effector functions, including recognition and lysis of infected, stressed, or transformed cells and production of immunoregulatory cytokines, particularly IFN-γ (Vivier et al., 2011). Human NK cells account for 10–20% of peripheral blood lymphocytes and are defined by the presence of the CD56 and NKp46 molecules and the lack of CD3 and CD19 expression. Their activity is regulated by both positive and inhibitory signals from a wide range of germ line encoded cell surface receptors. Two major subsets of NK cells have been identified in humans according to CD56 and CD16 intensity expression and also in terms of chemokine receptors and adhesion molecules expression that differ in phenotype and function (Caligiuri, 2008). Functionally, $CD56^{bright}$ cells are effective cytokine producers, whereas $CD56^{dim}$ cells are efficient effectors of natural and antibody-dependent target cell lysis (Hanna & Mandelboim 2007). Together with the classical NK functions (i.e. cytotoxicity and cytokine production), novel skills have recently been described in niche-specific and in vitro-activated human NK cells. These unconventional capabilities include angiogenesis and tissue remodeling, immunological memory, functional cross-talk with T cells and direct pathogen recognition (Cooper et al., 2009; Di Santo, 2008; Vivier et al., 2011).

During the last years we have begun to characterize the phenotype and function of pleural NK from TB patients. In contrast to peripheral blood (PB) counterpart that are mostly composed by $CD56^{dim}CD16^{+}$ resting cells, pleural NK population is enriched in activated $CD56^{bright}CD16^{neg}$ cells that quickly and strongly respond to *Mtb* stimulation by producing IFN-γ (Schierloh et al., 2005a, 2007). Besides, *Mtb* stimulated IFN-γ production by NK shows Ag-specific features, given that pleural NK cells derived from non TB patients' lack of this response. According to these findings, we realized that pleural NK cells properties could be utilized in an innate immune cell based assay for differential diagnosis of tuberculous pleurisy (Schierloh et al., 2008).

2. Phenotype of pleural NK cells: Activated $CD56^{bright}$ NK cells

Along several studies, phenotype of tuberculous pleural fluid derived NK cells have been extensively analyzed as starting point in the understanding of their role at the site of active *Mtb* infection (Alvarez et al., 2010; Fu et al., 2011; Okubo et al., 1986, 1987; Pokkali et al.,

2009; Schierloh et al., 2005a, 2007, 2009). The finding that immunoregulatory $CD56^{bright}$ NK subset showing cellular activation features are strongly enriched among pleural NK cells was interesting given that these cells are known to link innate with adaptative immunity in a number of intracellular infections (Artavanis-Tsakonas et al., 2003; Culley, 2009; Fehniger et al., 2003). Along the present section we summarize these findings.

2.1 Enrichment of $CD56^{bright}$ NK cells in tuberculous pleural fluid

In tuberculous patients, T helper ($CD4^+/CD3^+$) is the predominant cell population among pleural fluid derived mononuclear cells (PFMC), which has been associated with a selective recruitment of antigen specific TH1 effectors cells to the site of infection (Li et al., 2010; Mitra et al., 2005). This increased T helper abundance account for the reduced percentage of pleural fluid NK cells ($CD3^-/CD56^+$) close to 5% in TB patients (Schierloh et al., 2005a). In contrast, in pleural effusions caused by cancer or paraneumonic infections, the percentage of NK among PFMC tends to be constant compared to peripheral blood (Dalbeth et al., 2004). However, when we analyzed the composition of NK cell populations, a drastic change in the proportion of these subsets was found (Schierloh et al., 2005a). In the case of tuberculosis, the cytotoxic $CD16^+CD56^{dim}$ NK subset, which represents more than 95% in the circulation, is reduced to less than 50% in the pleural effusions with a concomitant enhancement of the immuneregulatory $CD16^{dim/-}CD56^{bright}$ NK subset.

Phenotypic differences between $CD56^{brigth}$ NK with $CD56^{dim}$ include higher expression of the C-type lectin CD94/NKG2 family, weak expression of killer cell immunoglobulin (Ig)–like receptors (KIRs) and high levels of L-selectin (CD62L) and CCR7, both of which are involved in trafficking of immune cells to lymph nodes (Caligiuri, 2008). In agreement, the levels of CD94/NKG2A, CD62L and CCR7 were all augmented in tuberculous pleural NK cells (Schierloh et al., 2005a). On the other hand, the percentages of NK cells expressing the fractalkine receptor (CX_3CR1), the cytotoxic granular protein perforin and the HLA-C2 receptor KIR2DL1/S1, which are all specific markers for the cytotoxic $CD16^+CD56^{dim}$ NK subset, are reduced in the tuberculous pleural effusions (Figure 1).

2.2 Pleural NK cells exhibit activated phenotype

NK cells activation can be triggered via two primary mechanisms: cytokine stimulation and engagement of activating NK receptors. Together or in isolation, both activation signals can result in NK cell responses (Vivier et al. 2011). Because tuberculous pleural microenvironment is plenty of soluble mediators and cells with stimulatory potential (Shimokata et al., 1991; Valdés et al., 2009; Vankayalapati et al., 2000), we hypothesized that NK cells arriving to this site may turn activated. Indeed, we found an elevated percentage of NK cells expressing the early activation markers CD69 and HLA-DR together with enhanced expression of the lymphocyte function-associated antigen 1a integrin (LFA-1/ CD11a) and its ligand, intercellular adhesion molecule-1 (ICAM-1, CD54) (Schierloh et al., 2005a, 2005b, 2009). Simultaneously, we observed a subpopulation of pleural NK cells expressing Toll-like Receptor 2 (TLR2), a molecule undetectable in resting NK cells but up-regulated in response to IL-12 and protozoan glycolipids (Becker et al., 2003; Lindgren et al., 2010; Schierloh et al., 2007). Furthermore, we and others recently identified a subpopulation of pleural NK that down-modulates the CD45RA and up-regulates CD45R0 isoform,

resembling the well known phenomenon that takes places during memory differentiation of T lymphocytes (Warren et al., 1994; Fu et al., 2011).

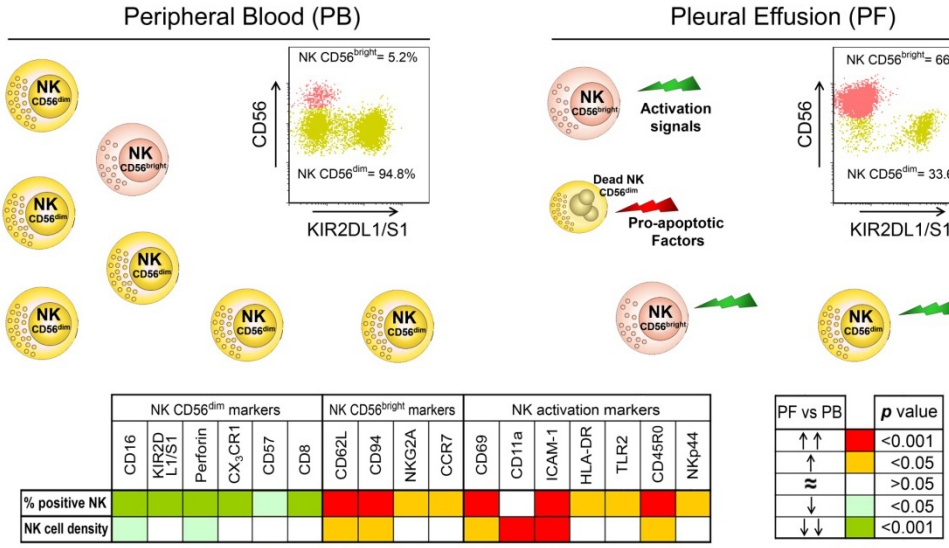

Fig. 1. Enrichment of NK CD56^{bright} cells with activated phenotype in tuberculous pleural effusions.

Immunophenotypic analysis of peripheral blood (PB) and pleural fluid (PF) NK cells (CD56^{+}/CD3^{-}). Dot plots are from one representative TB patient. Color scale indicates relative variations of several markers between PF and PB samples: invariant (white), augment (orange), strong augment (red), reduction (light green) and strong reduction (dark green).

2.3 Pleural fluid factors inducing CD56^{dim} apoptosis explain the altered NK subset ratio

In order to understand the causes of altered CD56^{bright}/CD56^{dim} subset ratio observed on pleural NK population, we hypothesize that soluble factors (i.e.: chemokines, cytokines, pathogen derived factors, immune complexes, etc.), present at the site of *Mtb* infection, may differentially affect the migration, the differentiation, the proliferation or the apoptosis of NK subsets. Our experimental approaches directed to test this were conducted by incubating peripheral resting NK cells with tuberculous cell-free-pleural fluid or purified factors (Schierloh et al., 2005a). Indeed, CD56^{dim}CD16^{+} cells show an increased susceptibility to pleural fluid induced caspase 9 dependent-apoptosis, explaining the predominance of the CD56^{bright} population. These finding were later confirmed in different experimental settings. These studies demonstrate that NK CD56^{bright} subset has larger resistance to oxidative stress (Harlin et al., 2007; Thorén et al., 2007). Our experiments directed to test if NK CD16^{+} cells could be differentiated to CD16^{-} NK cells by cytokines present at the site of infection gave negative results, in accordance with other group (Dalbeth et al., 2004). Additionally, a recent

report indicates that differential migration of NK cell subsets to the site of infection also take place (Pokkali et al., 2009).

3. Function of pleural NK cells: IFN-γ production and TH1 cell co-stimulation

Considering that type 1 cytokine and chemokine profile is a hallmark of tuberculous pleurisy (Dheda et al., 2009a; Kroegel & Antony 1997; Li et al., 2010; Mayanja-Kizza et al., 2009; Trajman et al., 2008), and given that pleural NK cells exhibit an endogenously induced activation state together with an enrichment of immunoregulatory NK CD56^{bright} subset, we asked whether these cells were polarized to the production of pro and/or anti-inflammatory cytokines. Also, we evaluated if these cells were capable to modulate other cells function. The answers to these questions gave us important clues for better understanding the immunopathogenesis of TB infection and will be discussed during the present section.

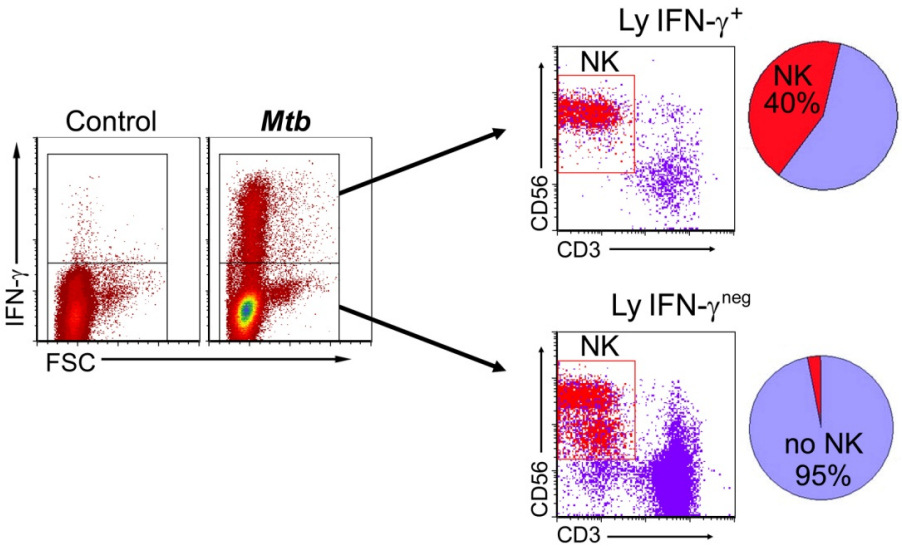

Fig. 2. Pleural NK cells are a major early source of IFN-γ upon ex vivo Mtb-stimulation.

PFMC were stimulated with *Mtb* for 24h .Then cells were gated on the basis of IFN-γ-positive (Ly IFN-γ^{+}) and IFN-γ-negative (Ly IFN-γ^{neg}) cells and their surface phenotype were determined according to CD56 and CD3 expression by flow cytometry. Dot plots are from one representative TB patient. NK cells are highlighted in red. Pie charts indicate mean values of 30 TB patients.

3.1 Pleural NK cells strongly produce IFN-γ after *Mtb* stimulation

Experiments varying the quality and quantity of signaling input for NK cell activation have revealed a hierarchy in requirements for induction of chemokines and cytokines (Fauriat et al., 2010). Furthermore, recent data indicate that anatomical niche and developmental stage of NK cells strongly determine its cytokine profile production giving rise to NK1, NK2 and

NK22 cells (Di Santo, 2008; Spits & Di Santo, 2011). In this sense, pleural fluid CD56^{bright} NK did not produce significant levels of IL-10, IL-17A or TNF-α spontaneously or after γ-irradiated *Mtb*-stimulation. However, the percentage of IFN-γ$^+$ NK cells (NK IFN-γ+) was strongly increased under the same experimental conditions (Schierloh et al., 2005a). It is interesting to note that, in spite of its reduced numbers in TB pleural fluid (~5% of PF lymphocytes), NK constitute a major source of IFN-γ together with CD3$^+$ cells (Figure 2). *Mtb* derived culture filtrate proteins (CFP), TLR2 and 4 agonist and recombinant IL-12 also induced IFN-γ among pleural NK but to a lesser extent than *Mtb* does (Schierloh et al., 2007). Interestingly, pleural fluid NK cells derived from other etiologies (i.e: cancer, paraneumonic or helmintic infections) did not give *Mtb*-stimulated IFN-γ responses, suggesting a paradoxical "Ag specific response" induced in an innate immune cell (see Figure 4). This finding is in accordance with recent data provided by experimental mice models which clearly demonstrate the adaptative properties of NK cells (Cooper et al., 2009; Paust & von Andrian, 2011; Vivier et al., 2011). Instead, this apparent "Ag specific" NK cell responses may be produced by T cell-secreted IL-2 (Horowitz et al., 2010; Fehniger et al., 2003).

3.2 Pleural NK cells co-stimulate local TH1 response

Among novel skills described for human activated NK cells, it has been shown that they may stimulate T cells by cell contact-dependent mechanisms (Hanna & Mandelboim 2007). In this line, we were able to demonstrate that, in TB pleurisy, a functional ICAM-1-dependent cell to cell interaction among pleural fluid NK and T cells lead to T cell activation (Schierloh et al 2009). Likewise, peripheral blood human NK cells can instruct *in vitro* cytotoxic CD8$^+$ T cells from PPD responsive donors to lyse *Mtb*-infected monocytes (Vankayalapati et al., 2004). Taken together, these findings suggest a previously unappreciated role of NK cells in the maintenance and/or activation of T cell functions during the immune response in tuberculosis.

4. Cellular and molecular factors controlling pleural NK cells functions

Having observed that NK cells were the main early source of IFN-γ within the pleural space, we investigate extracellular events and signaling pathways that drive this process. The mechanisms involved reveal classical and particular ways of NK cell activation and signaling. Three environmental signals act in concert to fulfill IFN-γ response in pleural NK cells: cytokines, activation ligands expressed on accessory cells and direct *Mtb* recognition (Schierloh et al., 2007).

4.1 Pleural NK response involve Ca^{2+} influx and Calcineurin, ERK and p38 MAPK signaling pathways

NK cells express on their surface an array of germ-line encoded inhibitory and activating receptor as well as cytokine receptors that, upon activation, mediate intracellular signaling pathways for IFN-γ together with other functional responses. It is well known that interaction of NK receptors cells with their activating ligands on target and/or Ag presenting cells (APC) induce a quick increase of cytoplasmic calcium (Ca^{2+}) concentration, a universal second messenger (Maghazachi, 2005). Employing divalent cation chelators we

demonstrate a pivotal role for this process during *Mtb*-induced pleural NK IFN-γ response. Downstream the Ca^{2+} influx, signaling proteins and their target transcription factors are activated, including calcineurin, a calmodulin-dependent serine/threonine phosphatase, and its target NFAT (nuclear factor of activated T cells). By mean of Cyclosporin A treatment, we confirm the involvement of this pathway too. Similarly, using several protein kinase specific inhibitors and phospho-specific monoclonal antibodies, we also address the participation of other two phosphorylation cascades, p38 MAPK and ERK1/2 (Schierloh et al., 2007). Figure 3 summarize the most important events.

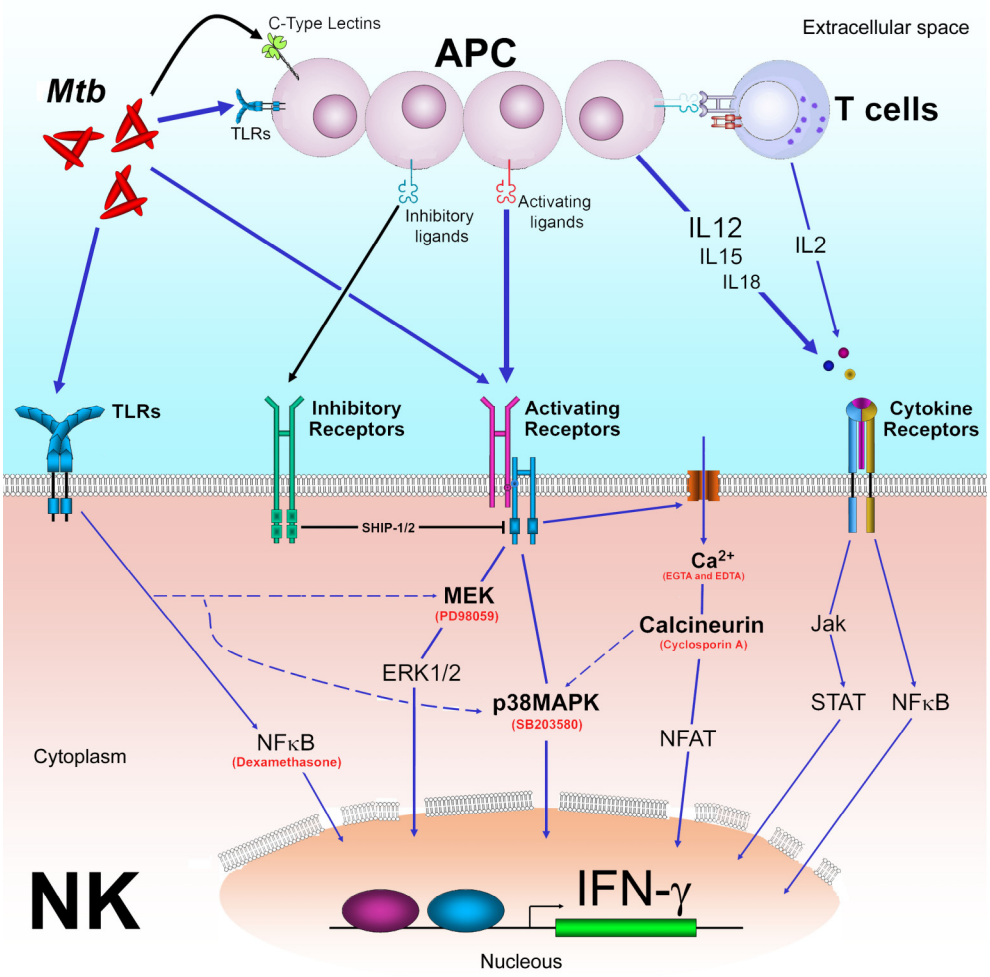

Fig. 3. Extracellular signals and intracellular pathways in IFN-γ production by pleural NK cells.

Picture describe interactions among activation signals (i.e: cytokines, APC expressed activating ligands and *Mtb*), modulating/inhibitory signals (i.e: APC expressed inhibitory

ligans and *Mtb* derived lipoglicans recognized by C-type lectins) and cellular receptors together with their corresponding intracellular signaling pathways. Blue arrows indicate positive interactions. Dotted blue arrows denote cross-talk between pathways. Black arrows denote inhibitory interactions. Pharmacological inhibitors used in our experiments are written in red within brackets under its target molecule.

4.2 Pleural NK response is dependent on IL-12 and accessory cell contact

Numerous *in vitro* and *in vivo* studies show that a wide variety of APC secreted cytokines, especially IL-12, IL-15, and IL-18, can activate NK cells to produce IFN-γ. By means of cytokine neutralization, we showed that IL-12 is necessary for *Mtb*-induced NK IFN-γ production in tuberculous pleurisy (Schierloh et al., 2007). This requirement is not surprising because IL-12 has been shown to mediate bystander activation of NK cells in response to a number of different pathogens (Artavanis-Tsakonas et al., 2007; Culley, 2009, Lindgren et al., 2011). However, IL-12 alone does not allow the same level of IFN-γ^+ NK cells than *Mtb* does. Indeed, costimulatory signals delivered by APC such as receptor-ligand interactions are required as demonstrated by Ab blockade and APC depletion experiments. Among activating ligands ICAM-1, CD86 and Vimentin has been shown to be engaged. At the same time, these signals could be counter-balanced by MHC class I, PD-L1 and PD-L2 inhibitory ligands (Alvarez et al., 2010; Garg et al., 2006; Schierloh et al., 2007).

At the level of pleural APC, we also observed that *Mtb* induced stimulatory signals mediated by TLR2 and TLR4 are counter-modulated by C-type lectin receptors like mannose receptor (MR) and DC-SIGN (Schierloh et al., 2007). Both molecules bind mycobacterial derived mannosilated lipoglycans and may indirectly inhibit NK cell response by limiting IL-12 production and/or down-modulating activating ligands expression (van Kooyk Y & Geijtenbeek, 2003).

4.3 Pleural NK cells directly recognize *Mtb*

Recent studies have pointed out the capacity of NK cells to bind and been directly activated by *Mycobacterium* species (Esin et al., 2004; Evans et al., 2011; Watkins et al., 2008). This recognition seems to be mediated, at least, by two putative activating receptors: TLR2 and NKp44 (Esin et al., 2008; Marcenaro et al., 2008). Interestingly, these receptors are both up-regulated among pleural NK cells (Figure 1). Consistently we observe that, compared with its PB counterparts, pleural NK cells have enhanced capacity to bind *Mtb* and that p38 MAPK phosphorilation on pleural NK occurs shortly after *Mtb*-NK coculture, independently on bystander cell derived signals (Schierloh et al., 2007). Furthermore, it has been observed that NK $CD56^{bright}$ cells are more reactive to direct BCG stimulation than $CD56^{dim}$ NK (Batoni et al., 2005). Altogether, these results strongly indicate that direct interaction between NK and *Mtb* play a significant role during functional response of pleural NK $CD56^{bright}$ cells.

5. Pleural NK application: Immunodiagnosis of tuberculous pleurisy

Conventional diagnostic tests for pleural TB include microscopic examination of pleural fluid for acid-fast bacilli and differential cytology; mycobacterial culture of pleural fluid, sputum or pleural tissue; pleural fluid Adenosin Deaminanse (ADA) activity as well as

determination and histopathological examination of pleural tissue looking for granulomatous inflammation (Porcel, 2009; Light, 2010). These tests have limitations for clinical use; however, in combination, they have been recognized as the best reference standard for evaluation of the accuracy of novel tests (Trajman et al., 2008). Although detection of serum antibodies against *Mtb* antigens is known to have poor and highly variable sensitivity and specificity, attempts have been made to detect antibodies in pleural fluid by ELISA. Even though, these tests show high specificity they are limited by the very poor sensitivity (Weldingh & Pai 2007). Several T cell based assays have been employed for diagnosis of TB pleurisy such as in vitro stimulation of lymphocytes with PPD or RD-1 encoded antigens leading to T-cell proliferation and/or IFN-γ release by ELISPOT and ELISA assays (TIGRAs) (Hooper et al., 2009). For example, using a commercially available *Mtb*-specific ELISPOT for peripheral blood mononuclear cells and pleural fluid mononuclear cells from patients with exudative pleurisies, its sensitivity in active tuberculosis was very high (95%); however, the specificity was suboptimal (76%) (Losi et al., 2007). The high coverage of BCG vaccination as well as the high prevalence of latent TB infection (PPD^+ individuals) at the population level might impair the results when employing T and B cell based immunodiagnostic methods. On the other hand, immunocompromised or HIV^+ infected patients may lead to false negative results due to the ablation or reduction of cellular immunity (Trajman et al., 2008). In this context, we thought that pleural IFN-γ^+ NK cells could be a promising target for immunodiagnostic method that circumvent these memory related problems in differential diagnosis of tuberculous pleurisy.

5.1 Preliminary trial for testing clinical value of NK cell based assay in Argentina

In order to provide evidence that support or reject the clinical diagnostic utility of NK IFN-γ^+ cell based assay in differential diagnosis of TB pleural effusion, we have performed a retrospective, single-center preliminary study in a reference center of the city of Buenos Aires, Argentina. To do this, PFMC were stimulated with *Mtb* and the percentage of NK and T cells expressing IFN-γ were determined by flow cytometry as described in Figure 2. In this trial we included 40 consecutive patients with profuse exudative pleural effusion (TB n=28 and No-TB n=12; Cancer=6, Paraneumonic infection=5, Helmintic infection=1) admitted and diagnosed at the Tisioneumonolgy service of the Hospital Muñiz during 2006-2009. According to epidemiological data provided by medical staff, all the patients were at high relative risk of TB infection. As can be observed in table 1 and Figure 4, pleural NK shows

	Median Δ % IFN-γ^+ (25–75% percentil)	**Cut off** (Max. likelihood ratio)	**Area under ROCurve** (95% CI)	**Sensitivity** (95% CI)	**Specificity** (95% CI)
NK assay	30.69% (9.23–42.77%)	> 1.555% (11.57)	0.9643 (0.9002–1.028)	96.43% (81.65–99.91%)	91.67% (61.52–99.79%)
T assay	2.14% (1.04– 4.63%)	> 1.230% (8.14)	0.9435 (0.8670–1.020)	67.86% (47.65–84.12%)	91.67% (61.52–99.79%)

Table 1. ROC analysis for NK cell and T cell assays. Data values summarized above are derived from ROC analysis depicted in Fig.4.

better performance than pleural T cell based assay in discriminating TB and no-TB pleural effusions. These differences may reflect the polyclonal vs oligoclonal nature of IFN-γ response in NK compared to T cells (Schierloh et al., 2008).

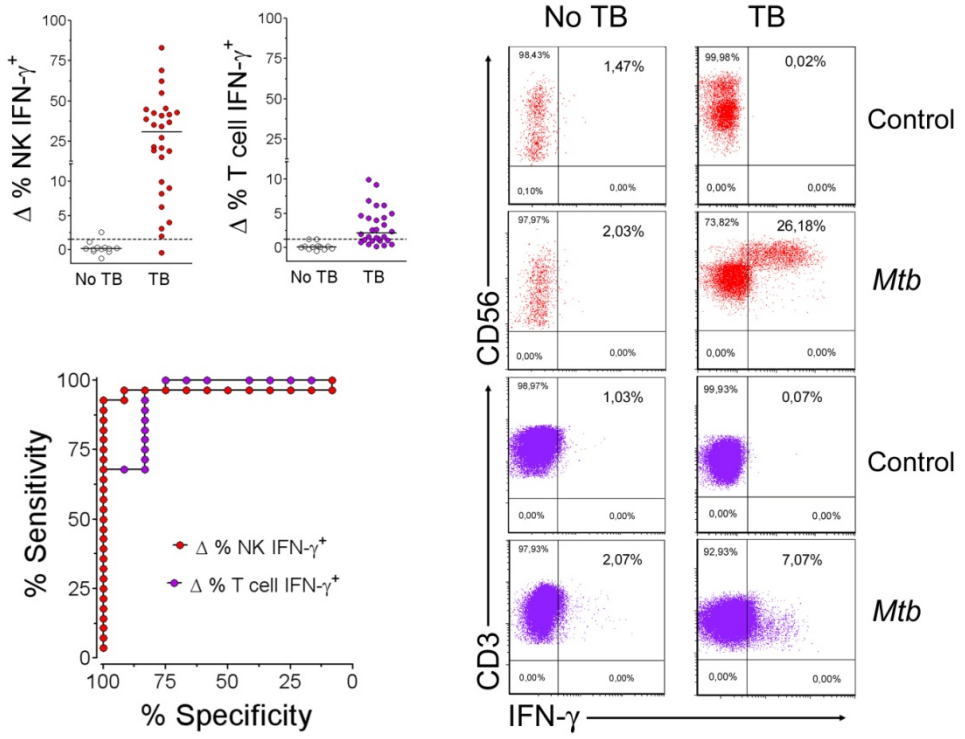

Fig. 4. Mtb-induced IFN-γ response by NK and T cells in the diagnosis of tuberculous pleurisy.

PFMC were incubated 24h with medium alone (Control) or stimulated with *Mtb*. Then, *Mtb* specific IFN-γ response was obtained from gated NK ($CD56^+/CD3^-$) and T cells ($CD3^+$) by subtracting the percentage of spontaneous production: **Δ% IFN-γ⁺= % IFN-γ⁺ *Mtb* - % IFN-γ⁺ control**. Upper left scatter plots depict the results of the study population (TB patients n=28; No-TB patients n=12). Lower graph present ROC analysis for NK and T cell based assays. Flow cytometry dot plots analyses are from one representative TB patient and one patient with helmintic (*Echinococcus granulosus*) infection (No TB). Red dots are gated NK ($CD56^+/CD3^-$) and purple dots are T cells ($CD3^+$).

5.2 Rational design for NK based IFN-γ release assay: "KIGRA"

Flow cytometers are sophisticated equipments that may be not available in almost all public health laboratories. Therefore, in order to make our NK derived IFN-γ based assay more applicable for common clinical settings, we attempt to introduce experimental modifications that could direct the development of a NK based IFN-γ release assay.

Unlike flow cytometry, cytokine release detection devises did not allow the identification of secreting cell. This fact represents an important challenge in our particular case, where the cellular source of IFN-γ is the basis of the diagnostic improvement. One way to avoid this issue is blocking IFN-γ production by pleural T cells, leaving NK as the main producer cells. To do so, we employed anti-HLA class I and class II monoclonal antibodies (mAbs), which block Ag presentation to $CD8^+$ and $CD4^+$ T cells. As can be observed in Figure 5, most T IFN-γ^+ cells were inhibited when both mAbs were present during *Mtb* stimulation; however, NK cells still remain expressing IFN-γ^+ under the same treatment. Similarly, when we analyzed IFN-γ release by ELISA, the presence of mAbs diminished but not abolished the secretion of this biomarker. This result, together with other adjustments that are under current testing, may constitute the rational for a more accurate assay.

Furthermore, we think that in the context of diagnosis of tuberculous pleurisy, commercially available IGRA, which employ ELISPOT (Losi et al., 2007), ELISA (Losi et al., 2011) or immunochromatography (Corstjens et al., 2008), could be easily adapted in order to obtain a Natural Killer IFN-gamma release assay or "KIGRA".

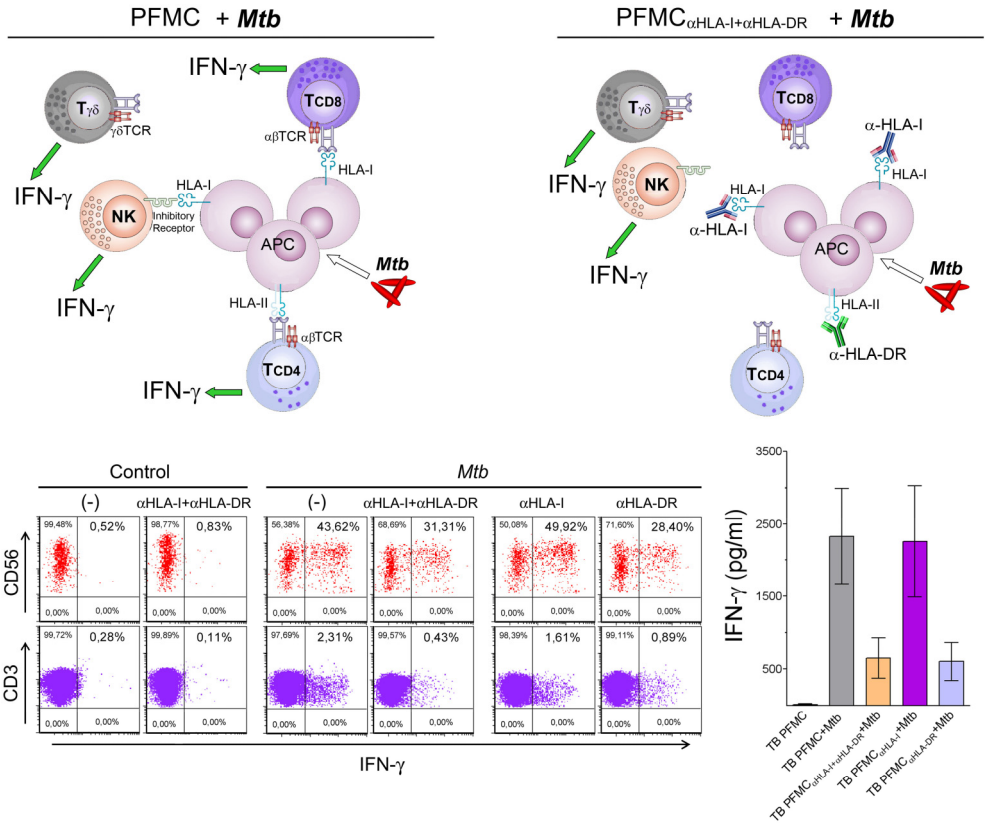

Fig. 5. Rational design for development of NK IFN-γ release assay.

PFMC were *Mtb*-stimulated as indicated in Fig.4 with or without the addition of anti-HLA-class I and/or anti-HLA-class DR mAbs. A schematic explanation of how mAbs interfere on antigen presentation to $CD4^+$ and $CD8^+$ T αβ cells without affecting NK or T γδ cells activation (upper cartoon). Flow cytometry dot plots analyses are from one representative TB patient. Red dots are gated NK ($CD56^+/CD3^-$) and purple dots are T ($CD3^+$) cells. Bar graph of IFN-γ ELISA assay (TB n=3).

6. Conclusion

Tuberculous pleurisy, one of the most common extrapulmonary manifestations of tuberculosis among young adults, is characterized by strong delayed type hypersensitivity reaction mediated by effector lymphocytes. Our data demonstrate that a substantial part of these cells are indeed NK cells. Herein, we have discussed phenotypic and functional features of this local innate immune cell population and the factors involved in its regulation. Furthermore, we identify NK cells as the main source of IFN-γ, the most widely used TB biomarker, in the context of tuberculous pleurisy.

In the context of high antigen experienced population the diagnosis that allow the discrimination between tuberculous pleurisy from other exudative pleural effusions remain as an unresolved clinical issue (Dheda et al., 2009b; Hooper et al., 2009; Salazar-Lezama et al., 1997). Hence, we propose an NK cell IFN-γ based assay as complementary procedure. To our knowledge, no previous NK inmunodiagnostic were reported for TB or any other infectious diseases. The advantageous characteristics of this functional assay are: (i) short time result output (1 day) and ii) very good performance in terms of specificity and sensitivity.

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13

Are Polyfunctional Cells Protective in *M. tuberculosis* Infection?

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1. Introduction

Tuberculosis (TB) continues to claim almost 2 million lives each year, and causes active TB disease in over 9 million new cases yearly. Control of TB is further impeded by the strong increase in TB morbidity and mortality due to HIV co-infection, and the rise of multi-drug resistant (MDR) and extensively drug-resistant (XDR) Mycobacterium tuberculosis (Mtb) strains (WHO. Global tuberculosis control: surveillance, planning, financing: WHO 10 report 2008). Clinical disease does not develop in the vast majority (90-98%) of all Mtb infected individuals, providing compelling evidence that the human system is capable of controlling the pathogen. However, these clinically asymptomatic subjects do not achieve sterile eradication of the pathogen and consequently remain latently infected lifelong, but 2-10% of them will progress to developing TB during their lifetime.

Evidence from both animal and human studies suggest an important role for both CD4 and CD8 T cells in successful control of Mtb infection. Notably CD4 T cells of Th1 type (CD4 Th1 cells), dominate protective immunity and participate in the formation and maintenance of granuloma (Russell, 2007; Russell et al, 2010; Cooper 2009; Van der Wel et al. 2007); upon activation, CD4 T cells secrete interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which activate antimycobacterial mechanisms in mononuclear phagocytes. IFN-γ effects on the macrophage extend beyond oxidative stress products, and also include induction of autophagy, which has been demonstrated in both human and mouse to be an essential antimycobacterial mechanism (Gutierrez et al. 2004; Singh et al. 2006; Harris et al. 2007).

Besides CD4 T cells, other T-cell subsets, such as CD8, γδ and CD1-restricted T cells influence disease outcome. On the contrary, Th2 cell inhibit autophagy-dependent killing of intracellular Mtb (Harris et al. 2007). Notably, $CD8^+$ T cells contribute to host defense, not only by cytokine production, but also by perforin- and granzyme-mediated cytotoxic activity against the pathogen and infected phagocytes. In contrast to $CD4^+$ T cells, Mtb-specific CD8 T cells are primed after transfer of mycobacterial Ags into the cytosol (Khader and Cooper 2008; Harding and Boom 2010; Kurt et al. 2010), or through crosspriming mediated by uptake of apoptotic vesicles from mycobacteria-infected macrophages by dendritic cells. Remarkably, cross-presentation also is subject to inhibition through bacterial evasion strategies that utilize eicosanoid pathways (Divangahi et al. 2010). Recent evidence suggests progressive dysfunction of CD8 T cells in chronic Mtb infection; for example, CD8

T cells in Mtb-infected mice gradually lose lytic potential during progression to the chronic phase of infection (Parida and Kaufmann 2010) and CD8 T cells from individuals with pulmonary TB display decreased cytolytic activity and expression of cytotoxic molecules, compared with these cells from uninfected healthy controls (Ho et al. 2009; Franco-Paredes et al. 2006).

Furthermore, the involvement of lymphocytes in host defense against an infection leads to the development of a memory response that normally rapidly elicits a secondary response after re-encounter of the pathogen (Pichichero 2009). In the case of chronic TB, however, the memory response must be tightly controlled in order to master the delicate tightrope walk between immunopathology and host integrity.

In addition, certain individuals, or strains of mice, may develop inappropriate (e.g., Th2) (Bold et al. 2011; Flynn et al, 1995; Wangoo et al. 2001) or imbalanced effector phenotypes such as Th1/Th17 (Chen et al. 2010) in response to infection. However, even in humans or mice that develop Th1 responses, failure of CD4 effector T cells to recognize infected cells may preclude their optimal activation and limit induction of effector functions in the lungs. Furthermore, host regulatory mechanisms that limit immune pathology, such as T regulatory cells (Tregs, Scott-Browne et al. 2007) or production of inhibitory cytokines (Turner et al. 2002), and, possibly, onset of T cell exhaustion (Yi et al. 2010; Reiley et al. 2010), may inhibit the activity of effector T cells at the site of infection. Moreover, even when CD4 effector T cells are activated, the efficacy of these responses may be limited by the impaired ability of infected cells to respond to IFN-γ (Ting et al. 1999; Banaiee et al. 2006; Pai et al. 2003), induce phagosome maturation (Rohde et al. 2007; Clemens et al. 1995) or undergo apoptosis (Hinchey et al. 2007; Miller et al. 2010).

Finally, vaccination is a key strategy in reducing the incidence of TB and unfortunately the only licensed TB vaccine, BCG, consistently protects against disseminated TB in children but fails to protect against pulmonary disease, which accounts for the burden of TB mortality and morbidity. A total of 11 vaccine candidates have entered clinical trials within the last several years (Kaufmann et al., 2010). Considering that about 2 billion humans are presumably infected with Mtb, with only 10 percent developing active disease, it is obvious that vaccination strategies follow two different approaches: pre-exposure vaccination in order to prevent disease in individuals that have so far not encountered Mtb versus post-exposure vaccination that aims at inhibiting disease outbreak in individuals that are already infected. Up to now, the majority of novel candidates belongs to the first group. A widely held view considers infection synonymous with disease, i.e., we consider disease the unequivocal sequelae of infection with a pathogen. It is the 10% of individuals at risk of developing disease who represent the targets for novel vaccines against TB. Under these circumstances, future vaccines are satisfactory if they induce an immune response in susceptible individuals comparable to that evoked by natural Mtb infection in resistant ones. Alternatively, if one aims at sterile Mtb eradication, future vaccines need to perform better than natural immunity in resistant individuals, which only contains infection. Furthermore, the factors that determine whether or not an individual is protected against natural infection with Mtb or whether someone is at risk of developing TB at a later stage are already unknown.

2. In vitro tests for diagnosis of TB

A recent break through in TB diagnosis is the introduction of IFN-γ release assay (IGRA), in which the production of IFN-γ in response to Mtb specific antigens is measured. Three commercial kits based on the IGRA principle are available: T-SPOT.TB, QuantiFERON-TB Gold, QuantiFERON-TB Gold in-tube (QFT-IT). T-SPOT.TB and QuantiFERON-TB Gold assays use only Early Secretory Antigenic Target (ESAT)-6 and Culture Filtrate Protein (CFP)-10, whereas an additional antigen TB 7.7 is incorporated in QuantiFERON-TB. The currently available data suggest that IGRAs are less influenced by prior Bacille Calmette-Guerin (BCG) vaccination and environmental mycobacteria infection (Pai et al. 2006), rendering them more specific than the tuberculin skin test (TST). In addition, with active tuberculosis as a surrogate for LTBI, it appears that the ELISA-based assays have a similar sensitivity to the TST, whereas the ELISPOT assay is more sensitive. Recent longitudinal data have demonstrated the prognostic power of positive IGRA results in recent contacts for the subsequent progression to active TB. Deployment of IGRAs, driven by new guidelines internationally, will impact on clinical practice in several ways. Their high specificity means that BCG-vaccinated individuals with a false-positive TST will not receive unnecessary preventive treatment, whereas improved sensitivity in individuals with weakened cellular immunity at highest risk of progressing to active TB (for example HIV-infected individuals) enables more reliable targeted testing and treatment. In addition, longitudinal studies during treatment of active TB and LTBI have shown that serial IGRA testing cannot be used for treatment monitoring or as test of cure. However, simultaneous measurement of IL-2 and IFN-γ correlates with therapeutic response and may allow treatment monitoring and potentially test of cure. Studies are also exploring whether measuring alternative, downstream chemokines secreted by IFN-γ-activated macrophages such as inducible protein 10 (IP-10), in combination with IFN-γ, may serve as a more amplified readout than IFN-γ alone thereby resulting in higher sensitivity. A recent study conducted in urban hospitals in United Kingdom using ELISPOT, reported that IGRA in combination with TST can be used to rule out the suspicion of active TB disease among clinically suspected subjects (Dosanjh et al. 2008). However, the data from high TB endemic countries are limited. Thus, further studies are needed to validate the role of IGRA in the diagnosis of TB in high endemic settings.

3. Pathogenesis of TB

Upon inhalation of aerosol droplets, Mtb is phagocytosed by alveolar macrophages, lung parenchyma macrophages, and dendritic cells. Subsequently, these cells elicit local inflammatory responses, leading to the recruitment of mononuclear cells from the blood, which in turn become potential targets for infection (Cooper 2009). Inside the phagosomal compartment, the mycobacteria employ their first immune evasion strategy as they prevent phagosome acidification and thus survive within this compartment (Russel 2007). Second, Mtb apparently can escape into the cytosol and thus evade phagosomal effector mechanisms (van der Wel et al. 2007). The pathogen is eventually controlled by granuloma formation, which is the histopathologic hallmark of protective immune responses. The granuloma, first being an aggregate of macrophages, neutrophils, and monocytes, develops into a more organized structure with the initiation of an adaptive immune response. Immune cells and a fibrotic wall surround the granulomas in order to prevent bacterial spreading (Russell et al.

2009), and in this form, disease outbreak can be prevented over long periods of time unless the immune response weakens. Massive cell death leads to caseation of the granuloma, and Mtb can no longer be enclosed. Mtb exploits cell necrosis to leave its host cells and spread, whereas apoptotic cell death sustains plasma membrane integrity and thus impedes Mtb exit. Here again, the bacteria apparently have developed an evasion strategy, since virulent Mtb blocks apoptosis by inhibiting prostaglandin E2 (PGE2) production (Divangahi et al. 2010).

As previously outlined, T cells are crucial for granuloma formation and containment of Mtb (Cooper 2009, Quesniaux et al. 2010; Khader and Cooper 2008). Importantly, the bacteria have developed a further immune evasion strategy to interfere with this process, since they are capable of inhibiting MHC class II molecule expression and antigen presentation. This evasion strategy is based on innate immune recognition of the bacteria via Toll-like receptor 2 (TLR2), indicating that, during the course of evolution, Mtb has found a way to turn the spear and exploit the host's innate defense mechanisms to its own advantage (Harding and Boom 2010). APCs have important instructive functions and play a central role in polarizing T-cell functions and/or lineage commitment. Efforts have been made to decipher the site of and the cell types responsible for T-cell priming (Cooper 2009). Using the mouse model of aerogenic TB infection, several groups have shown that the draining lymph nodes accommodate the priming events (Chackerian et al 2002; Wolf et al. 2008). Moreover, using antigen-pulsed cells and transfer systems, DCs were recognized to accomplish antigen presentation. IL-12p40 promotes DC migration (Khader et al. 2006), while IL-10 limits it (Demangel et al 2002). However, despite this partial success, there still exists an inability to define precisely the exact cell population delivering Mtb to the draining lymph nodes. Intriguingly, priming requires extended time in TB in comparison to many other infections. Specific T cell responses occur in the mediastinal lymph nodes 10 days after aerogenic exposure at the earliest. The reasons for the delay in priming naive T lymphocytes are still ill-defined but included slow Mtb multiplication rates, regulatory phenotype of lung-resident APCs (Cooper 2009) or natural Tregs (Shafiani et al. 2010).

4. Memory T cells and their role in Mtb infection

The study of memory T cells in individuals that have developed an adaptive immune response against a given pathogen can provide detailed information about the recognized target antigens and the class of the response. This information is relevant to define the quality of the response, to dissect the mechanisms of immunity versus immunopathology, and to design preventive and therapeutic vaccination strategies.

Generally, the induction of T cell memory is characterized by a number of distinct phases (Sallusto et al. 1999). Following Ag priming, Ag-specific T cells undergo massive proliferation and clonal expansion followed by a contraction phase in which the vast majority of the activated effector cells are eliminated by apoptosis (Lanzavecchia and Sallusto 2005; Zanetti and Franchini 2006). During this primary response, memory T cells start to emerge and are maintained for extended periods either by retained antigen, repeated stimulation/boosters, or homeostatic proliferation, hence providing a pool of cells that can rapidly respond to subsequent encounters with the pathogen. The induction of such a pool of memory T cells of adequate size and duration by vaccination procedures against intracellular pathogens has proven a major challenge for the development of new vaccines.

In humans, it is easily study the properties and functions of memory T and B cells, at least of those which circulate in the blood, using specific cell surface markers. The combinatorial expression of adhesion molecules and chemokine receptors allows for tissue specific homing of memory and effector cells and thus a segregation of the immunologic memory in terms of tissue localization (Butcher et al. 1996; Sallusto et al. 2000). Initial studies in humans led to the notion that two functionally distinct subsets of memory T cells can be identified based on the expression of lymph node homing receptors (Sallusto et al. 1999). T central memory (CM) cells express CCR7 and CD62L and, like naive T cells (TNaive), patrol the T-cell areas of secondary lymphoid organs. TCM have limited effector function but have a low activation threshold, retain high IL-2 production and proliferative capacity, and can rapidly differentiate to effector cells upon encountering the specific antigen. In contrast, T effector memory (EM) cells lack CCR7 and CD62L and express receptors for homing to peripheral or inflamed tissues, such as CCR6, CCR4, CXCR3, or CCR5. TEM cells are heterogeneous in terms of homing receptor expression and effector functions and comprise the classical T-helper cell subsets Th1, Th2, Th17, as well as cytotoxic CD8 T lymphocytes. Surface molecules other than homing receptors can be used to further dissect memory subsets. The costimulatory molecules CD28 and CD27 are expressed by TCM and by some TEM cells and are lost on the most differentiated TEM cells (Romero et al. 2007; Hamman et al. 1997). The relative distribution of antigen-specific T cells within TCM and TEM subsets may represent a useful correlate of protection; in fact, an increased frequency of antigen specific TCM cells producing high levels of IL-2 is characteristic of individuals that control chronic infectious agents such as HIV-1, hepatitis C virus (HCV), and Mtb (Harari et al. 2004; Younes et al. 2003; Semmo et al. 2005; Millington et al. 2007).

The lineage relationship between TCM and TEM has been the subject of intense investigation. The initial finding that antigenic stimulation leads to an irreversible differentiation from TCM to TEM led to the proposal of a linear differentiation model, suggesting that TCM cells are differentiation intermediates that retain proliferative capacity and differentiation potential, while TEM cells are more differentiated cells with limited proliferative potential and differentiation capacity. According to this model, T cells differentiate along a one-way linear pathway, the progression being determined by the cumulative strength of stimulation received by T cells. The stochastic interaction with antigen-presenting DCs and the different concentrations of cytokines, to which proliferating cells are exposed, would account for the generation of different fates, even within a single clone. This proposition has been corroborated by new methods that facilitate the analysis of the progeny of single T cells (Stemberger et al. 2007; Gerlach et al. 2010). In several experimental systems, it has been shown that TCM cells confer long-term protection upon adoptive transfer, while TEM cells have only limited reconstitution capacity (Gattinoni et al. 2005). Moreover, the response of TCM and TEM cells to cytokines has been initially characterized in the human system (Unutmaz et al. 1994). Using this approach, it was shown that TEM cells readily proliferate *in vitro* in response IL-7 and IL-15 but fail to expand substantially due to a high degree of spontaneous apoptosis. In contrast, TCM proliferated and spontaneously differentiated to TEM-like cells, even in the absence of polarizing cytokines (Geginat et al. 2001; Geginat et al. 2003). These findings are consistent with the notion that the TCM population contains uncommitted precursors with self-renewing capacity as well as cells that are committed to differentiate into Th1 or Th2 in an antigen-independent fashion (pre-Th1 and pre-Th2). The sustained antigen-independent generation

of TEM from TCM cells provides a plausible mechanism for the maintenance of a polyclonal and functionally diverse repertoire of TCM and TEM cells, in spite of rapid attrition of the latter.

The delineation of T cells into distinct functional populations defines the quality of the response. New evidence suggests that the quality of T-cell responses is crucial for determining the outcome of various infections. It has been postulated that T cells progressively gain functionality with further differentiation, until they reach the stage that is optimized for their effector function (such as the production of IL-2, IFN-γ and TNF-α) (Seder et al. 2008). Continued antigenic stimulation can lead to progressive loss of memory potential as well as cytokine production, resulting in terminally differentiated T cells that only produce IFN-γ and are short-lived. Following antigen stimulation, any of these stable differentiated subsets can also develop into activated effector T cells, leading to their death. The amount of initial antigen exposure or innate-immune factors in the microenvironment will govern the extent of differentiation (Figure 1).

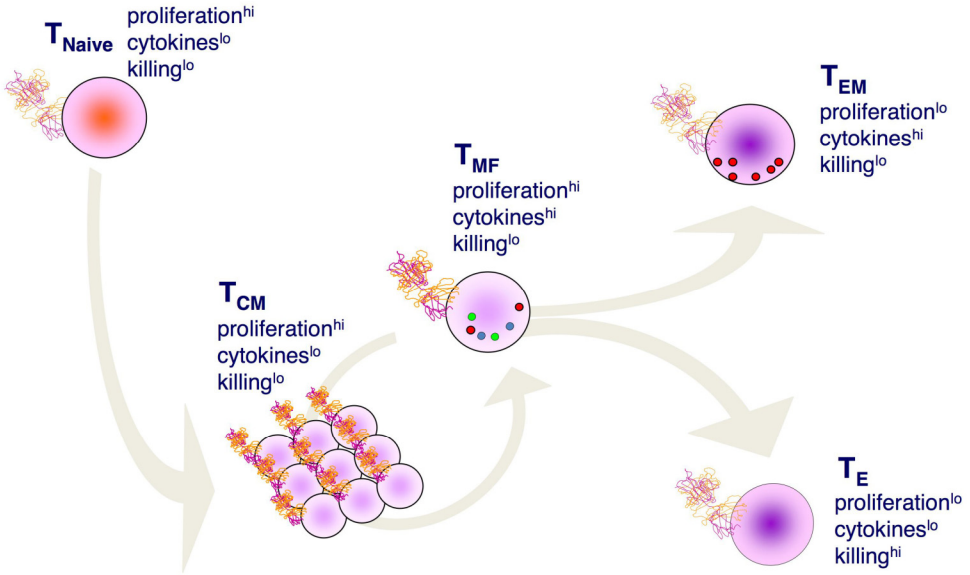

Fig. 1. Model of differentiation of memory T cells.

Unlike CD4 TEM cells, CD8 TEM cells may be able to re-acquire IL-2 expression and become CD8 TCM cells (Figure 1). An inverse correlation was observed between the frequency of multifunctional CD8 T cells and persistence of antigen load in chronic viral infection (Seder et al. 2008). Another functional state of memory cells has been observed in settings of chronic infections by pathogens that have evolved strategies to resist acute innate and adaptive immune attacks. As a result of pathogen persistence, specific CD8 T cells exhaust their cytokine production and proliferative capacity (Brooks et al. 2005; McNeil et al. 2001; Day et al. 2006). However, sustained negative signaling by the inhibitory receptor PD-1 has been mechanistically implicated in T-cell exhaustion. The role of PD-1 is illustrated by the

reversal of T cell exhaustion and concomitant increase in proliferation, cytokine secretion and cytotoxicity, and pathogen clearance upon blockade of PD-1 signaling with anti-PD-L1 antibody (Barber et al. 2006). Besides PD-1, other negative regulators, including CTLA-4, 2B4, and LAG-3, are expressed in chronically activated T cells and have been implicated in T-cell exhaustion (Crawford et al. 2009).

In summary, determining the quality of a T cell response in combination with the cell surface phenotype increases the fundamental understanding of T cell memory and effector differentiation by defining the T cell functional capacity, durability, history of antigen exposure and their capacity to traffic to lymphoid and non-lymphoid organs. Hence, a combined phenotypic and functional analysis of T cells should allow greater insight into whether a response is protective, than either measurement alone.

CD4 and CD8 T cell differentiation upon antigen stimulation, is regarded as a linear process, in which naive T cells (T_{Naive}) progressively gain functionality until they reach the stage that is optimized for their effector function (such as cytokine production or cytotoxicity). Persistent and prolonged antigen stimulation can lead to progressive loss of the pool of long-lived central memory (T_{CM}) and multifunctional (T_{MF}) T cells, resulting in terminally differentiated, short-lived T cells that only produce a single cytokine (T_{EM}) or exert cytotoxic activity(T_E). It is commonly accepted that the amount of initial antigen exposure will govern the extent of such a differentiation pathway.

5. Multifunctional CD4 T cells in Mtb infection

The capacity of antigen-specific T cells to produce simultaneously multiple cytokines (i.e., multifunctional or polyfunctional cells) has been associated with superior functional capacity (Kannanganat et al. 2007) and has been correlated with control of human chronic viral infections such as HIV (Makedonas et al. 2007; Kannanganat et al. 2010; Betts et al. 2006) and hepatitis C virus (Ciuffreda et al. 2008). Moreover, polyfunctional T cells have been associated with protection against disease progression in mouse models of Leishmania major (Darrah, et al. 2007; Darrah et al. 2010) and Mtb (Forbes et al. 2008). Polyfunctional T cells producing IFN-γ, IL-2, and TNF-α have been described in studies of Mtb infected adults (Sutherland et al. 2009; Caccamo et al. 2010; Streitz et al. 2011; Harari et al. 2011) although with differing conclusions. Overall, polyfunctional T cells that secrete multiple cytokines and are able to proliferate, are more likely than single cytokine secretors to represent correlates of protective antiviral immunity in chronic infections (when antigen load is low), while single IFN-γ-secreting CD4 and CD8 T cells are characteristic of acute infections (when antigen load is high). If chronic infection ensues after failure of complete immune control, the balance of responding T cells tends to shift towards the single IFN-γ-secreting phenotype. This process is particularly skewed in the case of HIV-1 infection, as the HIV-1-specific CD4 and CD8 T-cell response is overwhelmingly dominated by a single IFN-γ-secreting effector response during both the primary and chronic phases of infection. On the other hand, the cellular immune response to intracellular pathogens comprises a spectrum of T cell subpopulations characterized by distinct cytokine secretion profiles and surface marker phenotypes. Three main subsets are recognized and can be identified on the basis of T-cell cytokine profiles: TEM cells that secrete only IFN-γ, TEM cells that secrete both IFN-γ and IL- 2 and TCM cells that secrete only IL-2. The relative proportions of these

three T cell subsets correlate with antigen load in chronic viral infections (Sester et al. 2008; Pantaleo and Harari 2006). It has been previously shown that the relative proportions and frequencies of these three T cell subsets among Mtb antigen-specific T cells correlated with pathogen burden and antigen load in TB patients. The significant shift in cytokine profiles after anti-TB treatment led to propose this as a new approach for monitoring anti-mycobacterial treatment but the technical approach was labour intensive and the number of patients was small (Millington et al. 2007). These studies have shown that IFN-γ and IL-2 production, and the proliferative capacities of CD4 and CD8 T cells are key functions that define different aspects of the protective response. Multifunctional Mtb-specific CD4 T cells have been detected in peripheral blood of children with active TB disease and LTBI (Mueller et al. 2008), and are maintained in HIV-1-positive individuals in the absence of active disease (Day et al. 2008), although their functional capacity is affected by HIV-1 disease status both in peripheral blood (Day et al. 2008) and in the lungs (Kalsdorf et al. 2009). Therefore, quality rather than quantity of Mtb-specific T cell responses has been assumed to indicate protection and the capacity to generate long term memory. We have analyzed multifunctional CD4 T cells, expressing simultaneously three cytokines (IFN-γ, TNF-α and IL-2), in response to three Mtb antigens (ESAT-6, Ag85B and 16 kDa) in adults with active TB disease, and compared these with responses in LTBI subjects. Surprisingly, and in contrast to what has been assumed to be the hallmark of a protective CD4 T cell response, we found a significantly higher proportion of multifunctional CD4 T cells simultaneously producing IFN-γ, IL-2 and TNF-α in subjects with active TB disease, compared with LTBI subjects, while in the latter, IFN-γ single and IFN-γ/IL-2 dual secreting CD4 T cells dominated the anti-mycobacterial response. Moreover, these distinct IFN-γ, IL-2 and TNF-α profiles of Mtb-specific CD4 T cells may be associated with bacterial loads, as suggested by their decreased frequency in TB patients after completion of anti-TB chemotherapy. Lending further support to our results is the observation that the pattern of distribution of cytokine producing CD4 T cells was consistently observed in response to three different Mtb antigens, Ag85B, ESAT-6 and 16 kDa antigen. Our starting hypothesis was to find increased proportions of multifunctional T cells in LTBI subjects, since they are, to a certain level, protected against disease development, while a decreased frequency was expected in those individuals who developed disease (Caccamo et al. 2010). However, our data show the opposite pattern, namely, an increased frequency of multifunctional T cells in patients with current or historic-active TB disease and almost undetectable levels in LTBI subjects. In line with our observations, another study in the Gambia also showed that TB cases had significantly higher levels of CD4 T cells secreting simultaneously IFN-γ, IL-2 and TNF-α, compared to exposed household contacts (Sutherland et al. 2009). Collectively, the results from two different ethnic populations are in agreement, and together suggest that this particular ''multifunctional'' CD4 T cell population may be the hallmark of active TB disease. Furthermore, our results suggest that the bacterial load is related to the functional patterns of the CD4 T cell response, in fact the frequencies of Ag85B-, ESAT-6- and 16-kDa antigen-specific CD4 T cells, which simultaneously produce IFN-γ, IL-2 and TNF-α, were significantly increased during active disease, but decreased after 6 months of curative TB treatment to undetectable levels. To our knowledge, our study provides the first evidence for pre/postchemotherapy changes of ''multifunctional'' CD4 T cells, simultaneously secreting three different cytokines, IFN-γ, IL-2 and TNF-α. In our study, we also found that although multifunctional CD4 T cells were undetectable in LTBI individuals in a short-term

in vitro stimulation assay, they could be detected, although at a very low frequency, after long-term *in vitro* stimulation. Moreover, using the long-term stimulation assay, we were also able to detect significant proportion of these cells in cured TB patients. It has been hypothesized that in the short-term assay only the recently primed CD4 T cells, the product of residual antigen, would be detected, but a major reservoir of Mtb-specific CD4 T cells that returned to the resting state (Andersen et al. 2000; Bell et al. 2008) would be missed. Consequently, in individuals who have been infected with Mtb in the past, multifunctional CD4 T cells may persist but in a resting state, and hence causing negative results in a short-term incubation assay, but positive responses after a prolonged incubation. In line with our study, some authors have examined the effects of SIV infection on T cell cytokine responses in cynomolgus macaques from latent Mtb infection, acute SIV infection, and through reactivated TB in order to investigate the dynamics of multifunctional T cell responses and granuloma T cell phenotypes (Matila et al. 2011). Coinfected animals experienced increased Th1 (IL-2, IFN-γ, TNF-α) cytokine responses to Mtb Ags above the latent-response baseline 3–5 wk post-SIV infection, that corresponded to peak plasma viremia. Thus, it appears that Mtb-specific multifunctional T cells are better correlates of Ag load (i.e., disease status) than of protection. Increased cytokine responses reminiscent of amplified cytokine responses observed during acute HIV infection (Stacey et al. 2009) included significantly elevated frequencies of Mtb-specific IFN–γ, IL-2–, and TNF-α-expressing T cells. Therefore, the authors have postulated that SIV depletes T cells coordinating anti-mycobacterial responses in stable granulomas during acute infection, releasing immune pressures that normally limit bacterial replication, and leading to an increased abundance of mycobacterial antigens (Matila et al.2011). The data presented in that study suggest that the increased antigen load stimulates proliferation of Mtb-specific T cells, which are detected as Th1 cytokine-positive cells in the peripheral blood. Thus, the abundance of Mtb-specific multifunctional T cells during acute SIV infection may represent higher Ag loads, and the earliest reactivating animals had both the highest frequencies of IFN-γ^+L-2^+TNF-α^+CD4 T cells during acute infection and the highest bacterial burden at necropsy (Diedrich et al. 2010). Other authors have analyzed the cytokine profile (IFN-γ, TNF-α and IL-2) of Mtb-specific T cells by polychromatic flow cytometry and studied Mtb-specific CD4 T cell responses in subjects with latent Mtb infection and active TB disease (Sester et al. 2011). The results showed substantial increase in the proportion of TNF-α single-positive Mtb-specific CD4 T cells in subjects with active disease, and this parameter was the strongest predictor of diagnosis of active disease versus latent infection. In detail, tuberculin (PPD), ESAT-6 and CFP-10 were used as stimuli to determine antigen-specific cytokine profiles in CD4 T cells from 24 patients with active TB and 28 patients with successfully treated TB using flow-cytometry. Moreover, 25 individuals with immunity consistent with latent Mtb infection and BCG-vaccination, respectively, were recruited. When assessing cytokine profiles, PPD specific CD4 T cells secreting both IFN-γ and IL-2 predominated in treated TB, latent infection and BCG-vaccination, whilst in active TB the cytokine profile was shifted towards cells secreting IFN-γ only. A receiver operator characteristics (ROC) analysis revealed that a percentage of less than 56% of dual cytokine-secreting cells identified patients with active tuberculosis with a specificity of 100% and a sensitivity of 70%. The detection of less than 56% PPD specific dual cytokine-secreting T-cells is strongly indicative of active tuberculosis, whereas frequencies of PPD reactive dual cytokine-secreting T-cells above 56% were observed in all non-active disease states as well as active TB patients (Sester et al. 2011). These results

indicate that the analysis of cytokine profiles in Mtb-specific CD4 T cells by polychromatic flow cytometry is a major immunological measure discriminating between active and latent Mtb infection.

Other authors have recently shown a significant increase in the proportion of both CD4 and CD8 T cells expressing CD107a, instead of IL-2, in combination with IFN-γ and TNF-α in patients with active TB disease prior to treatment compared to post-treatment responses, following stimulation with ESAT-6/CFP-10, suggesting this phenotype is not protective in the TB setting (Sutherland et al. 2010). Following successful TB treatment, the proportion of cytokine positive cells was reduced to levels equivalent to those seen in healthy contacts. Furthermore, subjects with active TB disease had significantly higher levels of T cells producing 2 or more factors which were again reduced following treatment. Apart from cytokine profiling, other immune-based approaches exist to distinguish active disease from non-active states, such as the comparative analyses of Mtb-specific T-cell responses from blood and specimens from the sites of disease. However, these approaches are invasive and depend on the availability of specimens from the sites of disease (Wilkinson et al. 2005; Jafari C et al. 2009; Thomas et al. 2008; Jafari et al. 2008; Strassburg et al. 2008).

Not surprisingly, the differences seen in active disease depend on disease severity and the site of analysis: increased antigenic load in advanced pulmonary TB has been shown to correlate with decreased IFN-γ production in the blood, but increased production in the lungs. Determination of the T cell cytokine profile at specific stages of infection, disease and recovery is critical for development of new diagnostics and vaccine strategies.

A recent interesting study has attempted to correlate the relationship between Ag load in chronic Mtb infection and functional capacity of Mtb-specific T cells in three groups of adults from the Cape Town region of South Africa: 30 healthy asymptomatic adults with LTBI, and 54 individuals with pulmonary TB stratified into two groups, one acid-fast bacilli (AFB) sputum smear negative and the other sputum smear positive. Individuals with smear-positive TB displayed decreased proportions of PPD specific IFN-γ^+IL-2^+TNF-α^+ and ESAT-6-specific IL-2^+TNF-α^+CD4 T cells, but increased proportions of both ESAT-6- and PPD-specific IFN-γ^+TNF-α^+ and PPD-specific TNF-α single positive CD4 T cells, compared with other groups. This study suggests a possible selective decrease in production of IL-2 by Mtb-specific CD4 T cells in individuals with smear-positive TB, compared with smear-negative TB and LTBI, indicating a shift in the cytokine production profiles of specific CD4 T cells with increasing mycobacterial load, characterized by progressive decreases of polyfunctional cytokine production capacity, coincident with increased TNF-α production.

To determine whether reduction of bacterial load by antibiotic treatment was associated with enhanced functional capacity of Mtb-specific T cells, the authors followed longitudinally 13 TB patients after initiating anti-TB treatment; all them were sputum smear-negative by 6 months of treatment. With the exception of CFP-10–specific CD4 T cells, the total frequency of specific CD4 T cells producing any combination of cytokines was not different after 6 months of TB treatment, compared with pretreatment values. The proportion of IFN-γ^+IL-2^+TNF-α^+ and IL-2^+TNF-α^+ PPD-specific CD4 T cells increased significantly on TB treatment, coincident with a decrease in IFN-γ^+TNF-α^+ and TNF-α single-positive cells. The proportion of polyfunctional PPD-specific CD4 T cells increased in all subjects following 6 months of TB treatment. Polyfunctional Mtb-specific IFN-γ^+IL-

2^{+}TNF-α^{+} CD4 T cells were the only cytokine subset that showed a positive correlation with proliferative capacity, thus providing further evidence that polyfunctional cytokine production capacity may be associated with superior functional capacity in the context of a chronic human bacterial infection, and these results indicate that IL-2 production capacity specifically within the context of simultaneous IFN-γ and TNF-α production is indicative of Ag-specific CD4 T cell proliferative capacity. In contrast to polyfunctional Mtb-specific CD4 T cell responses, the proportion of *ex vivo* TNF-α single-positive CD4 cells, which were increased in individuals with smear-positive TB, were inversely correlated with proliferative capacity.

The populations of Mtb-specific CD4 T cells producing TNF-α, in the absence of IFN-γ and IL-2 coexpression, are expanded under inflammatory conditions of high mycobacterial load and may identify a short-lived population of effector cells with limited survival and ability to expand upon a repeated encounter with Ag.

A distinct cytokine profile consistent of simultaneously increased TNF-α, IL-6 and TGF-β was found in a TB patient cohort from Central Africa (Nemeth et al. 2010). Spontaneous TNF-α secretion as well as Mtb-specific-TNF-α production in CD4 T cells was increased in patients with active TB.

TNF-α blocking therapy is a risk factor to reactivate latent TB infection (Kaufmann et al. 2005), highlighting the role of TNF-α in granuloma maintenance during latent TB, even though the role of TNF-α during active TB is less clear and might not only be beneficial but responsible for immunopathology and contributive to the progression of disease (Quesniaux et al. 2010). These data suggest that T cells capable to produce two cytokines are a marker of active TB, a finding which is in line with the studies mentioned above (Caccamo et al. 2010; Millington et al. 2007). The authors report an excess of IL-6, TNF-α and TGF-β spontaneously secreted in the supernatant, suggesting that these cytokines are in part or completely produced by monocytes. This observation suggests that during active pulmonary TB a rather unspecific inflammation takes place which is absent in latent infection. Hence, the combination of Mtb-specific cytokines with markers of inflammation could lead to immune based diagnostics from peripheral blood which is able to discriminate between latent infection and active disease. In line, IL-6 together with TNF-α and IFN-γ have recently been used as markers to monitor TB treatment success. This observation could provide the rationale for novel immunological approaches to detect active TB.

In conclusion, possible differences in the cytokine production profiles of Mtb- specific CD4 T cells found in the reported studies may be due to differences in Ag specificity and type, methodological differences used for detection of cytokine-producing cells, and differences in study cohort characteristics. Further studies are warranted to determine particular phenotypes of Mtb-specific T cells, such as activation, memory, and inhibitory receptors and ligands, which are associated with functional capacity in different stages of Mtb infection.

6. Multifunctional CD8 T cells in Mtb infection

Following recognition of mycobacterial Ags on infected cells, CD8 T cells contribute to Mtb control through: 1) IFN-γ and TNF-α production (Flynn et al. 1992; Caccamo et al. 2009) 2) lysis of infected host cells (Cho et al. 2000; Kaufmann et al. 2005; Lalvani et al. 1998) and 3)

direct killing of mycobacteria (Klein et al. 2001; Ottenhoff et al. 2008; Stenger et al. 1997). One study demonstrated clonal CD4 and CD8 T cell expansion in granulomas from subjects with latent TB infection (Tully et al. 2005), and similar changes in the TCR repertoire were reported in peripheral blood versus pleural fluid in TB patients (Gambon-Deza et al. 1995). Furthermore, CD8 T cells specific for a number of mycobacterial Ags have been isolated from human and mouse models, consistent with the hypothesis that CD8 T cells are constantly being stimulated with Ag (Lalvani et al. 1998; Ottenhoff et al. 2008). Previously, we have reported that the frequency of Mtb-Ag85A-specific CD8 T cells correlated with therapy induced curative responses in children: Ag85A epitope-specific CD8 cells during active TB produced low levels of IFN-γ and perforin, which normalized after therapy (Caccamo et al. 2006). In a later study, we reported similar findings for CD8 T cells directed against six Mtb epitopes (two of which were newly identified). In that study, it was also found that Mtb-peptide-specific IL-2$^+$/IFN-γ$^+$ CD8 T cell responses were associated with natural protection against developing TB disease. In parallel studies, other authors (Jacobsen et al. 2007) found clonal expansion of effector memory CD8 T cells in older children with TB, with potential impact on the course and severity of disease. Other authors have tried to identify CD8 T cells that recognized a number of Mtb epitopes in the context of HLA-A and -B alleles, and ourselves reported Ag85A, B, and C epitopes activating human CD8 T cells (Klein et al. 2001; Geluk et al. 2000; Leyten et al. 2006; Lewinsohn et al. 2007). Despite these studies, little remains known about the size, quality, and specificity of Mtb-specific CD8 T cell responses in TB patients and their relevance to control of infection (i.e., prevention of progression to TB disease). The presence of mostly single and double cytokine positive T-cells, the latter mainly present in CD8 T-cells, support previous findings that single and double positive T cells are prominent in LTBI (Caccamo et al. 2010, Caccamo et al. 2009). This suggests that these double and single cytokine producing T cells play a significant role in Mtb immunity, although their precise nature and mechanisms of action requires more detailed studies. While most studies on polyfunctional T-cells have focused on highly expressed Mtb early phase proteins such as ESAT6 and Ag85B, instead, it remains possible that antigens expressed during different phases of infection may preferentially induce different patterns of single, double and polyfunctional T-cells. Regardless, the functionality of CD8 T cells was detected using peptide/tetramers, and identical-peptide stimulated PBMC of the same cured TB patients and controls were studied to determine fractions of specific CD8 T cells producing IFN-γ, IL-2, and/or TNF-α at the single-cell level. With very few exceptions, single-, double-, and, in some cases, also triple-positive. CD8 T cells could be detected in cured TB patients, but not controls, providing a wealth of new Mtb Ags that may use as targets for TB vaccine development, particularly in the view of mounting evidence that CD8 T cells are important in controlling TB.

Some authors, by using well-defined cohorts of stratified individuals with smear-positive and smear-negative TB and LTBI subjects, have investigated on the effect of mycobacterial load on the functional capacity of Mtb-specific response in peripheral blood (Day et al. 2011). They found that, compared to individuals with lower mycobacterial load, high mycobacterial load in individuals with smear-positive TB was associated with decreased polyfunctional and IL-2–producing cells and increased TNF-α single positive Mtb-specific CD4 T cells, as well as increased frequencies of specific (cytokine-positive) CD8 T cells, and impaired proliferative capacity of both Mtb-specific CD4 and CD8 T cell responses. Moreover, the presence of Mtb-specific CD8 T cells was specifically associated with

pulmonary TB disease status, showing that greater than 60% of individuals with smear-positive TB had detectable CD8 T cells responses compared with 38% and 20 % with smear-negative and LTBI, respectively, suggesting that antigen-driven expansion of Mtb-specific CD8 T cells are detectable *ex vivo* in peripheral blood in patient with pulmonary TB, respect LTBI subjects. These patients were followed during the anti-mycobacterial therapy and in 7 of the 13 individuals the proportion of triple positive CD8 T cells (producing IFN-γ, IL-2 and TNF-α) increase over time, and that this increase was coincident with a decrease in the proportion of IFN-γ single positive cells demonstrating that the cytokine production capacity of Mtb CD8 T cell responses is associated with mycobacterial load. The increase of specific polyfuntional CD8 T cells presumably, and the reduction of IFN-γ or TNF-α producing cells may be indicative to further define the association between CD8 T cells and TB disease progression, particularly in specific populations such as children or immunocompromised individuals where it may be difficult to distinguish Mtb infection from disease and that are at high risk for developing TB, but also to correlate the presence of this subset of cells as indicator of successful response to treatment.

Very recently, it has been proposed that Mtb DosR-encoded Ags (Leyten et al. 2006) expressed by Mtb during *in vitro* conditions mimics intracellular infection and represent rational targets for TB vaccination. In fact, immune responses to Mtb DosR-encoded Ags are prominently found in latently infected individuals, and are associated with LTBI in several ethnically and geographically distinct populations (Leyten et al 2006; Roupie et al. 2007; Schuck et al. 2009). Strong Mtb DosR antigen-specific CD4 and CD8 polyfunctional T-cell responses were detected in LTBI subjects. The highest responses were observed among single cytokine producing CD4 and CD8 T-cell subsets (either TNF-α^+, IL-2^+ or IFN-γ^+, depending on the stimulus) followed by double producing CD4 and particularly CD8 T cells. Of interest, the most frequent multiple-cytokine producing T cells were IFN-γ^+TNF-α^+ CD8 T cells. These cells were further characterized as effector memory (CCR7$^-$ and CD45RA$^-$) or effector (CCR7$^-$ and CD45RA$^+$) T-cells, which have the ability to perform immediate effector functions. This is compatible with an important role for CD8 T cells in Mtb infection (Bruns et al. 2009; Flynn et al. 1992). A striking observation was the wealth of epitopes that could be identified in Mtb DosR-encoded antigens, in accordance with their significant immunogenicity in a wide variety of HLA backgrounds (Ottenhoff et al. 1987).

In conclusion, the qualitative and the quantitative associations between Mtb- specific CD8 response would have the significance to better understand the progression of TB disease facilitating early diagnosis in order to reduce the rates of Mtb transmission and TB associated morbidity and mortality, but also to enhance the study aimed at evaluate the induction of CD8 T cells that are protective from TB disease designing antigen and/or peptide based vaccination approaches to TB.

7. Multifunctional T cells at the site of disease in TB

Tuberculous pleurisy (TBP) is the second most frequent manifestation of extrapulmonary tuberculosis (TB) after lymph node TB (Jafari et al. 2008) and remains a common form of disease both in HIV infected and uninfected subjects in developing countries (Luzze et al. 2001; Ozvaran et al. 2007; Heyderman et al. 1998). TBP resolves spontaneously in some patients without treatment and is thus thought to be a good model system for studying the

protective immune response at the site of infection (Jalapathy et al. 2004). At the site of active MTB infection, as opposed to other forms of TB, pleural mononuclear cells are readily accessible providing an opportunity to study aspects of TB pathogenesis on cells from the actual site of TB disease. There have been limited data regarding detailed analysis of CD4 T cell phenotypes in sites of active TB in humans. Some authors looked specifically at pleural fluid samples from subjects with TBP using single parameter IFN-γ ELISPOT methods and found the greatest proportion of IFN-γ producing cells were CCR7- effector cells (Wilkinson et al. 2005). In murine pulmonary TB models the composition of the CD4 T cells is dominated by terminally differentiated effector cells (Kapina et al. 2007; Reiley et al. 2010). A number of studies in humans have shown that polyfunctional T cells that secrete multiple cytokines may indeed mediate protection against TB (Beveridge et al 2007; Scriba et al. 2010; Abel et al. 2010; Day et al. 2008; Sutherland et al. 2010). Two recent studies have evaluated the presence of polyfunctional T cells at sites of pleural TB indicating that these results could be useful for evaluable markers for diagnosis of TB. A first study evaluated the functional profile, of Mtb-specific CD4 T cells in pleural fluid from HIV-uninfected individuals with active TBP (El Fenniri et al. 2011). It has been found that during active TB disease the greatest proportion of Mtb-specific cells in the pleural space, were TEM cells and that they also had the greatest polyfunctionality. In a second study, it has been found that in patients with TBP, Mtb-specific CD4 T cells from pleural fluid expresses IFN-γ, IL-2, TNF-α, IL-17 or IL-22 and display an effector or effector memory phenotype (Li et al. 2011).

8. Multifunctional T cells in response to vaccination

Protective immunity against Mtb results from a complex interaction between innate immune response, Th1, Th2, Th17 effector cells and cells Treg cells. It is becoming increasingly clear that there may be a difference between aspects of immunity known to be necessary for protection, and an immune response which correlates with protection. Recent studies have indicated that the ability of vaccines to evoke T cell responses of sufficient magnitude and quality for the successful containment of intracellular microbial infections is associated with the induction of multifunctional T cells which express multiple cytokines per cell (Chan et al. 1992; Flynn et al. 1995; Flesch and Kaufmann 1990; Bruns et al 2009; Chen et al. 2009; Moyo et al. 2010). Experiments in several disease models have shown that multifunctional CD4 T cells, which express IFN-γ, IL-2, and TNF-α, are functionally superior to their mono- or bifunctional counterparts. The induction of these multifunctional T cells has correlated with protection against leishmania infections in mice and the control of SIV viremia in non-human primates (Chan et al 1992; Chen et al. 2009). Interestingly, the presence of multifunctional T cells is characteristic of the immune responses seen in non progressive HIV patients, whereas HIV non-controllers elicit responses dominated by monofunctional IFN-γ secreting CD4 T cells (Moyo et al. 2010). The factors responsible for the optimized effectiveness of multifunctional cells are uncertain but probably include the capacity of these cells to secrete high levels of cytokines per cell, the synergistic intracellular killing resulting from the secretion of IFN-γ and TNF-α from the same cell, and the promotion of T cell expansion by cells expressing IL-2.

Some studies have highlighted the induction of the multifunctional T cells upon immunization with mycobacterial antigens with adjuvant formulations or with adenovirus vaccine potentiate with mycobacterial antigens.

Immunization with the Ag85/ESAT6 fusion protein in CAF01 adjuvant formulation evoked long-term protective responses characterized by high levels of persisting multifunctional cells (Lindenstrom et al. 2009). Immunization of adolescents or children with a MVA85A vaccine alone or as a BCG booster vaccine in mice induced multiple CD4 T cell subsets including cells which co-express IFN-γ, TNF-α, and IL-2 (Trunz et al. 2006). BCG vaccination of newborns also induced a complex profile of T cells expressing multiple cytokines (Tuberculosis vaccine pipeline-2009. Stop TB working group on new vaccines. 2009).

In another study the persistence of anti-TB protective immunity was investigated for five different types of vaccines – live attenuated, subunit, viral vectored, plasmid DNA and combination vaccines - during a 14-month study period. The extent of vaccine-induced protective immunity correlated with the magnitude and quality of multifunctional CD4 T cells expressing IFN-γ, TNF-α, and IL-2 that were elicited by immunization with these different TB vaccine preparations (Derrick et al. 2011).

MVA85A, a recombinant strain of modified vaccinia Ankara expressing antigen 85A (Ag85A) from Mtb (McShane et al. 2002), is the first new tuberculosis vaccine to be tested in children and infants. This vaccine, designed to enhance the BCG-induced immune response, has an extensive and promising safety and immunogenicity record in adults from different settings (Sander et al. 2009; Beveridge et al. 2008, Brookes et al. 2008; Hawkridge et al. 2008). The vaccine was also well tolerated in children aged 2–7 years from a tuberculosis endemic setting in South Africa (Scriba et al. 2010) and induced robust and durable T cell responses. Injection of three different doses of MVA85A in healthy, BCG-vaccinated infants induced a robust, long-lived, and predominantly polyfunctional CD4 T cell responses that peaked 1 month after vaccination and low frequencies of IFN-γ-expressing CD8 T cells that peaked later than the CD4 response. This response was also highly durable; magnitudes exceeded prevaccination levels up to 168 days after vaccination.

However, recent results from a large cohort of BCG-vaccinated South African infants have shown that the frequency of multifunctional T cells making IFN-γ, TNF-α and IL-2, 10 weeks post-vaccination was not associated with protection in this population. In another study, BCG-vaccinated adults significantly induced cytokine production, activation and proliferation of CD4 T cells. After polyclonal stimulation, BCG-specific CD4 T cells produced Th1-like cytokines. Importantly, the proliferation and cytokine production of CD4 T cells were inhibited by Treg and partially reversed by blocking of IL-10 production, demonstrating that BCG-specific CD4 T cells are persistent in BCG-vaccinated adults and their specific responses are modulated by Treg, implying the possibility of enhancing immune responses of TB infection by down-regulating the function of Tregs (Li et al. 2011).

Similar results were reported following human vaccination with the BCG booster AERAS-402 (recombinant replication deficient Adenovirus (Ad35) virus, expressing a polyprotein of Ag85A, Ag85B and TB10.4) (Abel et al. 2010). Finally, mice vaccinated with hybrid subunit vaccines H1 (Ag85-ESAT6) and H56 (H1+Rv2660) also had high numbers of triple cytokine producing CD4 T cells (Lindenstrom et al. 2009; Aagaard et al. 2011).

However, any immunological correlate may be vaccine and disease-stage specific. Given the diversity of vaccine candidates being developed, and the diversity of disease states in infants, adolescents and HIV-infected adults, it is unlikely that a single, simple immune correlate exists across all these different populations. Despite these intriguing results, it has

not been shown in animal models or in clinical studies that the induction anti-TB protective immunity correlates with the frequencies and/or quality of multifuntional cellular responses for different types of TB vaccines.

9. Conclusion

The induction of memory T cells with multifunctional properties appears to provide a good correlate for protection against a number of disease targets. This new, more comprehensive understanding of the full functional capacity of effector and memory T cell responses has major implications for vaccine design and development and to maintain an efficient immune surveillance for prolonged periods. Consequently, to evaluate memory formation, long-term memory responses should be examined in terms of frequency, phenotype, quality, and persistence of the memory T cells induced, which are all factors anticipated to contribute to a protective immune response.

Immune correlates of protection from TB disease progression are not well defined, although results from the above reported studies have provided that polyfunctional cells play a different role in individuals with LTBI and with pulmonary TB, with some studies indicating increased Mtb-specific polyfunctional T cell responses in TB patients and upon vaccination, while other studies have indicated either decreased polyfunctional responses in TB patients or no difference. In conclusion, these differing findings highlight the difficulties of studying human immunity to TB and the need to evaluate the polyfunctional T cells in longitudinal studies and in different clinical settings. Finally, possible differences in the cytokine production profiles of Mtb-specific T cells found in the different studies may be due to differences in antigen specificity and type, methodological differences used for detection of cytokine-producing cells, and differences in study cohort characteristics.

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14

MHC Polymorphism and Tuberculosis Disease

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1. Introduction

Mycobacterium tuberculosis (Mtb), the causal agent of tuberculosis (TB), remains a major public health throughout the world causing high mortality in humans. According to the report published by the World Health Organization in 2009, 9.3 million new cases of TB were declared in the world and 1.3 million HIV-negative people died by this infection (http://www.who.int/tb/publications/global_report/2010/en/index.html). One-third of the world's population is estimated to be infected with Mtb, but, only 1 in 10 subjects who become infected would develop clinical disease. Furthermore, until now it is not fully understood why this category of individuals develop different forms of TB, pulmonary and extra pulmonary TB. Several environmental factors principally malnutrition, HIV infection and a decrease of socio-economic level favours TB progression. Furthermore, it has been confirmed by numerous studies that the outcome of TB infection is under the influence of the host genetic background (Vannberg et al., 2011). In fact, twin studies have revealed the increased concordance of disease in monozygotic compared with dizygotic twins (Jepson et al., 2001; Maartens et al., 2007). In addition, numerous families and case-control studies have demonstrated the involvement of many genes in the control of immune response in the context of the susceptibility or resistance to TB. Among these genes Major Histocompatibility Complex (MHC) takes a substantial and central role in the control of TB infection (Kamath et al., 2004).

MHC is a genetic complex that encodes the antigen-presenting molecules and is involved in the recognition and cellular cooperation functions which the substratum is the T lymphocyte. Its principal function is to ensure the selection, the transport and the presentation of peptides generated in the antigen-presenting cells. MHC is characterized by an extensive polymorphism. There are currently 6810 HLA alleles described by the HLA nomenclature and integrated in the IMGT/HLA Database (http://www.ebi.ac.uk/imgt/hla/stats.html). This particularity allows to this genetic complex a strong impact in term of immune response efficiency. While some MHC variant genes play a role in the protection against TB others variants in contrast were considered as markers of susceptibility to TB. MHC is the first molecule tested for genetic associations with TB susceptibility. For more than four decades, several authors have described strong associations between some MHC specificities and several immunological disorder including infectious diseases such as TB infection (Yee, 2004; Hill, 2006). The tri-molecular complex of T cell receptor (TCR), antigenic peptide, and

MHC molecules represents the fundamental basis of the immune response. So, genetic variation that could occur in any genes which code one of these elements could have an impact on the functional levels. MHC system takes a great part of its responsibility in term of TB pathogenesis. For example, the impact of MHC class I alleles on the Mtb antigen-specific CD8+ T-cell response in patients with TB has been reported by several studies (weichold et al., 2007; Lewinsohn et al., 2007; Smith & Dockrell, 2000). In addition, according to the MHC class I specificity some important specific peptides selected from Mtb antigens as Ag85B and 19-kDa lipoprotein are identified to be recognized by CD8 positive T lymphocytes (Geluk et al., 2000; Lalvani et al., 1998; Mohagheghpour et al., 1998). These CD8 T cells subset has been suggested to control MTB infection.

The predictive value of MHC system takes a considerable importance concerning susceptibility or resistance to TB disease. Two situations are observed: First, certain MHC markers are positively associated with disease and thus they are considered as markers of susceptibility. Second, other MHC specificities are negatively associated and may have a role in the protection against TB.

In this context, MHC polymorphism has been also employed to identify the efficient peptide that can be used to improve sensitivity and specificity of diagnosis test of TB and vaccine development. On the basis of the prediction of Mtb antigen sequences that bind to MHC molecules, several authors have designed MHC-promiscuous T-cell multi-epitopic peptides (Seghrouchni et al., 2009; Zhang et al., 2010).

The present chapter will discuss the most important work relating, first the impact of MHC polymorphism in the outcome of TB infection, and in the second the improvement of diagnosis method of TB using reverse immunogenetic.

2. Major Histocompatibility Complex (MHC) Human Leukocyte Antigen (HLA)

Major Histocompatibility Complex (MHC) or Human Leukocyte Antigen (HLA) in human coding region is located on the short arm of chromosome 6 (6p21.3). It occupies a segment of about 4000 kb, containing over 220 identified genes (Robinson J et al., 2003). The strong proximity between HLA genes explains why we observe a low rate of genetic recombination within this region. Consequently, HLA genes are transmitted as haplotypes from parents to children. Each individual inherits two parental haplotypes which expression is codominant. The HLA system is divided into three regions (Figure 1)

- From the centromere, there is HLA class II region (about 900 kb) which includes at least 32 genes. The most functional histocompatibility genes are represented by HLA-DR, HLA-DQ and HLA-DP. Other genes as large multifunctional protease (LMP2 and LMP7) or Transporter associated with antigen processing (TAP1 and TAP2) are found in this region which play a crucial role, respectively, during antigen processing or in the active transport of peptides across the membrane of the endoplasmic reticulum.
- The intermediate region is HLA class III (about 1100 kb) and is composed of at least 39 genes. Among them there are tumor necrosis factor (TNF), complement components (C4A, C4B, BF, and C2), etc.
- The telomeric region covers HLA class I genes and spans 1600 kb. This area contains about 17 genes and is divided into two sub-classes:

HLA classical class I genes (class Ia) namely HLA-A, HLA-B and HLA-C and HLA non-classical class I (class Ib) namely HLA-E, HLA-F, HLA-G, Major Histocompatibility Complex class I chain-related A and B (MICA and MICB), etc. (Figure 1). In this region, there is others non histocompatibility genes as UNHCR (α-helix coiled coil rod homolog),and hemochromatosis gene (HEF).

HLA class I genes are composed of eight exons and seven introns. Exon 1 encodes the signal sequence, exons 2, 3, 4 respectively encode for the extracellular domain (α 1, α 2, α 3), exon 5 encodes the transmembrane portion and exons 6, 7, 8 encode the intra-cytoplasmic (Malissen et al., 1982). HLA class Ia genes are among the most polymorphic genes described in the human genome. According to the IMGT/HLA data base, 1698 alleles of HLA-A, 2271 alleles of HLA-B and 1213 alleles of HLA-Cw have been identified to date. This genetic characterization allows HLA molecule to bind a large repertoire of peptides, controlling T cell polarization and consequently the profile of the cytokines production. In contrast, this characteristic could subverted if the immune system is disturbed as observed in the auto-immune and also in some infectious diseases development (De Castro, 2009; Acharya et al., 2010).

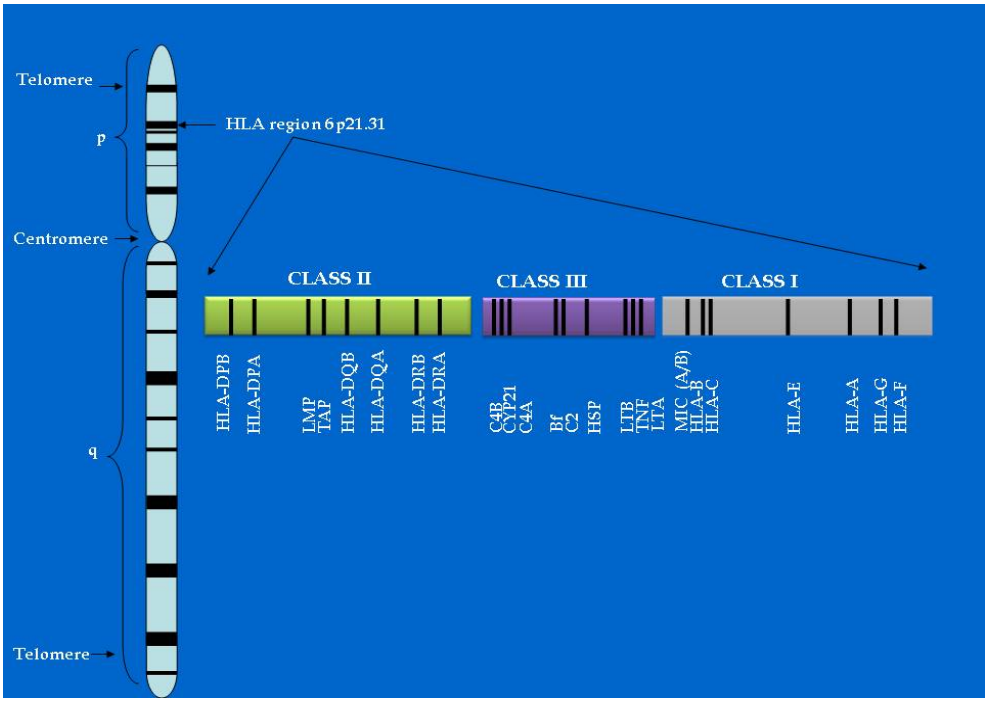

Fig. 1. Genomic organization of Major Histocompatibility Complex region.

At phenotypic level, HLA class I is represented by two chains: alpha heavy chain (α) and Beta 2-microglobulin (β2m) light chain, and the interaction between α1 and α2 domains generates a peptide binding cleft (figure 2). HLA class I molecule contains a series of six pockets, designated from A to F which can establish different interactions with antigenic

peptide residues (Bjorkman et al. 1987; Garrett et al., 1989). The conserved residues in pockets A and F are located at each side of the cavity and are responsible for the orientation of the binding peptide during antigen presentation step, while polymorphic residues located in the pockets B, C, D and E influence the specificity of peptide binding or site of peptide conformation within the cavity (Garrett et al., 1989; Madden et al., 1991; Matsumura et al., 1992).

Concerning HLA class II genes, HLA-DRB1 and HLA-DP1 encompass six exons whereas HLA-DQB1 includes five exons. HLA-DRB1 is the most polymorphic gene within HLA class II. HLA class II molecule consists of two polypeptides chains, alpha (α) and Beta chain (figure 2). Each chain includes two domains α1 and α2 for alpha chain, and β1 and β2 for β chain. The contact established between α1 and β1 domains creates the peptide binding site, which interact with TCR. These two domains play an important role during the presentation of antigenic peptide. Moreover, in this region where many genetics variations genetic variation are found, which characterize each HLA class II alleles thereby are influencing the outcome of the immune response.

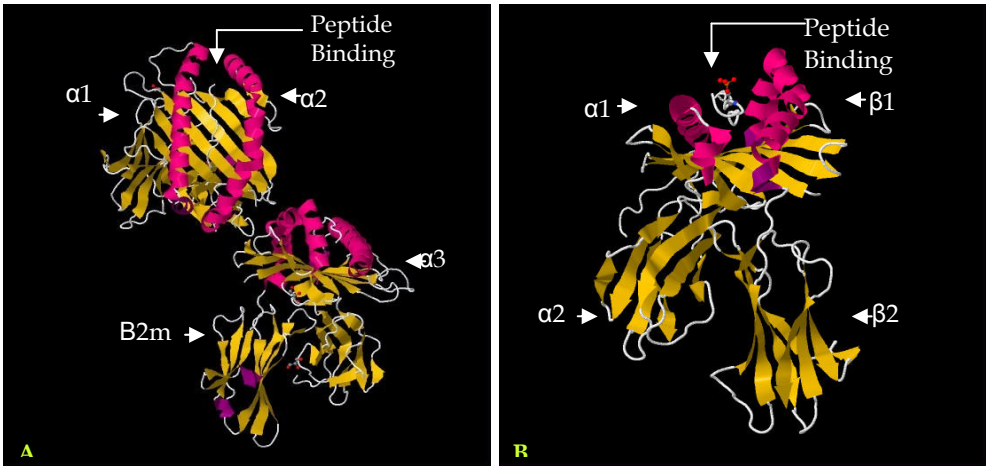

Fig. 2. Crystal structure of MHC class I (A) and class II (B) molecules in complex with antigenic peptide. (Accession numbers taking from Protein Data Bank are respectively 3PWJ and 3L6F) (Borbulevych et al., 2011; Li et al., 2010) **(A)** : HLA class I is comprised by two chains, alpha heavy chain (α) and Beta 2-microglobulin (β2m) light chain, with noncovalent interaction between them. The peptide binding groove shapes a short cleft by an interaction between α1 and α2 domains. **(B)**: HLA class II molecule consists of two polypeptides chains, alpha and Beta chain. Each chain includes two domains α1 and α2 for alpha chain and β1 and β2 for β chain. The interaction between α1 and β2 domains generates long peptide binding clefts which interact with TCR.

HLA molecules expression is different between the two classes. In fact, HLA class I molecules are expressed on most mononuclear cells, whereas class II molecules are expressed on antigen presenting cells (APCs): macrophages / monocytes, dendritic cells and B cells. Moreover, into the same class, different loci do not have the same level of tissue expression, such as HLA-C are naturally more weakly expressed than HLA-A or HLA-B.

3. MHC polymorphism and TB disease

One of the main roles of MHC is to regulate the immune response against all immunological abnormalities in normal physiological condition and during infection state. MHC is known as one of the important component of susceptibility and resistance to many infectious diseases and responsiveness to pathogens or vaccines. In the case of pulmonary TB (PTB) disease, case-control association studies have found significant associations between MHC genes polymorphism and this pathology (Kettaneh et al., 2006; Yim & Selvaraj, 2010). In fact, both genes encoding MHC class I and class II may play crucial roles in host susceptibility to PTB. table 1 summarizes the most important immunogenetic association studies related to MHC and PTB. Analysis of this table shows that first, the majors susceptibility locus are more within MHC class II region compared to those located in MHC class I section. Indeed, for MHC class I, almost all genetic association with TB are positive. In contrast, both negative and positive association are observed between TB and MHC class II polymorphism, suggesting a strong influence of MHC class II in the modulation of the immune response to MTB infection through cell-mediated immunity (Moss & Khanna, 1999; Kettaneh et al., 2006; Yim & Selvaraj , 2010).

However, the results are conflicting as reported by several studies. MHC polymorphism investigations have revealed, that the allele HLA-DRB1*04 is associated with TB in Syrian population (Harfouch-Hammoud & Daher, 2008), HLA-DRB1*07 and HLA-DQA1*0101 in Iranians (Amirzargar et al., 2004), HLA-DRB1*11 in Indonesians (Yuliwulandari et al., 2010), and HLA-DRB1*1302 in South Africans (Lombard et al., 2006). HLA-DRB1*0803 and HLA-DQB1*0601 were associated with PTB disease advancement in Koreans while a strong association with resistance to recurrent PTB is observed (Kim et al., 2005, Yuliwulandari et al., 2010) with HLA-DRB1*12 in Indonesians (Yuliwulandari et al., 2010). Finally, HLA-DR2 seems to be the main allele positively associated with PTB. This observation is replicated in populations with different genetic background, Indian (Brahmajothi et al., 1991, Ravikumar et al., 1999, Sriram et al., 2001), Chinese (Shi et al., 2011) and polish (Dubaniewicz et al., 2000). HLA-DR2 is divided into two subtypes, DRB1*15 and DRB*16. Except for the study reported by Dubaniewicz and his colleagues, DRB1*15, especially the DRB1*1501 allele, is strongly associated with PTB susceptibility (Dubaniewicz et al., 2000). This observation suggests that amino acids present in DR15 molecule but absent in DR16 could play an important role in the development of PTB. Certainly, this data does not exclude the involvement of other region parts of the protein and/or other immunoregulatory linked genes. The nucleotide sequence of the peptide presented by DRB1*15 are so different from those presented by DRB1*16 and may be recognized by $CD4^+$ T lymphocytes as inadequate form and consequently this situation could disturb the effective immune anti-TB response. At functional level, it has been suggested that HLA-DRB1*1501 and HLA-DRB1*1502 may be associated with down-regulation of perforin-positive cytotoxic cells (T-lymphocytes and natural killer) in PTB, supporting the potential role of theses alleles in the TB susceptibility (Rajeswari et al., 2007). On the other hand, HLA-DRB1*16 but not HLA-DRB1*15 are observed more frequently in Brazilian leprosy patients than in controls group (9.0% *vs*. 1.8%; P = 0.0016; OR = 5.81; CI = 2.05–16.46), underlying a difference in the impact of MHC polymorphism which may be related to the specificity of each pathology (Da silva et al., 2009).

Candidate allele	Genetic polymorphisms association			Population	P-value	OR	References
	Positive	Negative	Recurrent disease				
HLA class I							
A1		X		Indian	<0.001	ND	(Balamurugan et al., 2004)
B*1802			X	Indonesian	0,013	ND	(Yuliwulandari et al., 2010)
B*4001			X	Indonesian	0,015	ND	(Yuliwulandari et al., 2010)
B51	X			Indian	<0.0001	0.0	(Vijaya Lakshm et al., 2006)
B52		X		Indian	<0.0001	18.53	(Vijaya Lakshm et al., 2006)
Cw6		X		Indian	<0.001	ND	(Balamurugan et al., 2004)
Cw7	X			Indian	<0.001	ND	(Balamurugan et al., 2004)
HLA class II							
DR2	X			Indian	0,01	0.29	(Brahmajothi et al., 1991)
DRB1*1501	X			Indian	0.013	2.68	(Ravi kumar et al., 1999)
DRB1*15	X			Chinese	0,001	3.79	(Shi et al., 2011)
DRB1*16	X			Polish	<0.01	9.7	(Dubaniewicz et al., 2000)
DQB1*0301-*0304	x			South Africa	0,001	2.58	(Lombard et al., 2006)
DRB1*04	X			Syrian	0,01	1.77	(Harfouch-Hammoud & Daher, 2008)
DRB1*11		X		Syrian	0,003	0.51	(Harfouch-Hammoud & Daher, 2008)
DRB1*1101			X	Indonesia.	0,008	ND	(Yuliwulandari et al., 2010)
DRB1*1202			X	Indonesia.	0.0008	0.32	(Yuliwulandari et al., 2010)
DRB1*13		X		Polish	<0.001	0.04	(Dubaniewicz et al., 2000)
DRB1*1302	X			South Africa	<0,001	5.05	(Lombard et al., 2006)
DRB1*07	X			Iranian	0.025	2.7	(Amirzargar et al., 2004)
DRB1*0803			X	Korean	0.00009	5.31	(Kim et al., 2005)
DQA1*0301		X		Iranian	0.033	0.25	(Amirzargar et al., 2004)
DQA1*0601		X		Thailandaise	0.02	ND	(Vejbaesya et al., 2002)
DQB1*0301		X		Thailandaise	0.01	ND	(Vejbaesya et al., 2002)

Candidate allele	Genetic polymorphisms association			Population	P-value	OR	References
	Positive	Negative	Recurrent disease				
DQB1*0502	X			Thailandaise	0.01	2.06	(Vejbaesya et al., 2002)
DQB1*0601	X			Indian	0.008	2.32	(Ravi kumar et al., 1999)
			X	Korean	0.00003	5.45	(Kim et al., 2005)

Table 1. Genetic associations of important MHC gene variants with the susceptibility or resistance to tuberculosis and with disease recurrence. (OR: Odds ratio, ND: no Data)

Additionally, certain alleles like DRB1*11 and DRB1*13 are in contrast associated with protection against PTB (Harfouch-Hammoud & Daher 2008; Dubaniewicz et al., 2000). This conflicting results reported in theses studies could be due to the positive linkage disequilibrium (LD) observed between MHC class II alleles. DRB1*11-DQB1*03 haplotype was found in positive LD in controls polish patients (Dubaniewicz et al., 2005). In this case, DRB1*03 itself but not DRB1*11 may be linked to the resistance to TB. The hypothesis of the presence of other alleles in LD with DRB1*11-DQB1*03 haplotype is not excluded.

However, even if there are few studies reported in the literature concerning the impact of MHC class I polymorphism on the TB development, it seems likely that some MHC class I alleles are associated with PTB disease, as HLA-A1, HLA-B51, HLA-Cw6 and HLA-Cw7 in Indians (Balamurugan et al., 2004; Vijaya Lakshmi et al., 2006) and HLA-B*1802 and HLA-B*4001 in Indonesians (Yuliwulandari et al., 2010). Analysis of these results and others showed that HLA-B alleles may play the main role in PTB development comparing to the other alleles of MHC class I. HLA-B gene is the most polymorphic gene within the human MHC and the fundamental genetic variation occurs within exon 2 and exon 3, known by its determinant function during the presentation of antigenic peptide step. As cited above 2271 alleles of HLA-B are identified to date. In recent cellular immunological study, using IFN-g ELISPOT and following stimulation of T cell clones with specific Mtb synthetic peptide arrays, Lewinsohn and his colleagues have demonstrated that the immunodominant TB CD8 antigens was preferentially restricted by HLA-B (Lewinsohn et al., 2007). In the same way, it has been reported that the majority of epitope-specific CD8 T cells are HLA-B alleles restricted in patients with PTB and in addition these alleles found fast off-rates in peptide binding (Weichold et al., 2007).

4. Reverse Immunogenetic and TB diagnosis test development

For several years biologists used direct smear microscopy and culture for active TB diagnosis. But, until now the gold standard test remains the culture isolation of Mtb and it is the only test that confirms the diagnosis of TB disease. The control of the disease depends absolutely on early identification and treatment of active cases. However, direct microscopic examination as well as the culture doesn't have an adequate sensitivity and specificity, 20% and 80% for the first test and 60% and 99% for the second. For this reason, many teams interested in this topic have tried to improve these two parameters in bacteriological,

immunological and molecular biology techniques. At immunological levels, numerous studies have been reported on the cellular and humoral immunology field a significant improvement for the diagnosis of TB have been described (Seghrouchni et al., 2009; Panigada et al., 2002, Zhang et al., 2010). Unfortunately, none of the immunological methods reported in these studies was able to discriminate between active TB and latent TB infection. The use of Elispot technique and also the specific Mtb antigen have certainly played a good progress to resolve this problem. Among the panel of immunological technology used and the most relevant approach applied in this context is the reverse immunogenetic technique, based on in-silico identification peptide. This method allows the scientific community to better investigate the antigenic peptides that are presented by the relevant MHC molecules. Furthermore, this strategy offers the possibility to identify the specific T lymphocyte epitopes from living cells and provides a precious help for the development of vaccine candidates. This approach has been elegantly used to identify a specific T lymphocyte epitopes antigen in cancer (Liu et al., 2011; Imai et al., 2011) and in infectious disease (Kawashima et al., 2008; Hossain et al., 2003; Sobao et al., 2001; Seghrouchni et al., 2009; Wang et al., 2010). Various studies have been reported regarding the use of this strategy in TB disease. All immunological investigations are focused to produce a specific and synthetic peptide of Mtb in order to improve the sensitivity and the specificity of the diagnosis Kits. Several MTB-specific antigenic peptides demonstrated their potential application for TB diagnosis, (Ravn et al., 1999; Arend et al., 2002, Seghrouchni et al., 2009; Panigada et al., 2002, Zhang et al., 2010).

Both MHC class I and class II-restricted responses against Mtb are explored in this context with major importance for MHC class II. This importance takes its consideration regarding the roles played by CD4 T Lymphocytes in developing candidate vaccine for TB. In fact, Numerous Mtb specific antigens for CD4+ T lymphocytes have been identified and characterized up till now. We have previously identified Mtb specific peptide, selected from RD1 genomic region (Mahairas et al., 1996) and from proteins expressed during MTB growth in human macrophage (Cappelli et al., 2006; Mariani et al., 2000), and which are predicted to bind HLA–DR alleles (Seghrouchni et al., 2009. Baassi et al., 2009). IFN-γ ELISPOT after stimulation by Mtb selected peptide of peripheral blood mononuclear cells, extracted from TB patients and Healthy controls, have revealed an excellent result. In fact, using statistical algorithms we have identified a pool of specific Mtb immunodominant B and T cell epitopes, able to discriminate between active TB patients, tuberculin skin test positive (Mtb exposed subjects) and tuberculin skin test negative controls. A similar study has been reported recently using bioinformatic tools (chaitra et al., 2008). In fact, the authors of this work have designed some HLA class I binding epitopes of the PE (Pro-Glu) and PPE (Pro-Pro-Glu) proteins of Mtb, which are coded by Rv1818c, Rv3812 and Rv3018c genes, and have observed a significant difference in the responsiveness between healthy subjects and TB patients.

Likewise, other investigations have been reported concerning MHC class I and in-silico identification peptides. By means of appropriate bioinformatic tools several peptides are identified and could be used to improve both TB diagnosis and vaccine development. More recently, using HLA-peptide tetramers derived from Mtb peptides predicted to bind to HLA-A*0201, Tang and his colleagues have found a very interesting Mtb epitopes activating polyfunctional CD8+ T cells in human TB (Tang et al., 2011). Moreover, some specific

peptides to CD8 T Lymphocytes as HLA-B*35-restricted CD8(+) T-cell epitope in Mtb Rv2903c (Klein et al., 2002), HLA-A*0201-restricted T-cell epitope in the MPT51 protein (Aoshi et al., 2008) and HLA-B*35-restricted CD8 T cell epitopes in the antigen 85 (aa 204-212) WPTLIGLAM (Klein et al., 2001), were demonstrated to have a potential positive effect on Mtb -infected macrophages and produce significant level of gamma interferon and tumor necrosis factor alpha.

5. Conclusion

Taking all these data together reported in this review we can conclude that: MHC polymorphism and immunogenetic reverse studies offer a precious help at different level, in the identification of susceptibility/resistance genes or cluster of genes that are involved in the TB disease, and in the characterisation of a specific and relevant Mtb T lymphocyte epitopes for diagnosis improvement and vaccine development. The advancement of bioinformatic tools and immunological technologies could undoubtedly contribute to understand well the immunogenetic of TB, and consequently to improve the quality and the reliability (sensitivity/specificity) of immunological diagnosis Kits.

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15

Partial Mapping of the IL-10 Promoter Region: Identification of New SNPs and Association with Tuberculosis Outcome in Brazilians

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1. Introduction

Tuberculosis (TB) is one of the oldest infectious diseases which affect humankind. According to the World Health Organization (WHO), in 2009 the TB global burden reached 9.4 million of incident cases, 14 million of prevalent cases and 1.3 million deaths. Most cases were in the South-East Asia and Western Pacific regions. Concerning HIV-TB co-infection, 11-13% of the incident cases were HIV-positive, been the African continent accounted for approximately 80% of these cases (World Health Organization and Global Tuberculosis Programme 2010). Several factors are involved in susceptibility to active disease after infection by *Mycobacterium tuberculosis* including environmental and host genetic markers. Today, the search for functional genetic markers in target genes and association studies with different TB outcomes has been common in the literature (Amim et al, 2007, Calado et al, 2006). Several genes encoding different cytokines, receptors and transporters molecules are involved in the host immune response against *M. tuberculosis*, therefore playing crucial roles in TB susceptibility. The interleukin-10 (IL-10), one of the most important Th2 cytokine involved in the immunoregulation. It is a homodimeric molecule of 37kDa consisting of two monomers of 18.5kDa that interacts with the heterodimeric receptor complex to modulate the biological activities of several cells lineage such as T-cells and the myeloid cells, including monocytes, dendritic cells and macrophages. Interleukin 10 inhibits activation of these cells resulting in a reduced production of pro-inflammatory mediators, including different cytokines and chemokines (Groux et al, 2003; David et al, 2008, Lin et al, 2003; Westendorp et al, 1997).

Variability in IL-10 production has a hereditary component of approximately 75% and this variation is mainly due to polymorphisms within the promoter region of the IL-10 gene (Shin et al 2005; Eskdale et al, 1997).

The human IL-10 coding gene spans about 4.7 kb on chromosome 1q31-32 and contains five exons. It is a highly polymorphic gene, with sixty described single nucleotide polymorphisms (SNPs), spreaded along the promoter, coding and intronic regions (Ensembl Genome Browser April 2011, ENSG00000136634). According to the literature data, the genetic diversity of the IL-10 gene expressed by allele frequencies, varies depends on the ethnicity of the studied population (Moraes et al, 2003). Based in the admixture genetic background of Brazilians, the aim of this work was to map the proximal region of the regulatory sequence (promoter region) of the IL-10 coding gene to look for new SNPs and to evaluate the possible association between IL-10 gene polymorphisms and different TB outcomes after infection with *M. tuberculosis* in Brazilians.

2. Material and methods

2.1 Selection of subjects and sample collection

Four hundred and ninety two consanguineously unrelated individuals from Rio de Janeiro were enrolled in this study. They comprised 221 patients with culture-confirmed active TB (who had been treated at the University Hospital Complex: Thoracic Institute/Clementino Fraga University Hospital from Federal University of Rio de Janeiro-UFRJ and 271 close healthy contacts of TB cases, with no previous TB history and with information of Tuberculin Skin Test (TST) response, (TST+ and TST-) from the same Hospital. This study was approved by the ethics committee of Oswaldo Cruz Foundation.

After a written informed consent a volume of 5 mL of venous blood was collected from each volunteer and stored at -20°C. Genomic DNA was isolated from 100 µL of frozen whole blood using the FlexiGene DNA Kit (Qiagen Inc., USA), according to the manufacturer's specifications. After extraction, DNA samples were stored at -20°C.

2.2 IL-10 genotyping

The partial mapping of the promoter region of IL-10 coding gene was performed by PCR amplification followed by direct sequencing of a 1500pb region upstream of the transcription site. Two sets of primers, EF: 5` CTGTGCCTCAGTTTGCTCAC 3`, ER: 5´ACTCTGCTGAAGGCATCTCG3´, IF 5´GCAATTTGTCCACGTCACTG 3´ and IR 5´TTGGTTGAACATGAACTTCTG 3´ were used for amplification and sequencing of a DNA fragment of 1001pb (fragment 1) and one set, EF 5´ TTCCCCAGGTAGAGCAACAC3´ and ER5´GGCACATGTTTCCACCTCTT3´ for amplification and sequencing of a second DNA fragment of 565pb (fragment 2). Primers were design by using the Primers3 software (http://frodo.wi.mit.edu/cgi-bin/ primer3/primer3 www.cgi).

The PCR-mediated DNA amplification of the fragment 1 was performed from 100 ng of genomic DNA in a reaction mixture containing 200ng of each primer (IL10frag1) EF and (IL10frag1) EF, 0,2mM of dNTPs, 2,5mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Invitrogen by Life Technologies, USA) The cycling conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min., 70°C for 1 min. and 72°C for 1 min with a final

extension at 72°C for 5 min. For amplification of the fragment 2, 100ng of genomic DNA were used in a similar mixture except for the primers (IL10frag2) EF and (IL10Frag2) ER. Amplification conditions were also the same used for fragment 1 except for the annealing temperature of 63,3°C. Sequencing of the amplified fragments was performed in both DNA strands using a combination of the internal and external primers using ABI PRISM Big Dye Terminator v. 3.1 Kit (PE Applied BioSystems), according to the manufacturer's recommendations, on an ABI PRISM 3730 DNA Analyser (PE Applied BioSystems). All singletons and even new/rare mutation identified were confirmed by PCR and re-amplification for three times followed by re-sequencing.

2.3 Computational analysis

The SNPs identification in each individual sample was achieved after alignment with the reference sequences AF418271 and Z30175 for the fragments 1 and 2 (http://www.ncbi.nlm.nih.gov/GenBank). We have defined the DNA sequence of the transcription starting site of the human IL-10 gene from the first nucleotide immediately preceding the A of the ATG taken position -1 (Eskdale et al, 1997), through SeqScape v.s2.6 software (Applied Biosystem www.appliedbiosystems.com). Haplotype reconstruction were achieved through the of Software PHASE v.s2.1.1, development by Yongtao Guan and Matthew Stephens from University of Chicago department of human genetics and department of Statistics.

2.4 Statistical analysis

Pair-wise linkage disequilibrium was also tested for the loci studies. The Hard-Weinberg equilibrium using x^2 test. Statistics were performed in XLSTAT 2008.7 (Addinsoft Software Inc - New York USA). The magnitude of the associations was estimated by odds ratio values. All tests were performed at the 0.05 level of significance. Epi Info version 3.5.1 2008, (Centers for Disease Control and Prevention, USA) regarding the value of 5% ($p < 0.05$) as threshold for statistical significance in the association study of haplotypes between the numbers observed and expected separately in patients and contacts.

3. Results

Two epidemiological model of study were adopted in this work and the study population was stratified according the different models. Expected genotype frequencies were calculated from respective single allele frequencies and were consistent with Hard Weinberg Equilibrium using x^2 test.

3.1 SNPs profile of the human IL-10 promoter among Brazilian subjects

Using the direct PCR sequencing approach, analysis of the 492 DNA samples enrolled in this study revealed the presence of thirteen SNPs within *IL-10* promoter region, seven of which not reported yet and located at positions (-537G>T, -633G>A, -637G>C, -750C>T, -840A>C, -1162A>G/T and -1189C>T) respectively. The six remaining SNPs found, already deposited in GenBank-Entrez SNP database, were located at positions (-1117G>A, reported as -1082, -886G>A, -854C>T , reported as -819, -692G>A, -627C>A, reported as -592

Locus	Genotype	Subjects (N=492)	Absolute Frequency	Allele Frequency
-1189*	CC	483	0,982	**0,0090**
	CT	9	0,018	
	(fa) T	9	-	
-1162*	AA	485	0,986	**0,0060 (G)** **0,0030 (T)**
	AG	2	0,004	
	AT	3	0,006	
	GG	2	0,004	
	(fa) T	3	0,006	
	(fa) G	4	0,008	
-1117	GG	55	0,112	**0,6780**
	GA	207	0,421	
	AA	230	0,467	
	(fa) A	667	0,888	
-886	GG	464	0,943	**0,0295**
	GA	27	0,055	
	AA	1	0,002	
	(fa) A	29	0,057	
-854	CC	198	0,402	**0,3700**
	CT	224	0,455	
	TT	70	0,142	
	(fa) T	364	0,597	
-840*	AA	484	0,984	**0,0080**
	AC	8	0,016	
	(fa) C	8	-	
-750*	CC	473	0,960	**0,0213**
	CT	17	0,034	
	TT	2	0,004	
	(fa) T	21	0,038	
-692	GG	475	0,965	**0,0170**
	GA	17	0,035	
	(fa) A	17	-	
-637*	CC	487	0,989	**0,0050**
	CG	5	0,011	
	(fa) G	5	-	
-633*	GG	490	0,996	**0,0020**
	GA	2	0,004	
	(fa) A	2	-	
-627	CC	192	0,390	**0,3770**
	CA	229	0,465	
	AA	71	0,144	
	(fa) A	371	0,610	
-537*	GG	491	0,998	**0,0010**
	GT	1	0,002	
	(fa) T	1	-	

Locus	Genotype	Subjects (N=492)	Absolute Frequency	Allele Frequency
-464	GG	479	0,973	**0,0130**
	GT	13	0,026	
	(fa) T	13		

Table 1. Genotype and allele frequencies of SNPs within *IL-10* promoter in Brazilians from Rio de Janeiro.

and -464G>T. Upon genotype and allele frequency analysis, the three more frequent SNPs were the ones located at positions -627C>A, -854C>T and -1117G>A (Table1). Analysis of linkage disequilibrium (LD) showed that only two, SNPs -627C>A and -854C>T out of the thirteen SNPs identified in the total sample were in LD.

3.2 Haplotypes characterization

Haplotypes	*-1189*	*-1162*	*-1117*	*-886*	*-854*	*-840*	*-750*	*-692*	*-637*	*-633*	*-627*	*-537*	*-464*	*Alleles*	Frequency
1	C	A	G	G	C	A	C	G	C	G	C	G	G	265	0,270
2	**T**	A	G	G	C	A	C	G	C	G	C	G	G	2	0,002
3	C	**T**	G	G	C	A	C	G	C	G	C	G	G	2	0,002
4	C	A	**A**	G	C	A	C	G	C	G	C	G	G	274	0,280
5	C	A	G	**A**	C	A	C	G	C	G	C	G	G	28	0,028
6	C	A	**A**	G	**T**	A	C	G	C	G	C	G	G	10	0,010
7	C	A	**A**	G	C	**C**	C	G	C	G	C	G	G	2	0,002
8	C	A	G	G	C	A	**T**	G	C	G	C	G	G	6	0,006
9	C	A	**A**	G	C	A	**T**	G	C	G	C	G	G	2	0,002
10	C	A	G	G	C	A	C	**A**	C	G	C	G	G	2	0,002
11	C	A	**A**	G	C	A	C	**A**	C	G	C	G	G	2	0,002
12	C	A	G	G	C	A	C	G	**G**	G	C	G	G	2	0,002
13	C	A	G	G	C	A	C	G	C	G	**A**	G	G	1	0,001
14	C	A	**A**	G	C	A	C	G	C	G	**A**	G	G	11	0,011
15	C	A	G	G	**T**	A	C	G	C	G	**A**	G	G	2	0,002
16	C	A	**A**	G	**T**	A	C	G	C	G	**A**	G	G	311	0,316
17	**T**	A	**A**	G	**T**	A	C	G	C	G	**A**	G	G	7	0,007
18	C	**T**	**A**	G	**T**	A	C	G	C	G	**A**	G	G	1	0,001
19	C	A	**A**	G	**T**	**C**	C	G	C	G	**A**	G	G	6	0,006
20	C	A	G	G	C	A	**T**	G	C	G	**A**	G	G	1	0,001
21	C	A	**A**	G	**T**	A	**T**	G	C	G	**A**	G	G	10	0,010
22	C	A	G	G	C	A	C	**A**	C	G	**A**	G	G	2	0,002
23	C	A	G	**A**	C	A	C	**A**	C	G	**A**	G	G	1	0,001
24	C	A	**A**	G	**T**	A	C	**A**	C	G	**A**	G	G	10	0,010
25	C	A	**A**	G	**T**	A	C	G	**G**	G	**A**	G	G	2	0,002
26	C	A	**A**	G	**T**	A	C	G	C	**A**	**A**	G	G	2	0,002
27	C	A	G	G	C	A	C	G	C	G	C	**T**	G	1	0,001
28	C	A	**A**	G	C	A	C	G	C	G	C	G	**T**	7	0,007
29	C	A	G	G	C	A	**T**	G	C	G	C	G	**T**	2	0,002
30	C	A	**A**	G	**T**	A	C	G	C	G	**A**	G	**T**	3	0,003
31	C	A	**A**	G	C	A	C	G	**G**	G	**A**	G	**T**	1	0,001
32	C	**G**	**A**	G	C	A	C	G	C	G	C	G	G	6	0,006

Table 2. Haplotypes description and frequencies

3.3 Association of the IL-10 SNPs and TB outcomes

Frequencies of the *IL-10* polymorphisms in patients affected by active tuberculosis (n=221) and healthy controls TST+ (n=271) were compared among stratified groups to evaluate the possible association of the identified SNPs and the outcomes of susceptibility *per ser* to the occurrence of active TB, and disease severity. Table 3 shows that the most studied and well characterized SNPs at positions -1117, -854, -627, (also reported respectively as -1082, -819 and -592), did not show any association with the studied TB outcomes. Additionally, any of the remaining 10 SNPs showed to be associated with TB occurrence at neither genotype nor allele level.

Locus	Genotype	Pacients N=221	Controls TST+ N= 126	χ^2	*p*-valor	OR
-1189	CC	212	126	5,26	0,022	#
	CT	9	0			
-1162	AA	217	125	1,16	0,763	#
	AG	1	0			
	AT	2	1			
	GG	1	0			
-1117	GG	24	12	1,20	0,547	#
	GA	100	51			
	AA	97	63			
-886	GG	208	123	2,23	0,135	0,39
	GA	13	3			
-854	CC	89	45	1,67	0,432	#
	CT	100	66			
	TT	32	15			
-840	AA	213	126	ND	ND	ND
	AC	8	0			
-750	CC	221	114	ND	ND	ND
	CT	0	10			
	TT	0	2			
-692	GG	215	121	0,41	0,521	1,48
	GA	6	5			
-637	CC	216	126	ND	ND	ND
	CG	5	0			
-633	GG	221	125	ND	ND	ND
	GA	0	1			
-627	CC	85	44	0,95	0,619	#
	CA	102	65			
	AA	34	17			
-537	GG	220	126	ND	ND	ND
	GT	1	0			
-464	GG	211	123	1,02	0,311	0,514
	GT	10	3			

Table 3. Genotype distribution of the *IL-10* SNPs among TB patients and healthy controls (TST+)

Locus	Genotype	Pacients N=221	Controls (TST+/TST-) N= 271	χ^2	*p*-valor	OR
-1189	CC	212	271	**ND**	**ND**	**ND**
	CT	9	0			
-1162	AA	217	269	0,621	0,892	#
	AG	1	1			
	AT	2	1			
	GG	1	1			
-1117	GG	24	31	1,699	0,428	#
	GA	100	107			
	AA	97	133			
-886	GG	208	256	0.931	0.638	#
	GA	13	14			
	AA	0	1			
-854	CC	89	109	0,025	0,988	#
	CT	100	124			
	TT	32	38			
-840	AA	213	271	**ND**	**ND**	**ND**
	AC	8	0			
-750	CC	221	252	**ND**	**ND**	**ND**
	CT	0	17			
	TT	0	2			
-692	GG	215	260	0,659	0,417	1,51
	GA	6	11			
-637	CC	216	271	**ND**	**ND**	**ND**
	CG	5	0			
-633	GG	221	269	1,637	0,200	**#**
	GA	0	2			
-627	CC	85	107	0,299	0,861	#
	CA	102	127			
	AA	34	37			
-537	GG	220	271	ND	ND	#
	GT	1	0			
-464	GG	211	268	**5,528**	**0,019**	**4,23**
	GT	10	3			

Table 4. Genotype frequencies of the *IL-10* SNPs among TB patients and healthy controls (TST+/TST-)

Genetic diversity of the IL-10 gene, mainly in the promoter region has been studied in several populations and many studies have been associated polymorphisms in this region with differential IL-10 production. Among the sixty already described SNPs, the ones at positions -1117 G>A, -854C>T, -627C>A, also described as (-1082, -819 and -592) are the better characterized and taken as haplotypes are related to IL-10 production. The allele G at -1117, and haplotypes containing this allele have been associated with high IL-10 production, while the allele A and the haplotype ATA have been associated with low IL-10 production. Even so, association studies have been shown conflicting results depending on several factors,

including differences in laboratory experiments and ethnicity of the studied populations. As a result, different kinds of associations with immune response and diseases outcomes are commonly reported. Here, no association was found of any between the already described SNPs within IL-10 promoter with TB. This result corroborates at least in part with previous association study of these well characterized SNPs with leprosy, in which, only the only the -854C>T (-819) showed associated with leprosy susceptibility in Brazilians residents in Rio de Janeiro (Santos et al, 2002). No data concerning TB association are available in Brazil.

Locus	Genótipo	TBP (N=139)	TBE (N=43)	χ^2	*p*-valor	OR
-1189	CC	136	39	**4,532**	**0,033**	**0,215**
	CT	3	4			
-1162	AA	137	42	3,854	0,278	#
	AG	0	1			
	AT	1	0			
	GG	1	0			
-1117	GG	19	2	2,619	0,270	#
	GA	62	21			
	AA	58	20			
-886	GG	130	42	1,089	0,297	#
	GA	9	1			
-854	CC	61	16	1,193	0,551	#
	CT	58	22			
	TT	20	5			
-840	AA	134	42	0,010	0,565	0,640
	AC	5	1			
-692	GG	134	43	ND	ND	ND
	GA	5	0			
-637	CC	137	41	1,577	0,209	0,290
	CG	2	2			
-627	CC	60	14	1,802	0,406	#
	CA	59	23			
	AA	20	6			
-537	GG	139	42	ND	ND	ND
	GT	0	1			
-464	GG	134	39	2,274	0,132	0,363
	GT	5	4			

The influence of demographic characteristics such as gender and age in the studied outcomes were also evaluated, no significant difference was found for gender or age. (data not shown).

Table 5. Genotype distribution of the IL-10 SNPs among patients with pulmonary and extrapulmonary tuberculosis (TBP and TBE)

When genotype association was evaluated by comparison of the frequencies of TB patients against healthy controls without stratification by TST response (TST+/TST-), a significant increased frequency of the heterozygous -464 GT, showed to be associated with susceptibility *per se* to the occurrence of active TB (OR 4,23, *p* value 0,019). An interesting finding was the observation of the SNPs -750C>T and -633G>A, only the group of health

controls and on the contrary, the SNPs at positions -1189C>T, -840A>C, -637C>G and -537G>T, present only in the group of TB patients (Table 4).

Assessment of the possible evaluation of the *IL-10* SNPs found with the outcome of disease severity, were achieved by comparing the frequencies found in the group of pulmonary TB patients (TBP) and the group of extrapulmonary TB patients (TBE). Table 5 shows a significantly higher frequency of the allele variant -1189T in the group of patients with extrapulmonary TB in comparison with pulmonary TB suggesting an association of this allele variant with the outcome of severity. No statistically significant differences between the groups were observed for the other SNPs evaluated.

After haplotyping reconstruction and identification of thirty two different haplotypes, only the three more frequent, respectively, haplotype 1 (CAGGCACGCGCGG), haplotype 4 (CAAGCACGCGCGG) and haplotype 16 (CAAGTACGCGAGG) with frequencies of 27, 28 and 31.6 % respectively were considered for the association study. No haplotype association was found for any of the evaluated outcomes.

4. Conclusions

In this study, using a PCR direct sequencing approach, seven new SNPs and six already described were identified, in a region of one thousand and fifty hundred base pairs, proximal to the transcription starting site of IL-10 coding gene.

Evaluation of the possible association of the identified SNPs with different TB outcomes by comparison of allelic, genotype and haplotype frequencies between groups showed that none of the new SNPs were associated neither with susceptibility nor with severity. However, an interesting finding is that three of the new SNPs were present only among patients and one, only in the group of controls. Although it can suggest an association with susceptibility *per se* to TB occurrence and resistance to TB respectively, it is not conclusive. The functional role of these SNPs should be elucidated and sample size should also be increased.

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Part 2

Manipulating the Immune Responses to Favor the Host

16

Vaccines Against *Mycobacterium tuberculosis*: An Overview from Preclinical Animal Studies to the Clinic

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1. Introduction

More than a decade ago the World Health Organization (WHO) declared tuberculosis (TB) a global emergency and called on the biomedical community to strengthen its efforts to combat this scourge. The WHO predicts that by 2020 almost one billion people will be infected, with 35 million dying from the disease if research for new approaches to the management of this disease is unsuccessful (1). Designing a better TB vaccine is a high priority research goal. This chapter will review the various strategies currently being used to prevent and treat TB. In spite of the numerous new vaccine candidates in clinical trials, and several others in the preclinical pipeline, no clear TB vaccine development strategy has emerged.

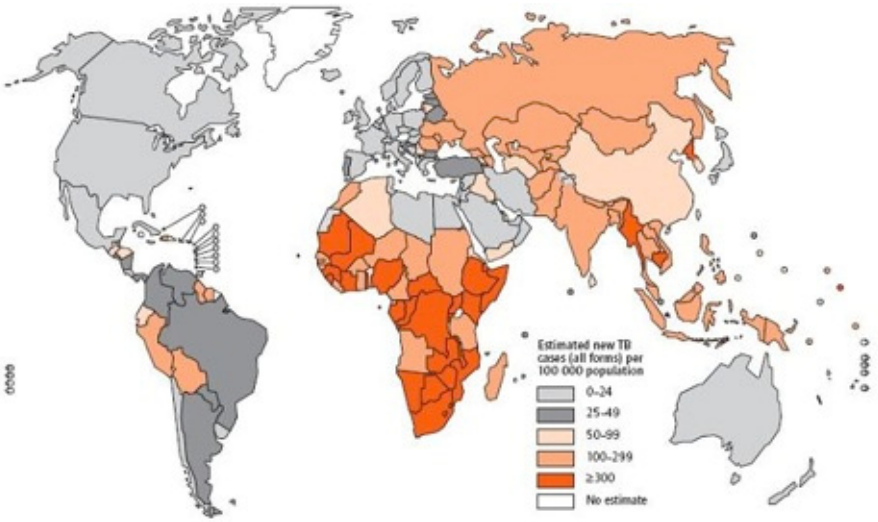

Fig. 1. Estimated TB incidence rates, by country, 2009 [http://para410.com/biophysical(2)].

Despite TB control programs, *Mycobacterium tuberculosis* (Mtb), a facultative bacterial pathogen, remains the most common cause of infectious disease-related mortality

worldwide. Nearly 2 billion people are estimated to be infected with TB. Figure 1 shows the global distribution of TB incidence rates in 2009. Nearly 10 million individuals developed active TB globally (range, 8.9 million–9.9 million; equivalent to 137 cases per 100,000 population), and 1.7 million HIV-negative and HIV-positive people died of TB or related complications (3). TB has now become the leading cause of death in HIV-positive patients and is thought to accelerate the progression of HIV disease (4). Worldwide, 1 in every 3 people is infected with Mtb (5) and may harbor *Mycobacterium* bacilli in their lungs, thus serving as an important reservoir (6). Most of these TB cases occur in India, China, Africa and Indonesia, where 1 in every 8 deaths is a result of TB (7).

Resistance to single anti-mycobacterial agents has long been recognized. Fortunately, the standardized use of multiple agents to treat active disease and the common use of directly observed therapy (DOT), where a health care worker ensures chemotherapy regimens are taken by patients as recommended, have made a significant impact on mitigating treatment regimens and mortality. Unfortunately, the evolution of drug resistance has led to the emergence of TB strains resistant to multiple agents, including those medications used as standard first-line therapies. Fifty million of those infected have multi-drug resistant (MDR)-TB, a disease caused by Mtb strains that are resistant to both isoniazid and rifampicin with or without resistance to other first-line drugs. The incidence of MDR-TB is rapidly growing, and the total number of estimated cases has steadily increased. The estimated global incidence of MDR-TB was 275,000 cases in 2000 and 440,000 cases in 2008 (8, 9). Nevertheless, the true prevalence of MDR-TB is likely under-recognized as many developing countries endemic for TB lack appropriate lab facilities, diagnostic resources and epidemiological capabilities (10). MDR strains do not appear to cause disease more readily than their drug sensitive counterparts, but HIV-positive individuals infected with MDR-TB have higher mortality rates, perhaps because HIV infection causes a malabsorption of TB drugs. This, and the fact that MDR-TB can require 24 months or more of drug therapy compared to 6-9 months for drug sensitive strains, can lead to acquired drug resistance and up to a 300-fold increase in drug costs (11).

Since the discovery of MDR-TB in the 1990s, the resistance pattern of TB has continued to evolve, and isolates resistant to both first- and second-line agents, termed extensively drug-resistant TB (XDR-TB), have been identified. Like MDR-TB, XDR-TB has been identified worldwide and now represents 2% of all cases of culture-positive TB (10).

Societal costs associated with MDR-TB are higher than for drug-susceptible TB due to longer hospitalization, longer treatment with more expensive and toxic medications, greater productivity losses, and higher rates of treatment failure and mortality. There have been recent reports of greater than 20% and 80% mortality attributable to MDR-TB and XDR-TB, respectively, with less than 60% of disease free MDR-TB patients after a mean drug treatment period of four years (12). In the U.S., where there are on average 300 newly reported cases of MDR-TB annually, this disease is very expensive to treat and current estimates suggest it is more than ten times as expensive as drug-sensitive infections (13-15).

2. BCG…then and now…

The bacille Calmette-Guérin (BCG) vaccine, derived from an attenuated strain of *Mycobacterium bovis*, has been used to vaccinate over 3 billion people throughout the world

for more than 80 years since 1928. BCG lacks the genomic 'Region of Difference' (RD1) which encodes the ESX-1 secretion system, including the immunodominant 6-kDa Mtb antigen ESAT-6, included in the Hybrid 1 (ESAT-6/Ag85) vaccine (described in more detail in a later section of this chapter) and in IFN-γ release assays (IGRA's) used to diagnose Mtb (16, 17). The overriding dogma is that BCG protects against primary childhood TB, but its role in consistently protecting against adult pulmonary disease is minimal (18). Indeed, the efficacy of BCG in several field trials has been variable (19). The suggested reasons for the variability observed include differences in the BCG strains – resulting from inconsistent laboratory culture conditions which caused gene deletions or attenuated organisms (20), poor handling of the vaccine, doses and vaccination schedules in the various field trials (21), interference from environmental mycobacteria (22-24), and poor nutrition or genetic variability in the populations immunized (25, 26). Several analyses have identified genetic changes within some BCG substrains such as in the *pho*P-*pho*R system that has occurred along the way since BCG Pasteur was first derived.

Except in cases where infants are HIV-seropositive, BCG is considered safe. This has led to development of other vaccines that either enhance the immune responses resulting from BCG immunization, for example by insertion of specific genes present in virulent *M. tuberculosis* but which have been lost in the avirulent BCG vaccine - the recombinant forms of BCG (rBCG) - or, more broadly, are capable of boosting the effects of BCG. Recent studies have demonstrated that the new rBCG vaccines are more immunogenic, inducing effector and memory T cells, however one potential concern is that many of these rBCGs encode antigens such as Ag85A, CFP-10 etc. that are immunodominant. Recent data suggest that these antigens are highly conserved and are used by the bacteria as a ploy to cause damage in the lungs resulting in escape of the mycobacteria bacilli and increased transmission. It is important to demonstrate whether the new rBCGs can protect against clinical strains. Furthermore, because BCG is designed to be administered only once, none of the rBCG strategies are likely to yield a successful vaccine superior to what we have now.

Over the last 10 years more than 170 TB vaccine candidates have been tested in mouse, guinea pig or non-human primate models of TB (27-31). These include: (i) subunit vaccines consisting of mycobacterial preparations (32-34), culture filtrates (CF) or secreted molecules (35-39), proteins (40-53), lipoglycoproteins (54), and glycolipids (55-57); (ii) DNA vaccines (58-72); (iii) live, attenuated, nonpathogenic/auxotrophic or recombinant bacteria (73-81); and (iv) attenuated, nonmycobacterial vectors such as *Salmonella* or *Vaccinia* virus (77, 82-87). In addition, attempts at improving BCG by administering lower doses (88-90), oral delivery (91), and prime/boost protocols are being explored (59, 85, 92-94). Currently, several candidate vaccines are being prepared for testing primarily as pre-exposure vaccines in humans (27, 95, 96).

Vaccine approaches currently in clinical trials also include altered forms of BCG to increase the effectiveness of the treatment. One of the vaccines, rBCG30, is an engineered form of BCG (rBCG) that over expresses Ag85B (97). It has shown much greater efficacy than the parental Tice BCG vaccine, perhaps due to loss of virulence in the current BCG vaccines, and was shown to increase Ag85B-specific T cell proliferation and IFN-γ responses in humans (97). Another rBCG in human clinical trials is a rBCG that is a urease-deficient mutant that expresses the lysteriolysin O gene from *Listeria monocytogenes* (98). Using this approach the vaccine increases phagosomal acidification in the absence of the ureC enzyme,

while expressing the lysteriolysin protein, Hly, which requires an acidic pH within the phagosome in order to damage/perforate the phagosomal membrane. This process allows the release of antigen into the cytoplasm and induces macrophage apoptosis, leading to enhanced $CD8^+$ T cell presentation through a cross-priming strategy. Other whole virus vaccine approaches have seen some success against TB. One, based on a recombinant modified vaccinia virus Ankara (MVA) vaccine which expresses the Mtb protein Ag85A, is currently in clinical trials (99). However, the complex nature of TB infections may very well require multiple weapons in our armamentarium. These may include not only the use of multiple Mtb antigens but also vaccines based on other adjuvant and delivery platforms.

A post-exposure vaccine, to be used in healthy individuals infected with Mtb or those recently exposed to MDR-TB, could also reduce the probability of going on to develop TB disease. It could work by limiting bacteria that cause TB or MDR-TB, that are residing in a dormant state, by preventing reactivation and/or by reducing the chance of reinfection by exogenous Mtb. Finally, a therapeutic vaccine could function alone, or alongside antibiotic regimens, for individuals with active TB disease and could potentially shorten the treatment period.

3. Immune responses required for development of a successful TB vaccine…

Advances in our knowledge of resistance to Mtb have emerged since the pioneering work of Mackaness (1960's, 1070's) who demonstrated a dependence on cellular immunity against mycobacterial infection (100, 101). Another key advancement to the development of vaccines against Mtb was made by Orme and Collins (1980's), who were the first to show that transfer of immunity against Mtb could be achieved with antigen-specific CD4 and CD8 T cells, and that metabolically active mycobacteria secreted key immunologically relevant antigens (102-106). A major new idea in the mid-1980's, that has shaped the development of vaccines against many different pathogens, was that of Mosmann with the discovery that there were two types of helper CD4 T cells: Thelper 1 and Thelper 2 cells, that secrete either IFNγ or IL-4 respectively (among other cytokines) (107). More recently, Sallusto et al. have defined memory T cell subsets which can be functionally separated based on their surface receptors, which further advance testing the capability of vaccine induction of long-lived immune responses (108, 109). Although our understanding of an effective immune response against Mtb is far from complete, some fundamentals have been identified, resulting in a number of TB vaccines that are now being tested in humans. Several of these advances in our knowledge of the host's resistance to Mtb are discussed in the remainder of this chapter.

Mycobacteria bacilli usually enter the host through aerosol droplets of 1-3 μM inhaled to the lung alveoli. Some bacilli remain in the lungs and evade adaptive immunity to persist in the lungs, often for the lifetime of the host, and some are transported to draining lymph nodes where dendritic cells (DC) prime T lymphocytes. Mtb undergoes an initial period of uninhibited growth within non-activated host macrophages (110). Cell mediated immunity (CMI) characterized by the expansion of antigen-specific T-lymphocytes that attract monocytes/macrophages to inhibit bacillary growth through the production of cytokines, plays a key role in the control of TB. Persistence of Mtb inside of mononuclear phagocytes and DCs during all stages of infection can occur via many mechanisms including down-regulating major histocompatibility complex (MHC) class II expression or presentation

(111), neutralizing the phagosomal pH, interference with autophagy, and by inducing the production of immunosuppressive cytokines such as interleukin (IL)-10 and tumor growth factor beta (TGF-β)(112-115). Mtb can also inhibit apoptosis through prostaglandin production (116) and can invade the cytosolic compartment (117). Recent data also showed that of the large number of CD4+ effector T cells recruited to the lungs of infected mice, few are stimulated to produce IFN-γ (118).

The hallmark of CMI to Mtb infection is the formation of solid granulomas from aggregates of mononuclear phagocytes and polymorphonuclear granulocytes in the lung with a center of infected macrophages surrounded by a marginal zone of lymphocytes (119, 120). The protective role of granulomas is confinement of bacilli in a space that is lacking in vascularity and alveolar air, preventing both replication and dissemination to other sites. Granulomas also serve as sites for priming of CD4+ and CD8+ T cells as well as germinal center B cells. Primed T cells are reported to be polyfunctional, secreting IFN-γ, TNF and IL-2 cytokines, and of the central memory lineage (Tcm) (121) (Figure 2). Studies in gene-deficient/knock out (KO) mice and through neutralization with antibodies, have demonstrated the importance of IFN-γ (122-131), CD4+, and CD8+ (132-141) T cells in the acquired immune response to Mtb.

$CD4^+$ T cells traffic to the lung within 7-14 days following infection and produce IFN-γ (142, 143). Depletion of $CD4^+$ T cells prior to Mtb infection leads to increased bacterial burden and shortened survival (138) and depletion of this subset in latently infected animals leads to rapid reactivation (144). In sublethally-irradiated mice, passive transfer of $CD4^+$ T cells mediates reduced susceptibility to Mtb infection (145). In contrast, CD4- and MHC Class II-deficient mice are extremely susceptible to Mtb. Finally, clinical conditions that impair $CD4^+$ T cell immunity, such as HIV infection, dramatically increase the likelihood of developing active TB.

Mice deficient in IFN-γ, an effector cytokine which defines Th1-type $CD4^+$ T cells, are highly susceptible to Mtb infection (127, 146). These mice fail to produce nitric oxide (NO) synthase (127) and develop a disseminated form of disease, characterized by irregular granulomas and necrotic areas. Patients in whom the gene for the IFN-γ receptor is mutated are prone to infection with atypical mycobacteria (147). Strong Th1-type, antigen-specific IFN-γ-secreting T cells are found in peripheral blood mononuclear cells (PBMC) from healthy individuals with latent TB infections (LTBI), but are diminished in individuals with pulmonary TB (148, 149). Recent results also indicate that CD4+ effector T cells are activated at suboptimal frequencies in tuberculosis, and that increasing effector T cell activation in the lungs by providing one or more epitope peptides may be a successful strategy for TB therapy (150).

The protective role of TNF in the immune response to Mtb was demonstrated in mice with defects in genes for TNF (151, 152). Its critical role for humans was also revealed by the occurrence of reactivation TB in rheumatoid arthritis patients who received long-term therapy with anti-TNF antibodies (153). Recently, both IL-23 and IL-17 were shown to be essential in the establishment of protective pulmonary CD4+ T cell responses, along with the concurrent expression of the chemokines CXCL9, CXCL10 and CXCL11 (154, 155).

Studies in mice and humans support an important role of $CD8^+$ T cells in TB immunity, particularly during LTBI. Adoptive transfer or *in vivo* depletion of $CD8^+$ cells demonstrated

that CD8+ cells could confer protection against subsequent Mtb challenge, although the effects were less pronounced than those seen with $CD4^+$ T cells (156-158). Mtb can egress into the cytosolic compartment of infected DCs resulting in direct loading of MHC class I (117). Cross-priming, which involves apoptosis of macrophages infected with Mtb, uptake of vesicles carrying Mtb antigens by nearby DC, and antigen presentation of the vesicular antigens by MHC I to CD8 is an additional mechanism by which CD8+ T cells are stimulated (159). Mice deficient in class I processing and presentation, including deficiencies in β2 microglobulin (160, 161), TAP1 (162), CD8 , or Class Ia ($K^{b-/-}/D^{b-/-}$)(163), are all more susceptible to Mtb infection than wild-type animals. In humans, Mtb-specific $CD8^+$ T cells have been identified in Mtb-infected individuals and include $CD8^+$ T cells that are classically (164-169), non-classically (170, 171), and CD1 restricted (172, 173).

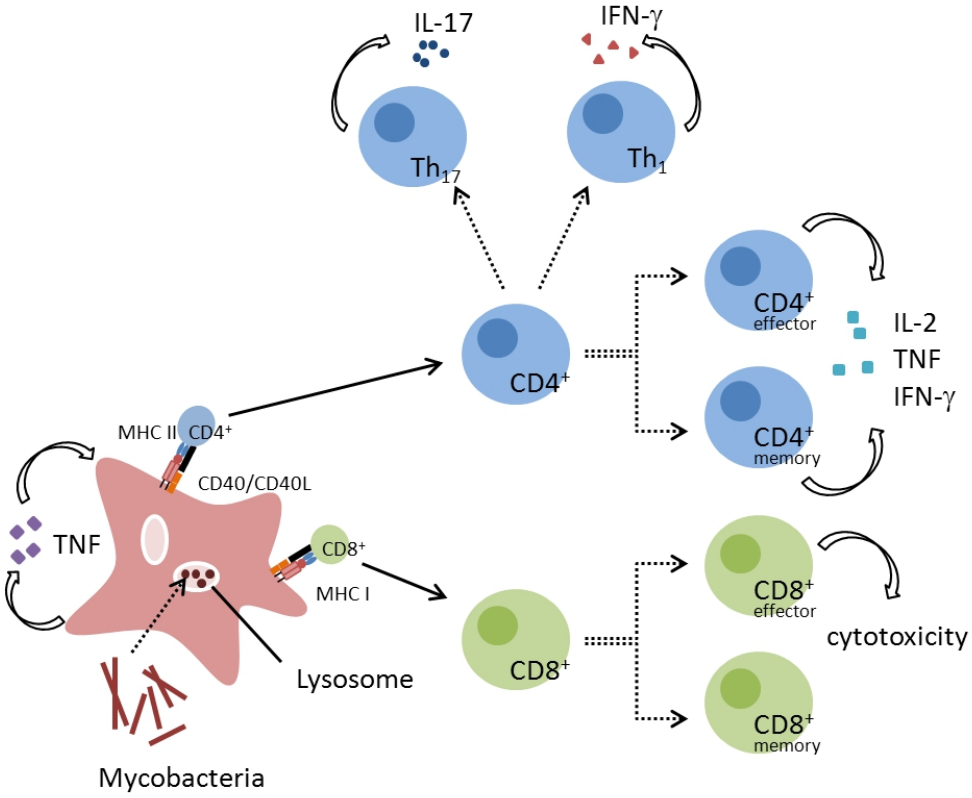

Fig. 2. The Cellular Host Response to TB. After infection of the host lung, macrophages and DCs infected with Mtb stimulate CD4+ and CD8+ T cells. CD4+ T cells are polarized into Th1 and Th17 effector cells or memory T cells secreting multiple cytokines including IFN-γ, TNF and IL-2. CD8+ memory T cells may be cytolytic and may secrete TNF and IFN-γ.

Infection with Mtb induces robust T cell responses yet adaptive immunity fails to eradicate *M. tuberculosis*. Mechanisms for the limited efficacy of the adaptive immune response in

tuberculosis are hypothesized to fall into two categories: either the T cell effector functions are not effective because of failed or inappropriate responses induced by the infected cells; or the T cells recruited to the site of infection do not optimally perform the effector functions required for immune clearance. The ability of *M. tuberculosis* to resist and inhibit the TNF and IFN-γ-induced microbicidal responses of the phagocytic cells it infects is one immune evasion strategy in vivo. Another is that only a small fraction of the CD4 + effector T cells in the lungs is activated to synthesize IFN-γ. Identification of the elements of this host-pathogen interaction may lead to the development of therapies that target antigen gene suppression and inhibition of antigen presentation and provide a novel strategy for overcoming bacterial persistence in vivo, leading to better outcomes in Mtb infected individuals.

4. Designing a sub-unit vaccine from start to finish…

This section highlights the development of a new subunit vaccine, ID93/GLA-SE, and briefly discusses the other human TB vaccine candidates in the pipeline (see Table I).

Preclinical studies with a new TB subunit vaccine, ID93/GLA-SE, have been conducted and this vaccine is ready for testing in Phase I human clinical studies. This vaccine now joins 14 others, which are currently being tested in humans (Table I). The selection of the proteins for ID93 involved the generation of an Mtb protein library based on H37Rv proteins that were within the known immunogenic EsX and PE/PPE classes, between 6 and 70 kDa and with low homology with the human genome (less than 30%) (174). A comprehensive analysis was then performed on over 100 potential candidate antigens selected based on genome mining and expression as recombinant proteins. These candidate antigens were then down-selected based on IFN-γ production from human PBMCs in patients that were PPD(+) and which were non-responsive in PPD(-) patient samples. In combination with the TLR9 agonist, CpG ODN 1826, the vaccine candidates were then tested for efficacy in the C57BL/6 mouse aerosol model of Mtb infection. The ID93 fusion protein consists of four selected Mtb proteins: Rv3619, Rv1813, Rv3620, and Rv2608 (the cumulative molecular weights of each individual protein define the "93" in ID93). Three of the proteins are associated with Mtb virulence (Rv2608, Rv3619, and Rv3620) and one with latency (Rv1813). Rv2608 is a member of the PE/PPE family, Rv3619 and 3620 are in the EsX family of proteins and Rv1813 is expressed under hypoxic conditions (174). Similar to other fusion proteins, including Mtb72f, Ag85B-ESAT6, Ag85B-TB10 and H56, the fusion of more than one Mtb antigen leads to increased vaccine efficacy. Another similarity of these subunit vaccines is the need for an adjuvant to elicit maximum efficacy.

The adjuvant selected for use with the ID93 vaccine is a synthetic toll-like receptor (TLR4) agonist called glucopyranosyl lipid adjuvant (or GLA). This molecule has been extensively characterized in many biological systems, including mice, guinea pigs, ferrets (unpublished results), hamsters, non-human primates (NHPs) and humans (52, 175, 176). Early on, the Mtb72F subunit vaccine, in Phase II human clinical trials, included AS02A as its adjuvant. AS02A consists of a biological TLR4 agonist called monophosphoryl lipid A (MPL), derived from *Salmonella minnesota* mixed with QS21 and an oil-in-water formulation (177).

Other TB vaccine candidates currently in clinical trials include four different categories of vaccines: a) recombinant protein vaccines; b) recombinant live vaccines; c) viral vectored

vaccines; and d) whole cell, inactivated or disrupted mycobacterial vaccines (Table 1). The recombinant subunit vaccines will be briefly described below.

	Protein/Vaccine	Adjuvant
Recombinant Proteins		
M72	fusion protein of Mtb32 and Mtb39 (72kDa)	AS02A: MPL and QS21
Hybrid 1	fusion protein of Ag85B and ESAT-6	IC31 (Intercell): ss oligodeoxynucleotide and peptide (KLKL5KLK)
Hybrid 1	fusion protein of Ag85B and ESAT-6	CAF01: cationic liposomes
HyVac4: AERAS-404	fusion protein of Ag85B and TB10.4	IC31 (Intercell): ss oligodeoxynucleotide and peptide (KLKL5KLK)
Recombinant Live Vaccines		
VPM1002: rBCG(delta)ureC:Hly	urease deficient; expresses listeriolysin (Hly) from *L. monocytogenes*	*NA*
rBCG30 (Tice strain): AERAS-422	rBCG30; overexpresses Ag85B	*NA*
rBCG (AFRO-1 strain): AERAS-422	rBCG30; overexpresses Ag85A, Ag85B and Rv3407 and expresses perfringolysin O	*NA*
Viral Vectored Vaccines		
MVA85A: AERAS-485	MVA (Modified vaccinia virus Ankara) expressing Ag85A	*NA*
Crucell Ad35: AERAS-402	Ad35 (non-replicating Adenovirus 35) expressing Ag85A, Ag85B and TB10.4	*NA*
Ad5Sg85A	Ad5 (non-replicating Adenovirus 5) expressing Ag85A	*NA*
Whole Cell Inactivated or Disrupted Vaccines		
M. vaccae	Inactivated whole cell mycobacteria	*NA*
Mw [*M. indicus pranii* (MIP)]	Whole cell saprophytic mycobacteria	*NA*
RUTI	Fragmented *M. tuberculosis* cells	*NA*
M. smegmatis	Whole cell extract	*NA*

Table 1. TB vaccines in human clinical trials (178), [TB vaccine candidates-2010; www.stoptb.org/wg/new_vaccines(2)].

The M72 (Mtb72F) + AS01 (or AS02A) vaccine was originally developed by Corixa and the Infectious Disease Research Institute (Seattle, WA) and clinical trials are currently being sponsored by GlaxoSmithKline (GSK) and Aeras. This vaccine is a fusion of tandomly linked proteins, Mtb32(C), Mtb39, and Mtb32(N) which showed efficacy in mice, guinea pigs, and NHPs (179-181) and is currently being evaluated in humans. This vaccine includes an AS01 adjuvant (GSK), which comprises the TLR4 agonist, monophosphoryl lipid A (MPL), QS21 and liposomes. In the first phase I clinical trial, Mtb72F combined with the AS02A adjuvant, which includes MPL, QS21, and an oil-in-water emulsion, the vaccine was locally reactogenic but the adverse events were mostly mild and transient and thus had an acceptable tolerability in humans (177). Immunologically, three doses of the Mtb72F/AS02A vaccine (given at 0, 1 and 2 months) induces both humoral and cellular responses in healthy PPD-negative adults (18-40 years of age); IL-2 and IFN-γ is elicited in PBMCs by ELISPOT and increased antigen-specific CD4+ T cells expressing CD40L, IL-2, TNF-α and IFN-γ by intracellular cytokine staining (ICS) are also induced.

The Hybrid-1 vaccine developed by the Statens Serum Institute, includes a fusion of the Mtb proteins antigen 85B and ESAT6. This vaccine, Hybrid 1, which is being evaluated in human clinical trials, is adjuvanted with either the Intercell adjuvant system, IC31 or with a liposomal adjuvant CAF01. CAF01 adjuvant is considered a cationic liposome, and is formulated with quaternary ammonium lipid N, N′-dimethyl-N,N′-dioctadecylammonium (DDA) plus a synthetic mycobacterial cord factor, α,α′-trehalose 6,6′-dibeheneate (TDB) (182-184). The IC31 adjuvant signals through TLR9, and contains the following KLK poly-peptide $KLKL_5KLK$-COOH and a non-CpG oligonucleotide ODN1a, consisting of a phosphodiester backbone ODN, 5′-ICI CIC ICI CIC ICI CIC ICI CIC IC-3′ (185). Both adjuvant systems, CAF01 and IC31, elicit strong Th1 inducing activities and protection in animal models of tuberculosis when combined with the Ag85B-ESAT6 fusion (185-189).

Another subunit vaccine in development by the same group that developed the Hybrid-1 vaccine is the H56 vaccine which includes a fusion of Hybrid 1 and a latency-associated protein, Rv2660c, which is activated during hypoxic conditions (50). The H56 vaccine, formulated in CAF01, shows a 10-fold reduction in lung bacterial load in the mouse model in a head-to-head comparison with their precursor subunit vaccine, the Hybrid 1 vaccine, containing only Ag85B and ESAT6. In addition, the authors demonstrate that the H56 vaccine is capable of protecting against reactivation when tested after Mtb exposure in a modified Cornell mouse model. HyVac4/AERAS-404 combined with IC31 is also in clinical trials, and includes a fusion of the Mtb antigens Ag85B and TB10.4. Replacement of the ESAT-6 protein with TB10.4 in this vaccine, conserves the use of ESAT-6 for diagnostic purposes (16, 190). This vaccine induces polyfunctional CD4 T cells, which express IFN-γ, TNF-α and IL-2, correlating with protective efficacy in the mouse model against Mtb (191) and guinea pig model using a BCG prime/subunit boost strategy (192).

5. Conclusion

Today, an ambitious portfolio of novel vaccines, drug regimens, and diagnostic tools for TB is being supported by various research funding agencies. Mathematical modeling of TB to evaluate the potential benefits of novel interventions under development and those not yet in the portfolio suggest that: neonatal vaccination with an effective portfolio vaccine would

decrease TB incidence by 39% to 52% by 2050, while drug regimens that shorten treatment duration and are efficacious against drug-resistant strains could reduce incidence by 10-27%. Clearly, TB elimination will require one or more effective vaccines. Importantly, new vaccines should have the potential to be effective against clinical strains representing all the major geographical regions.

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Immune Responses Against *Mycobacterium tuberculosis* and the Vaccine Strategies

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1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb). Robert Koch identified Mtb as a causative agent of TB at 1882 (Sakula, 1983). Since then, TB has been one of the most important infectious diseases for human being. According to the WHO report regarding the global burden of TB in 2009, there were 9.4 million incident cases of TB with approximately one third of the world total population being infected (World Health Organization, 2010). Especially, coinfection of Mtb and HIV is a serious issue in sub-Sahara Africa area. Distribution of multi-drug-resistant TB or extensively drug-resistant TB has been another issue in TB.

The epidemiological studies revealed that only 10 to 30% of people who exposed Mtb are infected with Mtb and that 90% or more of the infected people do not develop TB. Only 5% shows the symptoms within 1 year and 95% of the infected individuals are considered to remain infected latently for the long time. Further, only 5% of individuals who have persistently infected Mtb show internal reactivation, i.e., show the overt symptoms the long time later (North and Jung, 2004). These facts indicate that immune responses that are necessary to contain Mtb are induced in the majority of Mtb-infected individuals. Induction of appropriate immune responses against protective Mtb antigens in appropriate stages is necessary for preventing TB. In this chapter, we review the aspect of immune responses and the vaccine strategies against Mtb.

2. Protective immunity against Mtb

2.1 Immune cell effectors against Mtb

Mtb is a facultative intracellular bacterium that survives in phagosomes of alveolar macrophages. In general, effective immune responses against intracellular pathogens are based on the cellular arm (T cells), not on the humoral arm (antibodies) of immune responses (reviewed in Stenger & Modlin, 1999; Kaufmann, 2003). The followings are effective cell subsets that have been considered to be important for protection against Mtb infection.

2.1.1 $CD4^+$ T cells

Intracellular bacteria in phagosomes including Mtb are processed via major histocompatibility complex (MHC) class II-mediated antigen processing pathway and antigens of the bacteria are presented to $CD4^+$ helper T cells (reviewed in Kaufmann, 2003; Flynn & Chan, 2001; Cooper, 2009). Therefore, $CD4^+$ T cells are considered to be the principal effectors against Mtb. Mice that have a deletion in MHC class II or CD4 gene have been shown to be succumbed against Mtb challenge infection (Ladel et al., 1995; Caruso et al., 1999). $CD4^+$ T cells are divided to mainly two subsets depending on the difference of cytokines produced, type 1 helper T cells (Th1) and type 2 helper T cells (Th2). Th1 produces interferon (IFN)-γ, tumor necrosis factor (TNF)- α, and/or interleukin (IL)-2, and contribute to macrophage activation or granuloma formation through effects of these cytokines. IFN-γ has been reported to be the most critical for the protective immunity through analyses of mice or humans which have a deletion in genes encoding IFN-γ or the receptor (Cooper et al., 1993; Flynn et al., 1993; Ottenhoff et al., 1998). Therefore, Th1 has been considered to play a pivotal role for protection against Mtb infection. IFN-γ amounts or the number of IFN-γ-producing T cells has been considered to be a relevant marker for induction of a protective immunity against Mtb (Ellner et al., 2000; Walzl et al., 2011), although it is not the unique marker (Agger & Anderesn, 2001). This cytokine is also able to induce inflammation in the lesion, possibly to help aggravating the disease. Th2 produce IL-4, IL-5, and/or IL-10 and contribute to antibody-mediated immune responses or type 1 allergy development. Therefore, Th2 are considered to have inhibitory effects for protective immunity against Mtb.

2.1.2 $CD8^+$ T cells

Theoretically, $CD4^+$ T cells are major effecter cells against phagosome-localized pathogens. However, $CD8^+$ T cells also have been shown to play an important role in protective immunity against Mtb by analysis of Mtb infection experiments to mice that have deficiency in β2-microglobulin gene critical for MHC class I expression (Flynn et al., 1992; Ladel et al., 1995). Mtb are reported to be cross-presented to $CD8^+$ T cells via MHC class I antigen presentation pathway. Kaufmann's group reported that Mtb-infected macrophages induce apoptotic process and then cross-presentation via apoptotic vesicles (Winau et al., 2006). The process may lead to induction of antigen-specific $CD8^+$ T cells. van Pinxteren and colleagues (2000) reported that $CD8^+$ T cells are particularly important in protective immunity at the latent phase of TB.

$CD8^+$ T cells contribute to protective immunity against Mtb in the following mechanisms (Smith & Duckrell, 2000; Kaufmann & Flynn, 2005). (1) Secretion of cytokines such as IFN-γ and TNF-α. IFN-γ production is essential for $CD8^+$ T-cell mediation of protective immunity against Mtb (Tascon et al., 1998). (2) Lysis of infected host cells through perforin and granzyme B secretion. (3) Direct killing of bacteria infected through granulysin secretion (Stenger et al., 1997). Stegelmann and colleagues (2005) showed that a subset of $CD8^+$ T cells coordinately expresses CC chemokine ligand 5 (CCL5, RANTES), perforin and granulysin, attracts Mtb-infected macrophages and kill the intracellular Mtb (Stegelmann et al., 2005).

2.1.3 Th17 cells

Th17 is a subtype of $CD4^+$ T cells and produce IL-17. Th17 promote migration of Th1, neutrophils, and monocytes to TB lesion in the presence of chemokines and contribute to protective immunity against Mtb (Khader et al., 2007).

2.1.4 CD1-restricted T cells

A subset of T cells is antigen-presented through CD1 molecules, not through conventional MHC class I (Ia) or II molecules. Genes encoding CD1 molecules are mapped outside of MHC and are less polymorphic than conventional MHC genes. CD1-restricted T cells have been reported to recognize glycolipids in mycobacterial cell wall such as mycolic acid (Beckman et al., 1994).

2.1.5. γδ T cells

Most $CD4^+$ and $CD8^+$ T cells express T-cell receptors of α and β protein chains. A minor subset of T cells expresses T-cell receptor of γ and δ protein chains (γδ T cells). γδ T cells tend to distribute in epithelial tissues and contribute to early protection against pathogens invading through epithelium. The phosphoantigen-specific γδ T cells ($V\gamma2V\delta2^+$) displayed major expansion during BCG infection and a clear memory-type response after BCG reinfection in a macaque model (Shen et al., 2002)..

2.1.6 NK cells

NK cells have been associated with early resistance against Mtb (Junqueira-Kipnis et al., 2003). NK cells increased in the lung after aerosol infection of Mtb and produce IFN-γ and perforin. But in vivo NK cell depletion experiment had no influence on bacterial load within the lungs in mouse system, suggesting that NK cells do not play essential roles in early stage of Mtb infection.

2.1.7 Regulatory T cells

Regulatory T cells (Treg) are composed of a subset of T cells that express Foxp3 transcription factor and inhibit effector T-cell responses by both cell-to-cell direct contact and secretion of inhibitory cytokines such as IL-10 or transforming growth factor (TGF)-β. They also constitutively express CD25 molecule, α chain protein of the high affinity IL-2 receptor. So, this T-cell subset can be deleted in vivo with injection of anti-CD25 monoclonal antibody and the depletion experiments confirmed that it has been shown to be critical for prevention of autoimmunity and graft rejection. Treg have been reported to be induced in Mtb-infected mice and humans and this cell subset is considered to be important for the persistence of Mtb infection (Kursar et al., 2007).

2.1.8 Antibodies

In general, humoral immunity does not contribute to induction of the protective immunity against Mtb. However, antibodies specific for heparin-binding haemagglutinin (HBHA) and arabinomannan are reported to contribute to reduce the bacterial load in lung (Teitelbaum et

al., 1998; Pethe et al., 2001). Antibodies specific for antigens presented on the surface of Mtb may have some effect to prevent invading Mtb into the host cells.

2.2 Protective antigens of Mtb

The analysis of genome of Mtb H37Rv strain reveals that 3,985 open reading frames exist in the genome (Cole et al., 1998). Since the report, DNA vaccines are possible to be constructed through the genome information. Information of mycobacterial genes and proteins is available from several comprehensive databases (e.g., The Pasteur Institute TubercuList [http://genolist.pasteur.fr/tuberculist/], The Institute for Genomic Research (TIGR) Comprehensive Microbial Resource (CMR) [http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi]). The most important issue in developing effective TB vaccines is to clarify which genes or gene products are important for the protective immunity against Mtb. The fact that viable Mtb, but not killed Mtb, can induce the protective immunity (Orme, 1988) leads to the speculation that mainly secretory proteins would be the protective antigens. In fact, the majority of the target antigens of the cellular arm of immunity has been reported to be secretory or cell-membrane proteins (Andersen, 1994). Representative protective antigens of Mtb have been clarified by analyses of T-cell responses against protein fractions separated with the electrophoresis analyses (Belisle et al., 2005). The followings are the representative protective antigens of Mtb reported.

2.2.1 Antigen 85 complex proteins

Antigen 85 (Ag85) complex proteins are mycobacterial major secreted proteins of 30 to 32 kDa. The proteins have mycolyl transferase function that is necessary for synthesis of lipid components of mycobacterial cell wall and fibronectin-binding function (Wiker & Harboe, 1992; Belisle et al., 1997). Ag85 complex proteins are composed of Ag85A (p32A), Ag85B (p30, MPT59, α antigen), and Ag85C protein. These proteins are well conserved in *Mycobacterium* genus. MPT51 protein has some homology to these proteins (Nagai et al., 1991; Ohara et al., 1995) and has been shown to be a protective antigen (Miki et al., 2004).

2.2.2 Low-molecular-mass secretory proteins

Low-molecular-mass proteins (less than 20 kDa) in Mtb culture fluid proteins (CFPs) have been reported to be major antigens that evoke T-cell responses (Boesen et al., 1995; Demissie et al., 1999). Among a variety of low-molecular-mass secreted proteins, early secreted antigenic target 6-kDa protein (ESAT6) and culture fluid protein 10 (CFP10) have been well studied (Berthet et al., 1998). Recently, ESAT6 and CFP10 proteins are widely used in whole blood IFN-γ release assays for TB diagnosis (Andersen et al., 2000) (QuantiFERON® TB Gold; Cellestis, Ltd., Carnegie, Victoria, Australia).

2.2.3 Heat shock proteins

A variety of heat shock proteins have been shown to be targets for antibodies and T cells in murine and human systems. Among them, heat shock protein 65 (Hsp65) is a major stress protein Mtb produces in macrophage cells. Mtb Hsp65 protein has more than 50% homology with *Escherichia coli* GroEL or human Hsp60 protein (Lee & Horwitz, 1995). Heat

shock proteins have been shown to be expressed in Mtb in macrophages and induce protective immune responses.

2.2.4 Dormant phase proteins

Aforementioned proteins are expressed mainly in acute phage of TB. A different set of genes are expressed in late infection phase or chronic (dormant) phase of TB. DosR regulon is a unit of genes composed of 48 genes, the expression of which is regulated by DosR (Rv3133c). DosR regulon proteins are the major proteins expressed in chronic (dormant) phase of TB (Karalousis et al., 2004). HspX protein (Rv2031c) is one of the immunodominant antigens belong to DosR regulon proteins. It plays an important role in slowing the growth of Mtb as *hspX* gene-deleted Mtb mutants showed increased growth both in mice and in macrophages (Hu et al., 2006). T-cell responses specific for HspX were found in latent Mtb infection (Demissie et al., 2006). In addition, mycobacterial DNA-binding protein-1 (MDP1) has been shown to be expressed in the late phase of TB and induce humoral and cellular immune responses (Matsumoto et al., 2005; Suzuki et al., 2010). Analysis of dormant phase proteins is critical for development of therapeutic TB vaccines against persistent Mtb infection.

2.3 T-cell epitopes of Mtb proteins

T-cell epitopes are the peptides in antigenic proteins that bind to MHC molecules on antigen-presenting cells. In other words, T-cell epitopes are the peptides that stimulate T cells through the MHC-T-cell receptor interaction.

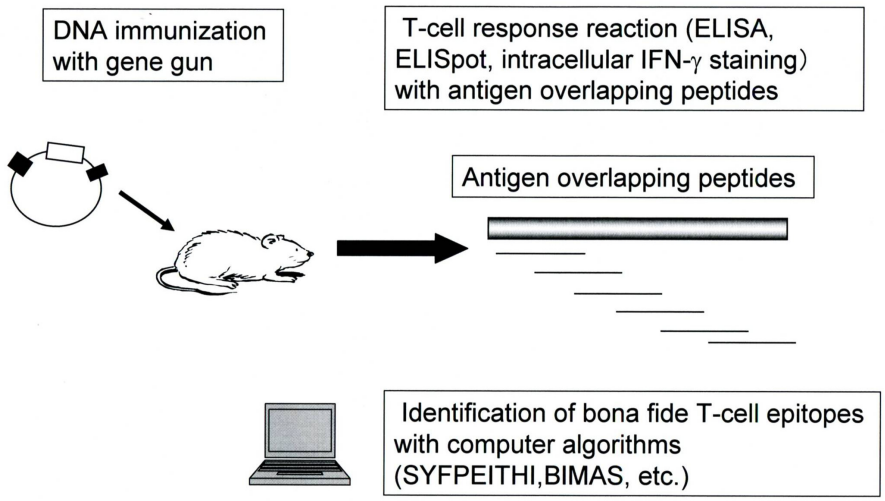

Fig. 1. Shematic diagram for identification of T-cell with DNa immunization

Identification of T-cell epitopes in Mtb antigens is indispensable for accurate analysis of T-cell responses against Mtb antigens with specific MHC tetramers or intracellular cytokine staining. We realized that DNA immunization with gene gun bombardment is an excellent method for identification of Mtb T-cell epitopes as it is highly reproducible and efficiently

Antigen	Epitope peptide	MHC restriction	Reactive T cells	References
Ag85B	p30-38 (9-mer)	A*0201	CD8	Tang et al., 2011
	p183-192 (10-mer)	A*0201	CD8	Geluk et al., 2000
	p239-247 (9-mer)	A*0201	CD8	Geluk et al., 2000
	p10-27 (18-mer)	DR3, 52, 53	CD4	Mustafa et al., 2000b
	p19-36 (18-mer)	Promiscuous	CD4	Mustafa et al., 2000b
	p91-108 (18-mer)	Promiscuous	CD4	Mustafa et al., 2000b
MPT51	p51-70 (10-mer)	A*0201	CD8	Aoshi et al., 2008
	p191-202 (12-mer)	DR4 (Promiscuous)	CD4	Wang et al., 2009
Hsp65	p369-377 (19-mer)	A*0201	CD8	Charo et al., 2001
	p3-13 (11-mer)	DR3	CD4	Geluk et al., 1992
ESAT6	p72-95 (24-mer)	DR52, DQ2	CD4	Mustafa et al., 2000a
HspX	p21-29 (9-mer)	A*0201	CD8	Caccamo et al., 2002
(16kDa Protein)	p120-128 (9-mer)	A*0201	CD8	Caccamo et al., 2002
(DosR regulon)	p91-105 (15-mer)	A*0201	CD8	Geluk et al., 2007
	p31-50 (20-mer)	DR3	CD4	Geluk et al., 2007
Rv1733c	p161-169 (9-mer)	A*0201	CD8	Commandeur et al., 2011
(DosR regulon)				
Rv1733c	p181-189 (9-mer)	A*0201	CD8	Commandeur et al., 2011
Rv2029c	p161-169 (9-mer)	A*0201	CD8	Commandeur et al., 2011
(DosR regulon)				

Table 1. Human T-cell epitopes of Mtb antigens (examples)

induces T-cell responses (Yoshida et al., 2000). Therefore, we have used gene gun DNA immunization method for identification of $CD8^+$ and $CD4^+$ T-cell epitopes of Mtb antigens (Fig. 1). After immunization with plasmid DNA encoding Mtb antigens, immune spleen cells were examined for their IFN-γ responses to overlapping peptides covering full-length Mtb antigens by measuring IFN-γ levels by enzyme-linked immunosorbent assay (ELISA) or by counting the numbers of IFN-γ-secreting cells by enzyme-linked immunospot assay (ELISPOT). We combined these methods with computer algorithms to predict T-cell epitopes. T-cell epitope prediction algorithm programs we used are as follows. They are able to access through their websites. (1) SYFPEITHI Epitope program (http://www.syfpeithi.de/) (Rammensee et al., 1999), (2) the National Institutes of Health Bioinformatics and Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform) (Parker et al., 1994), RANKPEP MHC binding peptide prediction algorithm (http://immunax.dfci.harvard.edu/Tools/rankpep.html) (Reche et al., 2004), and (4) ProPred HLA-DR binding peptide prediction algorithm (http://www.imtech.res.in/raghava/propred/) (Singh & Raghava, 2001). These programs are helpful for narrowing down the amino acid regions of T-cell epitopes. However, the algorithms are still not perfect for exact identification of bona fide T-cell epitopes. A peptide that shows the highest score in these algorithms is not necessarily the best T-cell epitope. Experimental validation is definitely necessary to determine actual T-cell epitopes. A variety of

T cell epitopes of Mtb antigens have been reported. A comprehensive analysis of T-cell epitope data regarding *Mycobacterium* genus in the immune epitope database (IEDB; http://immuneepitope.org) was performed (Blythe et al., 2007). Huygen and colleagues have reported identification of murine T-cell epitopes of Ag 85 family proteins (Ag85A, Ag85B, and Ag85C) (Denis et al., 1998, D'Souza, et al., 2003) using intramuscular DNA immunization. We have used gene gun DNA immunization method for identification of murine $CD8^+$ and $CD4^+$ T-cell epitopes of Mtb antigens including MPT51 (Suzuki et al., 2004), MDP1 (Suzuki et al., 2010), and low-molecular-mass secretory antigens (CFP11, CFP17, and TB18.5) (Eweda et al., 2010).

In addition to murine T-cell epitopes, human T-cell epitopes have been reported (some examples are shown in Table 1). HLA-A02 is the most frequent HLA molecule in Caucasians and HLA-A*0201 represents the most frequent allele. HLA-A*0201-restricted $CD8^+$ T-cell epitopes have been identified in a variety of antigens including those derived from cancers, viruses, bacteria, and protozoan. Mtb-derived HLA-A*0201-restricted $CD8^+$ T-cell epitopes have been reported, including epitopes in Ag85A (Smith et al., 2000), Ag85B (Geluk et al., 2000), ESAT6 (Lalvani et al., 1998), and Hsp65 (Charo et al., 2001).

T-cell epitope (10-mer)	Position 1 2 3 4 5 6 7 8 9 10	
Anchor residues	L, M — L, V, I	
MPT51 p53-62	T **L** A G K G I S V **V**	Aoshi et al., 2008
Ag85B p143-152	F I Y A G S L S A **L**	Geluk et al., 2000
T-cell epitope (9 mer)	**Position 1 2 3 4 5 6 7 8 9**	
Anchor residues	L, M — L,V, I	
Ag85B p30-38	G **L** A G G A A T A	Tang et al., 2011
Ag85B p183-192	K **L** V A N N T R **L**	Geluk et al., 2000
Ag85B p239-247	G **L** A G G A A T A	Geluk et al., 2000
Hsp65 p369-337	K **L** A G G V A V **I**	Charo et al., 2001
HspX (DosR reg) p13-21	L F A A F P S F A	Caccamo et al., 2002
HspX (DosR reg) p120-128	G I L T V S V A **V**	Caccamo et al., 2002
Rv1733c (DosR reg) p161-169	I A D A A L A A **L**	Commandeur et al., 2011
Rv1733c (DosR reg) p181-189	A **L** L A L T R A **I**	Commandeur et al., 2011
Rv2029c (DosR reg) p314-322	E **L** A A E P T E **V**	Commandeur et al., 2011

Table 2. HLA-A*0201-restricted T-cells epitopes od Mtb antigens (examples)

Most HLA-A*0201-restricted T-cell epitopes were nonamer peptides (Falk et al., 1991, Parker, 1994), but some epitopes were decamer peptides. We reported an immunodominant HLA-A*0201-restricted T-cell epitope in MPT51 antigen (Aoshi et al., 2008; Tables 1, 2). Main anchor amino acid positions are, position 2 (Leu) and position 9 (Val), which were conserved in MPT51 p53-62 (TLAGKGISVV) (Table 2). MPT51 p53-62 decamer peptide was capable of binding to HLA-A*0201 and of stimulating $CD8^+$ T cells of HLA-A*0201-transgenic mice, but MPT51 p53-61 nonamer was not. The conformational and electrostatic differences between the nonamer and the decamer would affect their binding affinity to HLA-A*0201 molecule and following T-cell responses. Ruppert and colleagues (1993) studied in detail the role of different amino acid residues on each position of nonamer or decamer peptides for binding to HLA-A*0201 molecule. They suggested that nonamer or decamer peptide has different preference of amino acid residues for binding to HLA-A*0201 molecule. They showed that, for example, Tyr, Phe, Trp residues at positions 1, 3, and 5 in nonamer peptides, Gly residues at positions 4 and 6 in decamer peptides are preferable for binding to HLA-A*0201. According to their speculation, MPT51 p53-62 peptide seems to have better A*0201 binding features than MPT51 p53-61 peptide (Gly residues at positions 4 and 6 in MPT51 p53-62 peptide are suggested to be associated with good A*0201 binding). Interestingly, MPT51 p21-29 peptide (FLAGGPHAV) was not immunogenic in terms of IFN-γ production and cytolytic ability although the peptide showed high affinity to HLA-A*0201 as predicted by MHC binding algorithms (Aoshi et al., 2008). Previous reports showed a strong association between immunodominance and HLA binding affinity (Geluk et al., 1998). But, binding of peptides to the restricted MHC molecules is a prerequisite for T-cell epitopes, but all the peptides which show high affinity binding for MHC molecules are not necessarily immunodominant epitopes.

3. Vaccine strategies against Mtb

3.1 Recombinant BCG vaccines

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the only approved attenuated live vaccine to date against TB. Original BCG strain was reported approximately ninety years ago (1921) by Calmette and Guérin (Bloom & Fine, 1994). Despite the fact that BCG is among the most widely used vaccine throughout the world since then, TB still poses a serious global health threat. Whereas BCG is believed to protect newborns and young children against early manifestations of TB (Rodgrigues et al., 1993), its efficacy against pulmonary TB in adults is still a subject of debate (Bloom & Fine, 1994; Andersen & Doherty, 2005) and was reported to wane with time since vaccination (Sterne et al., 1998). Variable levels of the protective efficacy ranging from 0 to 80% have been reported in different studies (Fine, 1995). Moreover, the viable nature of BCG makes it partly unsafe in case of immunocompromised people such as HIV-infected individuals. This highlights the need to develop more effective, safe and reliable vaccines against TB (Kaufmann, 2010).

To improve the immunoreactivity of conventional BCG vaccines, a variety of recombinant BCG (rBCG) vaccines have been tried and evaluated for the protective efficacy (Stover et al., 1991). One of the problems of conventional BCG vaccines in induction of protective immune responses is that BCG is not effective for induction of $CD8^+$ T-cell responses compared with Mtb. BCG is less effective in MHC class I-mediated antigen presentation, which is prerequisite for $CD8^+$ T-cell induction (Mazzaccaro et al., 1996). To improve $CD8^+$ T-cell

responses of BCG, Kaufmann and colleagues reported recombinant BCG (rBCG) introduced with listeriolysin O (LLO) gene derived from *Listeria monocytogenes* (Hess et al., 1998). *L. monocytogenes* escapes from the phagosomes into the cytoplasm shortly after infection into host macrophage cells. LLO, a thiol-activated cytolysin, has membrane-disrupting capability and plays a pivotal role in this process. The rBCG expressing LLO was reported to improve MHC class I-mediated antigen presentation of co-phagocytosed ovalbumin, suggesting that LLO endows BCG with an improved capacity to stimulate $CD8^+$ T cells. Further, the same group constructed urease C gene (*ureC*)-disrupted rBCG expressing LLO (Grode et al., 2005). Mycobacterial ureC increases pH value in the phagosome. The ureC deficiency induces low pH value in the phagosome, helping the LLO enzyme activity (to disrupt phagosome membranes), which leads enhancement of MHC class I-mediated $CD8^+$ T-cell responses.

Another promising rBCG is BCG overexpressing Ag85B protein (Horwitz et al., 2000). Ag85B is a major mycobacterial secreted protein and has been shown to be a protective antigen. Even though BCG does have endogenous Ag85B, overexpression of Ag85B in BCG was further enhanced the protective ability. In addition to these rBCG, attenuated auxotroph strains of Mtb have been examined (Guleria et al., 1996; Sambandamurthy et al., 2006; Larsen et al., 2009).

3.2 DNA Vaccination against TB

Many reports on DNA vaccination against Mtb have been published since 1996 (Huygen et al., 1996, Tascon et al., 1996). So far, a variety of Mtb antigen genes have been used for DNA vaccines, which include Hsp 65, Hsp 70, Ag85A, Ag85B, and ESAT6 (reviewed in Huygen, 2003 for early studies). DNA immunization with genes encoding dormancy regulon-encoded proteins has also been examined (Roupie et al., 2007). DNA immunization with naked DNA has been shown to efficiently induce cellular as well as humoral immune responses. DNA vaccines in most of these reports use needle injection through intramuscular or intradermal routes although some studies used gene gun (Sugawara et al., 2003). The DNA immunization with needle injection tends to raise predominant Th1 responses which are indispensable for induction of the protective immunity. On the other hand, gene gun DNA immunization is apt to produce "mixed type" (Th1 and Th2; producing IFN-γ and IL-4) T-cell responses which is not necessarily adequate for induction of the protective immunity (Tanghe et al., 2000). The difference is considered to be mainly due to the difference in the amount of antigen produced from the plasmids (high amounts in needle injection and low amounts in gene gun bombardment). Therefore, DNA vaccination with gene gun will need additional factors such as adjuvants for eliciting protective immunity against Mtb (D'Souza et al., 2002; Tollefsen et al., 2002; Li et al., 2006; Zhang et al., 2007).

Naked DNA vaccines have been evaluated as therapeutic TB vaccines as well as prophylactic TB vaccines. Lowrie and colleagues (1999) showed that intramuscular injection of mice with Hsp65 and MPT70 DNA vaccines reduced Mtb numbers in spleens and lungs after Mtb challenge by 1 to two $\log_{10}$ order compared with untreated mice. Further, they showed that three intramuscular injection of Hsp65 DNA vaccine eliminated residual Mtb after chemotherapy (isoniazid and pyrazinamid treatment) and immunosupprssive corticosteroid treatment. However, Orme's group reported that Ag85A DNA vaccine that was shown to induce protective immunity in mice when the vaccine was used as a

prophylactic vaccine, could not give any therapeutic effect on the course of the infection in the lungs in mice earlier infected by Mtb aerosol (Turner et al., 2000). Moreover, they reported that vaccination with DNA encoding hsp60 of *Mycobacterium leprae* induced cellular necrosis throughout the lung granulomas when the DNA was given in a mouse immunotherapeutic model (Taylor et al., 2003). Repique and colleagues (2002) also reported that vaccination with a DNA vaccine cocktail containing ten Mtb antigen genes which had showed significant protective responses in mice could not prevent reactivation of disease in a murine latent TB model. These reports indicate that therapeutic TB vaccines still have room for further studies in terms of safety.

3.3 Improvement in immunization regimen: prime-boost immunization

Evaluation of vaccination has indicated that the repeated injection of the same vaccine has a limitation in terms of its overall immunological effects. Especially, DNA immunization has been reported to induce considerably strong immunological responses in the rodents, but not in the primates including human (Li et al., 1993). Instead of the repeated injection of the same vaccine, the heterologous prime-boost regimen including DNA vaccination, which is primed with naked DNA vaccination and boosted with recombinant viral vectors such as vaccinia virus and adenovirus, has been shown to evoke superior levels of immunity to DNA vaccine or recombinant virus alone (Ramshaw & Ramsay, 2000). The relatively low-level but persistent expression of immunogenic proteins in vivo by naked DNA vaccines has been suggested to be important for priming immunological responses and inducing enhanced cellular immunity (Ramshaw & Ramsay, 2000).

A variety of prime-boost regimens have been examined for Mtb infection (McShane & Hill, 2005). Many investigators examined the regimens in which priming with DNA vaccines and boosting with other immunization strategies. Feng and colleagues (2001) showed that priming with Ag85B DNA vaccine and boosting with BCG vaccine strengthened protective immunity against Mtb induced by BCG vaccine alone in mice. Skinner and colleagues (2003) also reported that priming with ESAT6 andAg85A DNA vaccines and boosting with BCG vaccine enhanced specific IFN-γ production from immune splenocytes compared with that by the DNA vaccine or BCG vaccine alone in mice. Ferraz and colleagues (2004) used DNA vaccines encoding mycobacterial Hsp70, Hsp65, and Apa antigens as priming vaccine and showed that the DNA vaccines enhanced BCG boosting effects. Romano and colleagues (2006) showed that immunization of BALB/c mice with Ag85A DNA vaccine first and boosting with BCG vaccine induced stronger protective immunity against Mtb challenge than that by Ag85A DNA vaccine alone. These results demonstrated that DNA vaccine priming and BCG vaccine boosting enhanced immune responses induced by BCG vaccine alone.

The regimens in which BCG vaccine was used as a priming vaccine also have been tried. As the BCG vaccine has been injected to people all over the world, this regimen seems to be reasonable. Derrick and colleagues (2004) showed that a polyvalent DNA vaccine encoding an ESAT6-Ag85B fusion protein protects mice against a primary Mtb infection and boosts BCG-induced protective immunity. Priming with BCG vaccine and intranasal boosting with MVA85A in mice enhanced Ag85A-specific $CD4^+$ and $CD8^+$ T-cell responses and strengthened protective immunity against aerosol Mtb challenge infection in mice (Goonetilleke et al., 2003). This regimen was reported in humans. McShane and colleagues

(2004) reported that in volunteers who had been vaccinated 0.5 to 38 years previously with BCG, vaccination with MVA85A induced substantially higher levels of antigen-specific IFN-γ-secreting T cells and that at 24 weeks after vaccination, these levels were 5 to 30 times greater than in vaccinees administered a single BCG vaccination.

3.4 Ongoing tuberculosis vaccine projects

A variety of TB vaccines have been evaluated (reviewed in Hoft, 2008; Kaufmann, 2010a, 2010b). WHO have showed a list of TB vaccine candidates (TB vaccine pipeline: http://www.stoptb.org/retooling/). These TB vaccine strategies are based on the prime-boost regimens and the vaccine candidates are categorized into three vaccine groups, namely, (1) priming vaccines, (2) boosting vaccines, and (3) therapeutic vaccines after Mtb infection. Some of vaccine candidates that have been ongoing are shown in Table 3.

Vaccine	Source	Explanation	References
Priming Vaccines			
rBCG30	UCLA (M. Horwitz)/NIAID	Ag85B recombinant BCG	Horwitz et al., 2000
rBCG Δ*ureC:Hly* (VPM1002)	Max Planck Inst. (S. Kaufmann) /VPM/TBVI	Listeriolysin O (LLO) recombinant BCG	Grode et al., 2005, Tchilian et al., 2009
mc^{2}6220, 6221, 6222, 6231	Albert Einstein College of Med.	ΔlysAΔpanCD (lysin/pantothetic acid-erquiring attenuated *M. tuberculosis*	Sambandamurthy et al., 2006
Booster Vaccines			
MVA85A/AERAAS-485	Oxford/Isis/Aeras/Ermergent	Ag85A recombinant Vaccinia Virus	McShane et al., 2001, Goonetilleke et al., 2003, McShcane et al., 2004, Scriba et al., 2010
AERAS-402/Crucell Ad35	Crucell/Aeras	Ag85A, Ag85B, TB10.4 recombinant Adenovirus type 35	Radošević et al., 2007 Abel et al., 2010
GSK M72	GSK/Aeras	PPE family protein Rv1196-Rv0125 fusion protein + AS01 adjuvant	Skeikey et al., 2004
SSI Hybrid I	Statens Serum Inst. (SSI)	Ag85B-ESAT6 fusion protein+IC31 adjuvant	Olsen et al., 2004 Aagaard et al., 2011
SSI HyVac 4/AERAS-404	SSI/Sanori Pasteur/ Intercell/Aeras	Ag85B-TB10.4 fision protein+IC31 adjuvant	Dietrich et al., 2005, Skeikey et al., 2010
rBCG30	UCLA (M. Horwitz)/NIAID	Ag85B recombinant BCG	Horwitz et al., 2000
HVJ-liposome/ Hsp65 DNA+IL-12 DNA	Kinki-chuo Chest Medical Center (Okada)	Hsp65 DNA+IL-12 DNA + HVJ-liposome	Yoshida et al., 2006
Therapeutic Vaccines			
MVA85A	Oxford	Ag85A recombinant Vaccinia Virus	McShane et al., 2001, Goonetilleke et al., 2003, McShcane et al., 2004
Hsp65 DNA vaccine	Cardiff Univ. (D. Lowrie)	hsp65 DNA Vaccine	Lowrie et al., 1999

Table 3. TB vaccine candidates (According to WHO TB Vaccine Pipeline)

BCG vaccines and rBCG vaccines are considered to be priming vaccines. For priming vaccines, BCG overexpressing Ag85B (rBCG30) and *ure*C-deleting BCG expressing lysteriolysin O (Δ*ureC hly*$^+$ BCG) have been evaluated. For booster vaccines, Ag85A recombinant vaccinia virus (MVA85A), Ag85B-ESAT6 fusion protein with adjuvant (SSI Hybrid I), and Ag85B-TB10.4 fusion protein with adjuvant (SSI HyVac4/AERAS-404) have been examined. MVA85A and Hsp65 DNA vaccine are candidate TB vaccine are candidate therapeutic TB vaccines. Human studies using the prime-boost regimens by these TB vaccine candidates have been publishing. Tchilian and colleagues (2009) reported that priming with Δ*ureC hly*$^+$ BCG and boosting with MVA85A induced protective immunity against Mtb infection in mice. The protective effects were much higher in Δ*ureC hly*$^+$ BCG vaccination than that in parental BCG vaccination. MVA85A boost immunization enhanced

Ag85A-specific T-cell responses, but did not affect bacterial numbers in the lung after Mtb aerosol infection. Scriba and colleagues (2010) reported that vaccination with MVA85A in healthy adolescents and children from a TB endemic region, who received BCG at birth, is safe and induces polyfunctional $CD4^+$ T cells co-expressing IFN-γ, TNF-α, and IL-2. Further, Abel and colleagues (2010) reported that vaccination with AERAS-402 (Adenovirus type 35 expressing a fusion protein created from the sequences of Ag85A, Ag85B, and TB10.4) is safe and immunogenic in healthy South African BCG-vaccinated adults.

4. Conclusion

Mtb, a causative agent of TB, is a unique facultative intracellular bacterium. The cellular immunity is essential for protection against Mtb. The main effectors are type 1 $CD4^+$ T cells and $CD8^+$ T cells. IFN-γ produced from them has been considered to be important as biomarker of TB. Induction of appropriate immune responses against protective Mtb antigens in appropriate stages is necessary for preventing TB. Identification of protective Mtb antigens and the T-cell epitopes are critical for clarification of kinetics of TB and development of effective TB vaccines. A variety of TB vaccine strategies have been examined including rBCG and DNA vaccines. Prime-boost strategies with combination of different TB vaccines are promising for prophylactic TB vaccines. Therapeutic TB vaccines for latent TB have also been examined.

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18

Towards a New Challenge in TB Control: Development of Antibody-Based Protection

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1. Introduction

Throughout history tuberculosis (TB) has been a health problem for humanity. In the beginning of civilization when human population densities were sparse, this disease may have been fairly harmless. However, with the increase in population densities, probably from the 17th to 19th centuries, TB took epidemic proportions [1].

Bacille Calmette Guérin (BCG), the only licensed vaccine against TB, has been shown to be effective in preventing meningeal and miliary TB in children. However, the efficacy of this vaccine in preventing adult pulmonary TB is questionable. Despite widespread vaccination with BCG, nearly 2 million people die each year from TB. Furthermore, the World Health Organization no longer recommends BCG vaccination of children with HIV or HIV-positive mothers due to safety concerns, leaving many infants without any protection against this disease. While drug therapies exist to combat TB infection, the implentation of suitable treatment is often difficult in the countries hardest hit by the disease and a fact complicated by the limited effectiveness of the current therapeutic schemes at treating drug resistant strains of TB [2-4].

Nowadays there is an increasing realization of the need of new animal models to test vaccine efficacy in more realistic scenarios overcoming the limitations of the current models in use. In addition, the elucidation of the significance of humoral defense against intracellular pathogens, in particular against *Mycobacterium tuberculosis*, constitutes an exciting new approach to improve the rational design of new vaccines, therapies and diagnostics.

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2. Reshaping the classical paradigm

In order to develop improved vaccines and new methods for the control of TB, an important element is the discovery of markers to measure the effector mechanisms of the protective immune response against *M. tuberculosis*. For many years Cellular Mediated Immunity (CMI) was attributed as the exclusive defence mechanism against intracellular pathogens. The Th1/Th2 classical paradigm prevailed for a long time and directed the development of vaccines according to this theory [5].

Based on this point of view, only intracellular pathogens could be effectively controlled by granulomatous inflammation induced by a Th1 response whereas a Th2 response induces antibody production that control extracellular pathogens and parasites. However, the question arises of what really constitutes the true demarcation between "extracellular and intracellular"? In the infectious cycle of several intracellular pathogens, they could be found in the extracellular space and *vice versa*. In the specific case of *M. tuberculosis*, it can be localized extracellularly at the beginning of the infection in the upper respiratory tract as well as in advanced stages of the disease after the rupture of granulomatous lesions [6]. In the case of *Erhlichia* spp specific antibodies could mediate protection against [7], possibly by blocking cellular entry or promoting the expression of proinflammatory cytokines [8;9]. It has been demonstrated that this obligate intracellular pathogen has also an extracellular phase that may include replication which could be targeted by specific antibodies [10].

For certain viral pathogens, the induction of Antibody Mediated-Immunity is sufficient to prevent infection, as has been clearly demonstrated by the almost complete eradication of smallpox with the use of vaccines that elicited antibody-mediated immunity [11]. There are several prokaryotic and eukaryotic intracellular pathogens for which antibody have been shown to modify the course of infection by different mechanisms, as reviewed extensively by Casadevall and colleagues [12-14]. Nowadays, it is well established that an efficient combination of both humoral and cellular immune mechanisms could be the best choice to control certain diseases produced by intracellular pathogens [15;16].

In 2005, de Valiere and colleagues reported for the first time that human antimycobacterial antibodies stimulates the Th1 response instead of diminishing it, as was thought previously [17].

3. Protective role of antibodies: Epidemiological evidence

There is accummulated evidence in the last few decades on the influence of antibodies in the development of pulmonary or disseminated TB. Children with low serum IgG against sonicated mycobacterial antigens and LAM, or those who cannot mount antibody responses to these antigens, were predisposed to dissemination of the bacteria [18]. In another report, Kamble and colleagues reported that *M. leprae* reactive salivary IgA antibodies could be quite important in a mucosal protective immunity [19]. In one study carried out on the Mexican Totonaca Indian population, the presence of high antibody titers to Ag85 complex antigens were observed in patients with non-cavitary TB and in patients who were cured with anti-TB chemotherapy. In contrast, patients without such antibodies had a poor outcome of the disease [20].

4. Experimental studies

4.1 Animal models for the evaluation of the role of antibodies in TB infection

One important criterion for the evaluation of the role of specific antibodies in the protection against TB is the use of animal models. Currently, there is no optimal model to reproduce the infection as it occurs in humans [21]. Several animal models have been used to evaluate different aspects. One crucial aspect is the delivery of inoculums, where several routes of inoculation have been employed as intravenous, intraperitoneal, intranasal, aerosol and intratracheal [22]. The geographical location, genetic factors of the host, the presence of environmental mycobacteria and other concomitant infections like helminthiasis, are factors that have to be considered when designing animal experiments [23].

The study of the distribution of monoclonal and polyclonal antibody formulations in different organs and tissues of mice after administration by different routes, including the use of backpack models have been reported [24-26]. Each model has its advantages and drawbacks. For example, the backpack model is very useful for the evaluation of the protective role of IgA, but poses ethical problems in long term experiments due to the increase in tumour size over time produced by the inoculated hybridoma [27].

In prophylactic and therapeutic models, antibody formulations have been administered via the intranasal, intravenous and intraperitoneal routes and combined with cytokines and antibiotics [28,29] before and/or after the infectious challenge.

The administration of *M. tuberculosis* pre-coated with antibodies [30,31] in different models of infection have also contributed to understanding the interactions between host and microbe.

Another approach has been the use of knockout mice models for IgA, polymeric immunoglobulin receptor (pIgR) and B cells, as will be discussed later.

4.2 Experimental studies with antibodies

A great number of studies involving antibodies as inoculum have been conducted as far back as the end of the 19th century. These experiments can be grouped in several categories: serum therapies, mouse polyclonal antibodies, human polyclonal antibodies including commercial human gamma globulins, secretory human IgA (hsIgA) and studies with monoclonal antibodies.

4.2.1 Serum therapies

Serum therapy experiments were conducted from the second half of the 19th century. Immune sera was generated by immunizing animals with different microbial fractions and administered either to animals or humans. The results obtained were either variable, inconclusive or contradictory, due to differences in the methods of serum preparation or its administration, and the lack of appropriate experimental controls [32]. These controversial results led to the perceived minor role of antibodies in the defence against intracellular pathogens.

Why these results were considered ¨controversial¨? Immune serum is a polyclonal preparation that includes antibodies to multiple specificities and isotypes; consequently, polyclonal sera may contain blocking antibodies [33] and antibodies of different functional categories that can affect the outcome of infection. For example, IgG3 murine monoclonal antibodies protects against *Streptococcus pneumoniae* and *M. tuberculosis* but fails to protect against *C. neoformans* [34]. Moreover, results from animal studies are not always reflective of the Ig isotype function in humans. Besides intrinsic factors associated to the antibody structure, other parameters such as the genetic background of the microbe and the immunocompetence of the host could alter the outcome of antibody protection experiments. For some microorganisms (*Legionella pneumophila* and *C. neoformans*), passive antibody therapy efficacy depends on the mouse strain used [35]. In the same way, some microbial strains are more susceptible to the effects of antibodies. The animal model used is another important parameter that varies between different experiments cited in the literature. Timing, the route of infection, the magnitude of the infecting inoculum and the variables to measure efficacy are some of the critical parameters in antibody protection studies [36].

Despite its controversial nature, the results obtained with serum therapy were valuable, demonstrating some beneficial effect of serum on the course of TB in humans, mainly in cases of early or localized TB [37]. Moreover, it was demonstrated that long periods of treatment were necessary to achieve a sustained effect [38].

4.2.2 Polyclonal mouse antibodies

A recent study re-examined the usefulness of immune serum in the context of a therapeutic vaccine against TB [39]. This vaccine, called RUTI, is generated from detoxified *M. tuberculosis* cell fragments that facilitate a balanced T helper response to a wide range of antigens along with intense antibody production. Local accumulation of specific CD8+ T cells and a strong humoral response after immunization are characteristic features of RUTI, features that contribute to its protective properties. In this study, immune serum was generated by immunizing mice with RUTI. Severe Combined Immunodeficiency (SCID) mice were infected with *M. tuberculosis* and treated with chemotherapy for 3–8 weeks. After chemotherapy they were treated for up to 10 weeks with intraperitoneal injections of immune serum. Mice treated with immune serum from RUTI vaccinated animals showed significant decreases in lung CFU as well as reduction in the extent of granulomatous response and abscess formation in comparisson with controls. These results suggest that protective serum antibodies can be elicited by vaccination, and that antibodies may be usefully combined with chemotherapy [29,40].

4.2.3 Human gammaglobulins

4.2.3.1 Human polyclonal antibodies

The first evidence of the stimulatory role upon cellular immunity of specific antibodies in experimental mycobacterial infections was reported by Valiere and colleagues in 2005. In this study, serum samples containing specific antimycobacterial antibodies were obtained from volunteers vaccinated twice with BCG by the intradermal route. Significant titres of IgG antibodies against lipoarabinomannan (LAM) were detected in the volunteers. Moreover, BCG internalization into phagocytic cells was significantly increased in the

presence of BCG induced antibodies as were the inhibitory effects of neutrophils and macrophages on mycobacterial growth. Furthermore, these antibodies induced significant production of IFN-γ by CD4+ and CD8+ T cells [17].

4.2.3.2 IgG formulations

Roy and colleagues demonstrated that the treatment of *M. tuberculosis*-infected mice with a single cycle of human intravenous Ig resulted in substantially reduced bacterial loads in the spleen and lungs when administered either at early or at late stage of infection [41].

The effect of the administration of a commercial preparation of human gammaglobulins in a mouse model of intranasal infection with BCG was evaluated by our group. We demonstrated the passage of specific antibodies to saliva and lung lavage following the intranasal or intraperitoneal administration of human gammaglobulins to mice. This treatment inhibited BCG colonization of the lungs of treated mice. A similar inhibitory effect was observed after infection of mice with gammaglobulin-opsonized BCG [42]. The same formulation was evaluated also in a mouse model of intratracheal infection with *M. tuberculosis*. Animals receiving human gammaglobulins intranasally 2h before intratracheal challenge showed a significant decrease in lung bacilli load compared to non-treated animals. When *M. tuberculosis* was pre-incubated with the gammaglobulin before challenge the same effect was observed. The protective effect of the gammaglobulin formulation was abolished after pre-incubation with *M. tuberculosis* [30]. These results suggest a potential role of specific human antibodies in the defence against mycobacterial infections.

Taken together these studies provide consistent support for the potential use of gammaglobulins and their beneficial immunomodulatory effects in tuberculosis. The results of certain knockout mouse studies and the gammaglobulin experiments indicate that B cells and their products mediate protection against *M. tuberculosis* [43-45]. However, the important question that remains is whether B-cell responses can be augmented to improve immunity against *M. tuberculosis* through immunotherapy or vaccination.

4.2.3.3 Purified human secretory IgA

Human secretory IgA (hsIgA) is the major class of antibody associated with immune protection of the mucosal surfaces [46]. Colostrum volume is above 100 mL in humans during the first three days after delivery [47]. The high percentage of (hsIgA) in human colostrum [48] strongly suggests its important role in passive immune protection against gastrointestinal and respiratory infections [49]. In one study performed by our group, hsIgA from human colostrum was obtained by anion exchange and gel filtration chromatographic methods, using DEAE Sepharose FF and Superose 6 preparative grade, respectively [50]. HsIgA was administered intranasally to BALB/c mice, and the level of this immunoglobulin in several biological fluids was determined by ELISA. The results showed the presence of this antibody in the saliva of animals that received the hsIgA, at all time intervals studied. In tracheobronchial lavage, hsIgA was detected at 2 and 3 hours after inoculation in animals that received the hsIgA [51]. Similar studies were performed by Falero and colleagues with monoclonal antibodies of IgA and IgG class [52]. Following demostration that hsIgA could be detected in several biological secretions after intranasal administration, the protective effect of this formulation against *M. tuberculosis* challenge was evaluated. Mice challenged with *M. tuberculosis* preincubated with hsIgA showed a statistically significant decrease in

the mean number of viable bacteria recovered from the lungs compared to control mice and to the group that received the hsIgA before challenge with *M. tuberculosis*. Moreover, an increased level of iNOS production was also reported (Alvarez et al., mannuscript in preparation). Consistently with this result, a better organization of granulomatous areas with foci of lymphocytes and abundant activated macrophages were observed in the lungs of mice of the group that received *M. tuberculosis* pre-incubated with hsIgA sacrificed at 2 months post-challenge. Untreated animals, however, showed an increased area of bronchiectasis and atelectasis as well as fibrin deposits, accumulation of activated macrophages and lymphocytes. The pneumonic areas were more prominent in the untreated animals than in the groups treated with hsIgA and *M. tuberculosis* pre-incubated with hsIgA (Alvarez et al., mannuscript in preparation)

4.2.4 Studies performed with monoclonal antibodies

Since the first report on the use of the monoclonal antibody Mab 9d8 against *M. tuberculosis*, many similar studies have been reported [53;54]. This IgG3 monoclonal antibody (Mab) generated against arabinomannan (AM) capsular polysaccharide, increased thesurvival of intratracheally infected mice when the *M. tuberculosis* Erdman strain was pre-coated with it. In this study, a longer survival associated with an enhanced granulomatous response in the lungs was found as compared to controls receiving an isotype-specific non-related Mab [31].

Another Mab, SMITB14, directed against the AM portion of LAM prolonged the survival of intravenously infected mice associated with reduced lung CFU and prevention of weight loss. In this study, the authors demonstrated that protection was independent of the antibody Fc portion, because the F(ab')2 fragment also conferred a similar protective effect [55].

In another study, mice receiving the Mab 5c11 (an IgM antibody that recognizes other mycobacterial arabinose-containing carbohydrates in addition to AM) intravenously prior to Mannosylated lipoarabinomannan (ManLAM) administration, showed a significant clearance of ManLAM and redirection of this product to the hepatobiliary system. This study strongly supports an indirect effect of certain antibodies on the course of mycobacterial infection, altering problably the pharmacokinetics of mycobacterial components and contributing to protection against TB [56].

Heparin Binding Hemagglutinin Adhesin (HBHA) is a surface-exposed glycoprotein involved in the mycobacterial binding to epithelial cells and in mycobacterial dissemination [57]. Monoclonal antibodies 3921E4 (IgG2a) and 4057D2 (IgG3) directed against HBHA were used to coat mycobacteria before administration to mice. In this study, spleen CFUs were reduced while lung CFUs did not [58]. These results suggest that binding of these antibodies to HBHA impede mycobacterial dissemination.

The protective efficacy of a monoclonal antibody, TBA61, IgA anti-Acr administered intranasally before and after the intranasal or aerosol challenge with *M. tuberculosis* was demonstrated in a previous work [59]. In another series of experiments carried out by López and colleagues, the protective effect of this Mab administered intratracheally before an intratracheal challenge with virulent mycobacteria was evaluated. At 21 days post-infection, pre-treatment of mice with TBA61 caused a significant decrease in viable bacteria in the lungs compared to control mice or those treated with the Mab against the 38-kDa protein (TBA84). Consistent with the reduction of viable bacteria following treatment with TBA61,

the area of peribronchial inflammation was also statistically smaller in this group compared to the control group [60].

When the lungs of mice were histologically examined, granulomas were better organized in the infected animals that had received TBA61 than in controls or mice treated with TBA84. The reduction of CFU in lungs of the treated group was associated with milder histopathological changes, as indicated by the organization of the granulomas and less pneumonic area. The fact that this Mab promotes granuloma formation in mice infected intratracheally with *M. tuberculosis* strongly suggests the close interaction between antibody-mediated immunity and cell-mediated immunity to induce protection against intracellular pathogens (61). Some of the results obtained in the evaluation of TBA61 monoclonal antibody under different conditions are listed in the Table 1.

MAb, delivery route and inoculation regime	Challenge	Days selected for Organ Harvesting	Parameter measured		References
			CFU reduction	Histopathology	
TBA61 i.n (-3h, +3h, 6h) TBA61 i.n (-3h) TBA61 i.n (+3h) TBA61 i.n (-3h, +3h)	H37Rv i.n, aerosol	9 days	Significant reduction of CFU post-challenge	nd	59
TBA61 i.n + IFN-γ i.n (-3h, -2h, +2h, +7h)	H37Rv i.n, aerosol	9, 21 and 28 days	Significant reduction of CFU post-challenge	Significant reduction of the granulomatous area in the lungs of treated as, compared to untreated mice	28
TBA61 i.t (-3h)	H37Rv i.t	24h, 72h, 21 days	Significant reduction at 21 days post-challenge	Less interstitial and peribronchial inflammation. Well-organized granuloma	60

Table 1. Results from different experimental approaches involving a monoclonal antibody against *M. tuberculosis* 16 kDa protein (TBA61). Note: i.n: intranasal; i.t: intratracheal

The 16 kDa protein (Acr antigen) has been defined as a major membrane protein peripherally associated with the membrane [62] carrying epitopes restricted to tubercle bacilli on the basis of B-cell recognition [63,64]. The Acr antigen is present on the surface of tubercle bacilli and is highly expressed in organisms growing within infected macrophages, allowing it to be potentially targeted by specific antibodies either inside infected cells as well as extracellulary.

A novel immunotherapy, combining treatment with anti-IL-4 antibodies, IgA antibody against 16 kDa protein and IFN-γ, showed the potential for passive immunoprophylaxis against TB. In genetically deficient IL-4-/- BALB/c mice, infection in both lungs and spleen was substantially reduced for up to 8 weeks. Reconstitution of IL-4-/- mice with rIL-4 increased bacterial counts to wild-type levels and making mice refractory to protection by IgA/IFN-γ [65].

More recently, Balu and colleagues reported a novel human IgA1 Mab, constructed using a single-chain variable fragment clone selected from an Ab phage library. The purified Mab monomer revealed high binding affinities for the mycobacterial α-crystallin Ag and for the human FcαRI (CD89) IgA receptor. Intranasal inoculations with the monoclonal antibody and recombinant mouse IFN-γ significantly inhibited pulmonary H37Rv infection in mice transgenic for human CD89 but not in CD89-negative littermate controls, suggesting that binding to CD89 was necessary for the IgA-imparted passive protection. The Mab added to human whole-blood or monocyte cultures inhibited luciferase-tagged H37Rv infection although not for all tested blood donors. Inhibition of the infection by the antibody was synergistic with human rIFN-γ in cultures of purified human monocytes but not in whole-blood cultures. The demonstration of the mandatory role of FcαRI (CD89) for human IgA-mediated protection is important for understanding the mechanisms involved and also for translating this approach towards the development of passive immunotherapy for TB [66].

In all the studies analyzed, it is possible to assert that different mechanisms of action of monoclonal and polyclonal antibodies are involved in the protection against TB. Some of these mechanisms will be discussed later in this chapter.

4.2.5 Studies performed in transgenic mice

Mouse models with deficiency in antibody production can be useful in understanding certain roles of the antibodies in protection against mycobacterial infections. However, knockout mouse studies can lead to premature conclusions regarding the role of a particular component of immunity, if not interpreted carefully. Additionally, experimental conditions can have marked effects on the results.

Rodríguez and colleagues reported that IgA deficient (IgA-/-) mice and wild type non-targeted littermate (IgA+/+) were immunized by intranasal route with the mycobacterium surface antigen PstS-1. These authors showed that IgA-/- mice were more susceptible to BCG infection compared to IgA+/+ mice, as revealed by the higher bacterial loads in the lungs and bronchoalveolar lavage (BAL). More importantly, analysis of the cytokine responses revealed a reduction in the IFN-γ and TNF-α production in the lungs of IgA-/- compared to IgA+/+ mice, suggesting that IgA may play a role in protection against mycobacterial infections in the respiratory tract. Furthermore, these authors demonstrated that immunized pIgR-/- mice were more susceptible to BCG infection than immunized wild-type mice [67].

In the attempt to elucidate whether humoral immunity has a special role in the defence against TB, different experiments with B cell knockout mice were performed by several authors. In 1996, Vordermeier and colleagues developed an infection model of TB in μ chain knockout Ig$^-$ mice. Organs from *M. tuberculosis* infected IgG$^-$ mice had three to eight fold

elevated counts of viable bacilli compared with those from normal mice. This result suggested that B cells play a role in the containment of murine tuberculous infection [68]. In another study, B cell gene disrupted mice (B cell KO) and controls were infected by aerosol with *M. tuberculosis* to allow the latter group to generate an antibody response in the upper respiratory tract. They were subsequetly given chemotherapy to destroy remaining bacilli and then re-challenged by aerosol exposure. The results of this study, however, revealed no differences in the ability of animals to control this second infection, indicating that, in this low dose pulmonary infection model at least, any local production of antibodies neither impeded nor enhanced the expression of specific acquired resistance [69].

In another series of experiments the role of B cells during early immune responses to infection with a clinical isolate of *M. tuberculosis* (CDC 1551) was evaluated. In this study, despite comparable bacterial loads in the lungs, less severe pulmonary granuloma formation and delayed dissemination of bacteria from lungs to peripheral organs were observed in BKO mice. Additional analysis of lung cell populations revealed greater numbers of lymphocytes, especially CD8+ T cells, macrophages, and neutrophils in wild-type and reconstituted mice than in BKO mice. Thus, less severe lesion formation and delayed dissemination of bacteria found in BKO mice were dependent on B cells, (not antibodies, at least in this study) and were associated with altered cellular infiltrate to the lungs [70].

This latter result differs to the study carried out by Maglione and colleagues in which B cell-/- mice had exacerbated immunopathology corresponding with elevated pulmonary recruitment of neutrophils upon aerosol challenge with *M. tuberculosis* Erdman strain. Infected B cell-/- mice showed increased production of IL-10 in the lungs, whereas IFN-γ, TNF-α, and IL-10R remain unchanged from wild type. B cell-/- mice had enhanced susceptibility to infection when aerogenically challenged with 300 CFU of *M. tuberculosis* corresponding with elevated bacterial burden in the lungs but not in the spleen or liver [43].

Together these studies reveal that B cells may have a greater role in the host defence against *M. tuberculosis* than previously thought.

5. Possible mechanisms of action

Secretions found on mucosal surfaces contain significant levels of Igs, particularly, IgA. This immunoglobin has direct and indirect functional roles to combat infectious agents such as viruses and bacteria that cross the mucosal barrier. Moreover, experimental evidences suggest that the IgA associated with the pIgR may neutralize pathogens and antigens intracellularly during their transport from the basolateral to the apical zone of epithelial cells [71,72]. In addition, as demonstrated previously, IgA may interact with Gal-3 (an intracellular binding β-galactosidase lectin), and interfere with the interaction of mycobacteria with the phagosomal membrane, resulting in the decrease of bacterial survival and replication in the phagosome [73].

As reported by several authors, antibodies may be critical, at least during the extracellular phases of intracellular facultative pathogens. Antibodies may act by interfering with adhesion, neutralizing toxins and activating complement. Moreover, antibodies may be able to penetrate recently infected cells and bind to the internalised pathogen, increasing the antigen processing (74). It is well accepted that antibodies play a crucial role in modulating the immune response

by activating faster secretion of selected cytokines that in turn, contribute to more efficient and rapid Th1 response [74,75], increasing the efficacy of co-stimulatory signals, enhancing Antibody Dependent Cellular Cytotoxicity responses (ADCC) and the homing of immune cells to the lungs after the respiratory infection [13,76-81].

Examples of relevant action mechanisms of antibodies have been discussed by Glatman-Freedman [82].

6. Potential applications

Future applications of antibody formulations for the control of TB may include treatment of patients infected with Multidrug Resistant (MDR) strains, combination with the standard treatment in order to achieve faster therapeutic effects, and administration to recent contacts of TB patients with special attention to risk groups [85].

On the other hand, the induction of specific antibody responses by vaccination in addition to the stimulation of cell mediated immunity could be a novel strategy for the development of new generation prophylactic and therapeutic vaccines against TB.

The prevailing dogma about the uncertain role of antibodies in the protection against TB has somewhat limited the study of B cell immunodominant epitopes which have been mainly related with the development of serodiagnosis assays [86]. Consequently, little information is available on B cell epitopes that could potentially contribute to protection or therapy. With the development of bioinformatics tools for bacterial genome analysis, it has been possible to predict *in silico* microbial regions that trigger immune responses relevant for protection and vaccine development.

Our group is currently developing a candidate experimental vaccine based on proteoliposomes from *M. smegmatis*. In one study, bibliographic search was used to identify highly expressed proteins in active, latent and reactivation phases of TB [87]. The subcellular localization of the selected proteins was defined according to the report on the identification and localization of 1044 *M. tuberculosis* proteins using two-dimensional, capillary high-performance liquid chromatography coupled with mass spectrometry (2DLC/MS) method [88] and using prediction algorithms.

Taking into consideration the cell fractions potentially included in the proteoliposome, from the previously identified proteins, the ones located in the cell membrane and cell wall, as well as those which are secreted and homologous to those of *M. smegmatis* were selected. The regions of the selected proteins containing promiscuous B and T cell epitopes were determined [87]. Thus the *M. smegmatis* proteolipomes were predicted to contain multiple B and T epitopes which are potentially cross reactive with those of *M. tuberculosis*. It is important to note that there could be conformational B epitopes and additional epitopes related with lipids and carbohydrates included in the proteoliposomes that could reinforce the humoral cross reactivity.

Considering the results of the *in silico* analysis, proteoliposomes of *M. smegmatis* were obtained and their immunogenicity was studied in mice [89]. In addition to cellular immune effectors recognizing antigens from *M. tuberculosis*, cross reactive humoral immune responses of several IgG subclasses corresponding with a combined Th1 and Th2 pattern

against antigenic components of *M. tuberculosis* were elicited. These findings were in concordance with the *in silico* predictions [87,89]. It is interesting to note that differences in the pattern of humoral recognition of lipidic components was dependent on the characteristics of the adjuvant used, which could have relevance for the development of vaccines which includes lipidic components [89]. Currently studies are underway to evaluate the protective capacity of *M. smegmatis* proteoliposomes in challenge models with *M. tuberculosis* in mice.

Bioinformatics tools for prediction of T and B epitopes were also employed for the design of multiepitopic constructions, which were used to obtain recombinant BCG strains. Based on this prediction, B cell epitopes from ESAT-6, CFP-10, Ag85B and MTP40 proteins were selected and combined with T cell epitopes of the 85B protein and fused to 8.4 protein [90]. A significant IgG antibody response against specific B cell epitopes of ESAT-6 and CFP-10 was obtained in mice immunized with the recombinant strain. After studying the specific response of spleen cells by lymphoproliferation assay and detection of intracellular cytokines in CD4 + and CD8 + subpopulations, the recognition of T epitopes was also observed. The response showed a Th1 pattern after immunization with this recombinant strain (Mohamud, R, et al. manuscript in preparation). In another series of experiments, recombinant BCG strains expressing several combinations of multiepitopic constructions were used to immunize BALB/c mice subcutaneously and challenged intratracheally with the *M. tuberculosis* H37Rv strain. Recombinant BCG strains expressing T epitopes from 85BAg fused to Mtb8.4 protein and BCG expressing a HSP60 T cell epitope plus different combinations of B cell epitopes from 85BAg, Mce1A, L7/L12, 16 kDa, HBHA, ESAT6, CFP10 and MTP40 and combinations of B cell epitopes alone produced significant reductions in lung CFU compared with BCG (Norazmi MN, manuscript in preparation).

The cumulative works reviewed above related with the use of antibody formulations and vaccines suggest that antibodies if present at the right moment at the site of infection can provide protection against *M. tuberculosis*. This concept opens the way to the development of a new generation of vaccines that elicit specific IgA and/or IgG antibodies able to protect at the port of entry against the infection and directed to bacteria in the infected tissues.

An antibody-based vaccine could be implemented against TB. Such antibodies should recognize the pathogen immediately after its entry into the host, mainly at the mucosal surfaces, where these antibodies must be strategically induced [91]. This vaccine has to induce IgA and IgG antibodies that can inactivate bacterial components essential for the microbial survival in the host, activate complement for direct lysis of the cells, opsonize bacteria to promote their capture by phagocytic cells and induce stimulation of specific cellular immune responses.

Although no serological tests for diagnosis of TB are recommended [92], due to the generation of false results as well as incorrect treatments, for many other pathogens, the availability of serological diagnostic tests has been of great value, in particular in poor countries. In some cases, it constitutes the best protection correlate [93].

In the specific case of TB, several studies of the antibody response have been developed [94]. A number of factors have been described to contribute to the variation of antibody response during the disease. Some of these factors are associated to the pathogen (strain variation,

micro-environment and growth state of bacteria). Not less important are the factors related to the host, mainly the previous exposure to antigen and host genetics [95].

On the other hand, only a small fraction of the genomic regions of *M. tuberculosis* encoding proteins has been explored. Currently, novel immunoassay platforms are being used to dissect the entire proteome of *M. tuberculosis*, including reacting protein microarrays with sera from TB patients and controls [96,97]. These studies could lead to the discovery of new antigens that may constitute a suitable diagnostic marker as well as to the identification of correlates of protection.

The study of the role of specific antibodies in the defense against tuberculosis is opening new possibilities for the future development of new vaccines, diagnostics and therapies against the disease. It is envisaged that new discoveries will arise from the ongoing studies in this area that will expedite the introduction of new strategies in the fight against tuberculosis.

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19

Identification of CD8$^+$ T Cell Epitopes Against *Mycobacterium tuberculosis*

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1. Introduction

Although the effective immune response against *Mycobacterium tuberculosis* (*M. tuberculosis*, Mtb) is primarily due to cell-mediated immunity, the mechanisms by which T cells participate in the control of infection are still not completely understood. The importance of CD8$^+$ T cells in the immune response to tuberculosis has been recognized by many researchers [1]. Experimental evidence from the murine model indicated that CD8$^+$ T cells could be important to the control of *Mycobacterium tuberculosis* infection *in vivo* [2-5]. The isolation of *M. tuberculosis*-specific CD8$^+$ T cells from infected mice and human clearly showed that this subset could be induced during infection [6-8]. Reports on epitope-specific *M. tuberculosis* reactive CD8$^+$ T cells, which are present at very high frequencies in the peripheral blood of PPD positive individuals and patients with active tuberculosis, support the importance of CD8$^+$ T cells to the immunity of *M. tuberculosis* and emphasize that the CD8$^+$ T cell subset should be considered to the design of new anti-tuberculosis vaccines [9, 10]. CD8$^+$ T cells may contribute to the control of *M. tuberculosis* infection through four mechanisms: (1) Cytokine release, such as IFN-γ and TNF-α; (2) Cytotoxicity via granule-dependent exocytosis pathway; (3) Cytotoxicity mediated through Fas/Fas ligand interaction; (4) Direct microbicidal activity.

The pathogen's antigens are processed by antigen presenting cells (APCs) and digested into short peptides. Consequently, these non-self peptides are presented to the surface of the infected cells or APCs through the loading of the major histocompatibility complex (MHC) or called human leukocyte antigen (HLA) in human. These peptide/MHC complexes are recognized by specific T-cells that perform immune responses, such as cytotoxicity (in the case of class I MHC). These peptides which act as the markers of the pathogens are called T cell epitopes [11]. The identification of novel cytotoxic T lymphocyte (CTL) epitopes is important to the analysis of the involvement of CD8$^+$ T cells in *M. tuberculosis* infection as well as the anti-Mtb vaccine development. However, only a small number of MHC-I-restricted CTL epitopes have been identified within a few of *M. tuberculosis* proteins. The step to develop epitope-based vaccines include: (1) antigen selection; (2) epitope prediction; (3) epitope identification; (4) vaccine prototype engineering; (5) immunization and challenge studies. In this chapter we mainly focused on the strategy to identify CD8$^+$ T cell epitopes against *Mycobacterium tuberculosis*. It

includes: selection of the antigens; Prediction the $CD8^+$ T cell epitopes; Synthesis and modification of the epitopes; Identification of epitopes by *in vitro* and *in vivo* assays; Development of $CD8^+$ T cell epitope-based vaccines.

2. Selection of antigens from *Mycobacterium tuberculosis*

An essential step towards the development of novel vaccines against Mtb is gaining more information on the antigenic architecture of *Mycobacterium tuberculosis* to identify T-cell epitopes responsible for eliciting protective immune responses. Determination of the complete genome sequence of *Mycobacterium tuberculosis* facilitated this step considerably. The H37Rv strain of *Mycobacterium tuberculosis* has been found extensive, worldwide application in biomedical research because it has retained full virulence in animal models. Cole et al reported the complete genome sequence of H37Rv in 1998 [12]. This progress in genomics makes it possible that we can find corresponding protein sequences through submitting to publicly accessible sequence databases such as GenBank and TB database [13].

The Mtb genome contains about 4,203 open reading frames, nearly half of which are categorized as 'hypothetical proteins' with unknown function. Although secreted proteins are generally associated with protective immune responses, no proteins or protein families have yet been shown to fully protect against the active Mtb infection. Screening each protein from these 4,023 open reading frames by using human serum and gel electrophoresis would be time-consuming and costly method of identifying new vaccine candidates. Bioinformatics tools provide more practical means to rapidly identify potential epitopes and antigens for Mtb vaccines development. A range of approaches have been utilized to select proteins for analysis prior to epitope mapping. For example, whole genome analysis can be performed in order to identify candidate vaccine components, or alternatively, bioinformatics tools that select proteins according to their secretion characteristics can be used to narrow the number of proteins evaluated in the next step [14-16]. Frieder et al used pepmixes created by Micro-Scale SPOT™ to map T cell epitopes in 389 proteins of *Mycobacterium tuberculosis* [17]. These proteins are sorted by their known or likely known function such as PPE/PE, cell wall, cell processes, virulence, detoxification, and adaptation. Some $CD8^+$ T cell epitopes identified by researchers were listed in **Table 1.**

So far, antigens in *Mycobacterium tuberculosis* from which $CD8^+$ T cell epitopes were identified are mainly focused on secretory proteins, lipoproteins, such as Ag85-complex, ESAT-6, CFP10, 16kDa protein, 19kDa lipoprotein and so on. But there are few studies focused on the membrane proteins like the drug efflux pumps which play important role in the drug-resistant of *Mycobacterium tuberculosis*. Researchers in our lab tried to identify novel $CD8^+$ T cell epitopes from these antigens and found several novel $CD8^+$ T cell epitopes which could elicit potent immune responses both *in vitro* and *in vivo* [32].

3. Prediction of the $CD8^+$ T cell epitopes

The lack of simple methods to identify relevant T-cell epitopes, the high mutation rate of many pathogens, and restriction of T-cell response to epitopes due to human lymphocyte

Protein name	Gene description	Amino acid position	HLA allele	Frequency	References
Ag85A	mycolyltransferases	48-56 242-250	HLA-A2 HLA-A2	1/20,693PBMCs (Elispot) 1/3,300 PBMCs (tetramer) 1/23,779 PBMCs (Elispot) 1/3,750 PBMCs (tetramer)	[18]
Ag85B	mycolyltransferases	143–152 199–207 264–272	HLA-A2 HLA-A2 HLA-B35	ND Undetectable (tetramer) ND	[19, 20]
Ag85C	mycolyltransferases	204–212	HLA-B35	1/13,700–1/22,200 PBMCs	[20]
ESAT-6	Early secretory antigen target	69–76 82–90 21–29	HLA-B53 HLA-A2 HLA-A6802	1/700 $CD8^+$ 1/2,500 PBMCs 1/2,100 $CD8^+$	[21, 22]
CFP10	10kDa culture filtrate antigen	2–9 2–12 3–11 75–83 2–11 49–58 85–94 71–79 76–85	HLA-B4501 HLA-B4501 HLA-B0801 HLA-B1502 HLA-B44 HLA-B44 HLA-B3514 HLA-B14 HLA-B3514	1/101 $CD8^+$ 1/125 $CD8^+$ 1/645 $CD8^+$ 1/145 $CD8^+$ 1/700 $CD8^+$ 1/7,000 $CD8^+$ 1/2,100 $CD8^+$ 1/438–1/1,602 PBMCs 1/437–1/2,427 PBMCs	[11, 23, 24]
CFP21	cutinase precursor	134-142	HLA-A2	3/5000-1/10000 PBMCs ((Elispot))	[25]

Protein name	Gene description	Amino acid position	HLA allele	Frequency	References
Mtb8.4	low molecular weight T-cell antigen	5–15 32–40	HLA-B1501 HLA-B3514	1/10,416 $CD8^+$ 1/1,190 $CD8^+$	[23]
Mtb9.8	Hypothetical protein	3–11 53–61	HLA-A0201 HLA-B0801	<1/25,000 $CD8^+$ 1/2,840 $CD8^+$	[23]
Mtb39	PPE family protein	144–153 346–355	HLA-B44 HLA-B44	ND ND	[26]
16kDa protein	heat shock protein	21–29 120–128	HLA-A2 HLA-A2	1/1,000 $CD8^+$ 1/800 $CD8^+$	[19]
19kDa lipoprotein	19 kDa lipoprotein antigen precursor	88–97	HLA-A2	ND	[27]
38kDa protein	periplasmic phosphate-binding lipoprotein	8–17	HLA-A0201	1/331 $CD8^+$	[28]
Hsp65	chaperonin	369–377	HLA-A2	ND	[29]
Rv0341	isoniazid inductible gene protein	33–42 33–44 33–45	HLA-A2 HLA-A2 HLA-A2	ND ND ND	[30]
Rv2903c	signal peptidase	201–209	HLA-B35	Undetectable	[31]
Rv1410c	aminoglycosides/tetracycline -transport integral membrane protein	510-518	HLA-A0201	3/2500 PBMCs	[32]
GlnA1	glutamine synthetase	308–316	HLA-A2	ND	[33]
SodA	superoxide dismutase	160–168	HLA-A2	ND	[32]
AlaDH	delta-aminolevulinic acid dehydratase	160–169	HLA-A2	ND	[32]
Hemolysin	cytotoxin/hemolysin	73–82	HLA-A0201	1/353 $CD8^+$	[28]

Table 1. $CD8^+$ T cell epitopes identified in *Mycobacterium tuberculosis*

antigen (HLA) polymorphism have significantly hindered the development of cytotoxic T-lymphocyte (CTL) epitope-based or epitope-driven vaccines. As the development of bioinformatics, computer-driven algorithms methods for predicting $CD8^+$ T cell epitopes are used as important tools to the vaccine design. These tools offer a significant advantage over other methods of epitope selection because high-throughput screening can be performed in silico, followed by consequent immunological experiments *in vitro* and *in vivo* [34]. Traditionally, the identification of T cell epitopes required the synthesis of overlapping

peptides that spanned the entire length of a protein, followed by experimental assays for each peptide, such as *in vitro* intracellular cytokine staining, to determine the T cell activation. This method is economically viable only for single protein or pathogen that consists of fewer proteins. As a result, alternative computational approaches have been developed for the prediction of T cell epitopes, which have significantly decreased the experimental burden that is associated with epitope identification. Computer-driven algorithms are now routinely employed to sort through protein sequences for linear strings of amino acids that confirm to previously established patterns known to be associated with the binding to antigen presenting molecules (such as MHC) and stimulation of T cells. These peptide strings, once proven to stimulate T cells *in vitro* or *in vivo*, are considered as T cell epitopes.

A lot of computational algorithms have been developed to predict CTL epitopes in pathogen protein sequences (some of these are listed in **Table 2**). In reality, using only one computational algorithm to predict CTL epitopes may lead to large number of false positives and false negatives. Later and more comprehensive validations using data from several different prediction softwares are accurate and of high value in reducing the cost for epitope identification. Researchers in our lab used several prediction servers and identified novel CTL epitopes successfully [25, 32].

4. Synthesis and modification of the epitopes

Solid phase peptide synthesis (SPPS) offers important advantages over the synthesis in solution, in which coupling reactions can be carried out more rapidly and nearly to completion using an excess of the activated amino acid derivative, which can be removed at the end of the reaction by simple washing operations. The introduction of Fmoc protecting group into SPPS by Carpino in 1970 allowed the entire process of SPPS to be carried out under milder reactive conditions [35]. As a result of this chemical progress, nowadays the synthesis of many peptides can be smoothly accomplished by manual or automate-assisted SPPS and even longer proteins can be synthesized by coupling protected segments or more efficiently by chemical ligation with unprotected peptide segments [36].

The immunogenicity of the native epitope is often weak. Substitutions at main anchor positions to increase the complementarity between the peptide and HLA binding cleft constitute a common procedure to improve the binding capacity and immunogenicity of the native epitope. In 1993, Ruppert et al determined that the 'canonical' HLA-A2.1 motif could be defined as leucine (L) or methione (M) at position 2 (P2) and leucine (L), valine (V), or isoleucine (I) at position 9 (P9) [37]. In 2000, Tourdot et al demonstrated that residue tyrosine (Y) at position 1 (P1) could enhance the affinity of epitope to HLA-I molecule [38]. These results suggested that an altered peptide ligand (APL) might be used to exploit a latent capacity of the T cell repertoire to respond more effectively to the native epitope. We also used this strategy to modify the related positions and found the binding capacity and immunogenicity of some epitopes improved [25, 32]. The second strategy is to modify the amino acids with a side chain protruding out of the peptide binding cleft of the HLA molecule, because these amino acids may participate in T cell receptor (TCR) recognition. If they are substituted by amino acids with other properties, a mutated epitope may bind more efficiently to TCR or elicit a more diverse TCR reservoir [39, 40].

ID	server	Abbreviation	Prediction algorithm
1	BIMAS	BIMAS	Matrix[a]
2	HLA Ligand	HLA_LI	Matrix[a]
3	IEDB (ANN)	IEDB_ANN	ANN[b]
4	IEDB (ARB)	IEDB_ARB	Matrix[a]
5	IEDB (SMM)	IEDB_SMM	Matrix[a]
6	MAPPP (Bimas)	MAPPP_B	Matrix[a]
7	MAPPP(SYFPEITHI)	MAPPP_S	Matrix[a]
8	MHC Binder Prediction	MHC_BP	Matrix[a]
9	MHC-BPS	MHC-BPS	SVM[c]
10	MHC-I (Multiple matrix)	MHCI_MM	Structure-based model
11	MHC-I (Single matrix)	MHCI_SM	Structure-based model
12	MHCPred (Interactions)	MHCP_I	Partial least square
13	MHCPred (Amino Acids)	MHCP_AA	Partial least square
14	MULTIPRED (ANN)	MULTI_ANN	ANN[b]
15	MULTIPRED (HMM)	MULTI_HMM	HMM[d]
16	MULTIPRED (SVM)	MULTI_SVM	SVM[c]
17	NetMHC (ANN)	NETM_ANN	ANN[b]
18	NetMHC (Weight Matrix)	NETM_WM	Matrix[a]
19	nHLAPred (ANNPred)	NHP_ANN	ANN[b]
20	nHLAPred (ComPred)	NHP_CP	ANN[b] and Matrix[a]
21	PepDist	PEPDIST	distance function
22	PeptideCheck (Matrix)	PEPC_M	Matrix[a]
23	Predep	PREDEP	Structure-based model
24	ProPred1	PROPRED	Matrix[a]
25	Rankpep	RANKPEP	Matrix[a]
26	SMM	SMM	Matrix[a]
27	SVMHC (MHCPEP)	SVMHC_M	SVM[c]
28	SVMHC (SYFPEITHI)	SVMHC_S	SVM[c]
29	SVRMHC	SVRMHC	SVM[c]
30	SYFPEITHI	SYFPEITHI	Matrix[a]

a: binding matrices
b: artificial neural networks
c: support vector machines
d: hidden Markov models

Table 2. Some epitope prediction servers

5. Identification of epitopes by *in vitro* and *in vivo* assays

The immunogenicity of epitopes predicted through appropriate prediction softwares should be identified by a series of *in vitro* and *in vivo* assays. Only epitopes which are proven to stimulate T cells *in vitro* or *in vivo* can be used as potential subunit components for the design of vaccines against tuberculosis. Because there are various techniques to identify specific T-cell epitopes, the question arises about how to select and combine these techniques to screen peptides more efficiently and rapidly.

According to the processing and presenting progress of T cell epitopes and their ability to specifically stimulate T cells in an MHC allele-restricted manner. The pathway of MHC-I eptiope presentation has been exhibited and the process comprise three main steps: firstly, endogenous proteins are digested into short peptides by proteasomes; secondly, special epitopes are selected to load onto MHC-I molecule within the endoplasmic reticulum; finally, the peptide/MHC complexes are transferred to cell surface and to stimulate specific T-cell receptors on T cells. Therefore, there are three essential elements for a random peptide to be considered as MHC-I restricted T cell epitope. First, the peptide must be naturally processed into an optimal length via a proteasome dependent pathway. Second, the peptide should have the affinity to bind to the corresponding MHC-I molecule. Finally, and perhaps the most important, the peptide must have the ability to induce a T-cell-specific response after its presentation on an MHC-I molecule [11]. Thus, the techniques to evaluate the three features of a given peptide can be classified into three corresponding types: MHC-binding assay; T cell specific antigenicity assay; Verification of the natural processing of the peptide.

5.1 MHC binding assay

Various methods have been developed to evaluate the binding affinity of peptides to MHC-I molecule. Standard peptide binding inhibition assay is a common method used to quantitatively measure the binding affinity of the target peptides [41]. The MHC-I transfected cell line binding assay was utilized in many labs for its simplicity, repeatability and quantifiability for determining peptide and MHC-binding affinities [25, 32].

5.2 T cell specific antigenicity assay

Peptides with atypical anchoring residues for a related MHC-I allele always display very low binding affinity for MHC-I molecule tested *in vitro*. However, some of these peptides may possess potent antigenicity to induce robust and specific T cell responses. Therefore, it is commonly considered that the most definitive means of defining an epitope is to test peptide-specific T cell responses. Numerous techniques are currently utilized in peptide specific T cell determination, including MHC tetramer staining, enzyme-linked immunospot (ELISPOT) assay, intracellular cytokine staining (ICS), cytotoxicity assay (such as lactate dehydrogenase release assay and ^{51}Cr release assay) and T-cell proliferation assay [18-33].

5.3 Verification of the natural processing of the peptide

Some artificially synthesized peptides have the potent immunogenicity to trigger specific T cell responses and binding affinity to the related MHC molecules *in vitro*. However, these

peptides cannot be generated through natural processing and presenting steps [42]. Therefore, demonstrating that a given peptide is processed naturally is a prerequisite to epitope identification. There are two conventional techniques by using reversed phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) to analyze if a peptide is naturally processed. One method is to manipulate the peptide through the following procedures: (1) Target cells are transfected with specific MHC-I allele; (2) The cells are harvested, and the MHC molecules expressed on the surface of the cells are purified; (3) The peptides bound to the MHC molecules are acid-eluted, purified and finally analyzed by MS [43]. The other technique is to cleave the longer peptides which include the potential epitopes with purified proteasome complexes *in vitro*. Then, production of peptides by the digestion is analyzed by RP-HPLC and MS [44]. However, the results of this method must be analyzed carefully, because proteasome digestion can only generate the proper C terminus of the MHC-loaded peptides [11].

6. Development of $CD8^+$ T cell epitope-based vaccines

One of the approaches to find effective and safe vaccines is epitope-based DNA vaccination that enables focusing of the immune response on important and highly conserved epitopes [45]. This provides the opportunity to use specific epitopes to shift the immune system toward a Th1 or a Th2-mediated immune response and eliminate the unwanted responses. Besides that, CTL epitope-based immunization has the advantage of eliciting immune response only against the protective epitope and avoidance of epitope drift in *Mycobacterium tuberculosis* infection [46].

In many cases, a single CTL epitope-based DNA vaccine could not be fully protective against the Mtb infection. Development of epitope-based vaccines has been hampered for its relatively low antigenicity. Thus, different vaccine patterns and delivery methodologies have been developed, which include synthetic multivalent peptide vaccine, recombinant protein vaccine, DNA vaccine, viral vector, protein carrier, and adjuvant, to solve the problem. Studies of Mtb in human indicate that the induction of broad T-cell mediated immunity to Mtb and Type 1 cytokines including interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) are essential for Mtb vaccine design [47]. Vaccines based on CTL epitopes represent a logical approach to generate effective cellular immunity in both the prophylactic and therapeutic settings because multiple epitopes can be incorporated into the vaccine design with the goal of inducing broadly reactive responses composed of multiple CTL clones directed against different epitopes. DNA vaccines derived from multiple epitopes have been reported to induce broad CTL responses against HIV, HBV, and SARS-CoV [48-50]. Although epitope-based vaccines are often thought to be limited with respect to HLA polymorphism and population coverage, the use of supertype-restricted epitopes, those capable of binding with significant affinity to multiple related HLA alleles, provides a means to address this problem [51]. There are several problems which need to be solved in the future studies of epitope-based Mtb vaccines, such as the safety of the DNA vaccine, the development of better adjuvants, the difference and mutation among the Mtb strains, complete clearance of the Mtb, the results from challenge models in rats or guinea pigs inconsistent with that of clinical trials.

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The Hidden History of Tuberculin

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1. Introduction

Robert Koch was an eminence on Tuberculosis and a german hero worldwide since he discovered and described the ethiologic cause of the white plague. Soon afterwards, he devoted his work to study the effect of inoculating *M.tuberculosis* bacilli (Mtb) to guinea pigs, either healthy or already infected. From his experiments, as reported by himself, he learnt the infection was able to develop protection against reinfection in some extent and that using small doses of dead bacilli could help to heal the tuberculous lesions in the infected animals, while large doses could kill them. The 4th of July, 1890, during the International Medical Congress in Berlin, he announced he might have found a cure, and pointed out it should not be used in severe cases as it could do more harm than good. However, tuberculosis was one of the main causes of death at that moment, and the word of Koch's finding its cure rapidly spread. Many physicians from all over the world traveled to Berlin to learn how to use the new remedy, called tuberculin for being obtained from Mtb bacilli, as Sir Conan Doyle did himself. Koch, probably scared of the great expectancy generated by his cure still not too well-known, decided to write a manuscript entitled "A further report on a remedy for tuberculosis" (Koch 1890), which was published on mid-November of the same year. With this paper, he intended to clarify and to give a review of the subject, in order to avoid the public to get distorted knowledge on the remedy proposed by him. In the manuscript, he tried to explain why tuberculin worked: he believed the treatment was able to destroy the necrotic tissue of the tuberculous lesions, and this was the cause for the bacilli to die subsequently. But still more important, he cautioned about the fact of existing reactions following the inoculation of tuberculin, which severity depended on the patients and their previous illness status, and suggested it should be applied as early as possible to obtain a positive outcome (Koch 1890).

2. Tuberculins

2.1 Koch's tuberculin

Tuberculin was fine powder of Mtb (obtained after mechanically comminuting the products from a tubercle culture) brought into suspension, used in dilutions and sterilised by heating it. T was injected subcutaneously in the back, between the shoulder-blades and the lumbar region, for being the location where less local reactions (including pain) were recorded(Koch 1890). High doses were given in a schedule based in the inoculation of increasing doses. The

inoculation of tuberculin was followed by the so-called „Reactions", which in some cases were large and quite severe, sometimes leading to death.

There were three types of reactions depending on where did they happen: local, general (which we nowadays would call systemic) or focal reactions (in tuberculous lesions)(Riviere and Morland 1913).

At the injection site, if existing, the local reactions included redness, inflammatory swelling and pain, signs and symptoms which used to appear 2 or 3 days after the inoculation, were well-tolerated and tended to disappear. Fever was the more constant sign among the general reactions, followed by headache, malaise, lost of appetite and nausea. The amount of fever varied among the individuals, and didn't previewed any outcome, neither good or bad (Ross 19--?).

Focal reactions included haemoptysis, pleuritic pain, greater cough, râles and swelling of lymphatic glands. These were the most feared reactions but also the most wanted ones, as giving T was endeavoured to help to solve the tuberculous lesions. People well-reacting to the treatment reached, in a period in between 8 weeks and 4 months, a cessation of sweating, cough and expectoration, an improvement of the general condition and a clearing up of the moist rales, which meant the necrotic and ulcerating lesion to become cicatricial (Ross 19--?).

But due to the sudden and massive use of Koch's new remedy, lots of cases with negative outcome reached the mass media as well as the scientific community.

Only one year after presenting T, Koch himself commented the reactions observed by other physicians when using it (Pottenger 1913).

In spite of the recommendations of Koch's to carefully select the patients to be treated, tuberculin was given to any patient, including greatly advanced cases of tuberculosis, with large cavities. While some physicians praised for the benefits on some clinical forms of tuberculosis, specially when combined to surgery (tuberculosis of joints, bones and lupus) (Ross 19--?; Anonymous 1891; Morris 1893), many deaths were also reported. Due to them, and to the sensation of the adverse effects being much impressive than the positive effects obtained, soon all the hopes placed on Koch's remedy seemed to vanish (Ross 19--?). In 1891, a report was issued commenting the 55 trials undergone in Prussia between last 1890 and early 1891. This report, published in the Klinisches Jahrbuch, registered about 20% of patients getting better with the treatment and, in summary, more deaths than positive results.

T was discredited as fast as Koch's reputation, even if the political-social-personal context that surrounded him played an important role in that (Ross 19--?; Sahli 1912; Daniel 1997; Gradmann 2001). Koch intended to be eximed of his academic duties to develop and sell the tuberculin, but the Prussian Government seemed to have other plans for him: to rule an Institute able to compete the Pasteur's French one, as the two empires were rivals. Koch competed directly with von Behring, a disciple of him which had success selling antitoxins against diphtheria and tetanus and could replace him as director of the Hygiene Institute. Von Behring and Ehrlich (the last by collaborating with Hoetsch) worked with biotechnological companies, as was in vogue among the eminent scientists (also for representing a good income of money). Koch also needed the money of selling his remedy to pay his former wife to get a divorce from her, as he fell in love with a young girl (Hedwig

Freiberg) and wanted to marry her. After the report of the Prussian Government was fully published, in April 1891, tuberculin was discredited and Koch, pushed by the Government, renounced to receive any economical compensation and accepted to be proposed for directing the new institute (Gradmann 2001; Cardona 2007).

2.2 New Tuberculins and other similar products

The first attempt to avoid the reactions was to produce new tuberculins and similar products, a thing that Koch did himself, all of them being obtained from tubercle products and differing in the manufacture and/or the excipients. The Old Tuberculin, as was commonly called the original Koch's remedy, was based on human tubercle cultures grown on nutrient broth with a 5% of glycerin, sterilised by steam, evaporated, filtered and adding a 0,5% of phenol to be further refiltered. The New Tuberculin and the Koch's bacilli emulsion were the newest products developed by Koch, intending to ameliorate the first version of tuberculin. To generate the New Tuberculin, the steamed cultures were ground and mixed with glycerin to obtain only the insoluble parts of bacillary bodies, and it was developed in 1897. The Koch's bacilli emulsion was from 1901, and was based on powdered tubercle culture suspended in a mixture of half part of glycerin and half of distilled water, in order to obtain an emulsion.

Many other tuberculins were designed, generally classified in exotoxins or soluble products (mere filtered extracts of tubercle bacilli), endotoxins (containing the less soluble substance of microorganisms, also differing according to the process of extraction), and a third group, including both products, the soluble and insoluble (Wilkinson 1909; Riviere and Morland 1913). Some authors considered the albumose to be responsible for the reactions, thus albumose-free tuberculin from cultures grown in albumose-free medium were generated. Beraneck tuberculin was one of this, based on a mixture of filtered culture of tubercle bacilli grown in albumose-free medium plus an extract of bacillary bodies in 1% of phosporic acid (Riviere and Morland 1913). Wolff-Eisner refined the Koch' s New Tuberculin by filtering the powdered body-substance of tubercle bacilli through a Berkefeld candle to substract all fragments of bacilli (Sahli 1912). The products differed in the way they were obtained and treated, thus how the cultures were killed (by mechanical, physical or chemical means), how and how much they were filtrated, how they were dissolved and how and with what they were treated after.

New tuberculins appeared all over the world: Hunter's modification B, von Ruck's Watery Extract, Behring's TC, and many others (Trudeau 1907). With the promise of avoiding reactions also other products were commercialized, as the Partigens of Much of Hamburg. Even if they weren't considered as tuberculins, they could in fact be so-called, as were supposed to be partial antigens of *M.tuberculosis* bacilli extracted from the non-soluble part of the cultures, further treated by alcohol and ether to obtain them (Rothschild 1921).

Friedmann thought the reactions could be avoided if using cultures of other Mycobacteria, and developed a vaccine from *M.chelonei* cultures. He gave it therapeutically with success, but he failed in obtaining reactionless vaccination. However, he even went further, proving the vaccine to be quite useful if given post-exposure in selected cases and prophylactically (Belmes 1937; Vilaplana and Cardona 2010).

Even the Medical Research Council (MRC) had its own tuberculin, supposedly better than other candidates, they finally had to admit in 1924 it was not better than other products (Bryder 1988).

At the end, the best definition for tuberculin seems to be the one given by Pottenger: all products made from tubercle bacilli which contain their bacterial proteins (Pottenger 1913).

Some products produced more local reactions, mostly by a depôt effect, as did the Koch's Bacillary Emulsion and the New Tuberculin, as remained as insoluble deposit at the site of the injection for more time. The more soluble tuberculins, on the other hand, were supposed to generate more general reactions (Riviere and Morland 1913).

The main problem of the products was its preparation, as they all required dilutions and these were performed by the physicians themselves, a fact that the manufacturer of the Beraneck tuberculin improved (as its dilutions could be already provided) ensuring a better uniformity of concentration (Sahli 1912). The concentration was important to graduate the doses of the vaccines, and this was indispensable to be able to organize to time the injections (Wright 1902).

Fig. 1. Partigens of Deycke-Much.

The Old Tuberculin begun to be considered as the best for a diagnostic use. However, as pointed out Sahli, the therapeutic value of the treatment with different tuberculins were not comparable, as no standard existed (Sahli 1912).

Sahli also wrote that good responses could be obtained with all the products available if the right technique was used, but unfortunately, irrespective of which 'new' tuberculin was used, the reactions appeared to be similar, though varying somewhat in intensity (Sahli 1912).

The Partigens were tuberculin products, supposed to achieve good outcomes with less reactions, for being partial antigens instead of the former tuberculins. The Partigens were obtained from the insoluble parts of *M.tuberculosis* cultures after treating them with alcohol and ether, to obtain the 3 partial antigens: the fatty-acid-lipoids (soluble in alcohol); the neutral and highly molecular fats (soluble in ether) and the non-soluble residuum, supposed to belong to the group of proteins (Rothschild 1921). The picture shows how the Partigens were commercialized, providing diluted concentrations for better adjust the doses.

3. Dosage schedules

3.1 The clue of the dosage

It has already been said that no tuberculin was able to avoid reactions, and the difference between them was the intensity of the adverse effects. As soon as this became evident, the scientists and physicians devoted their efforts to find a safe schedule of inoculations. They were mainly two schools of therapists: those believing in true immunization, and thus only to be obtained by large tuberculin doses; and those believing in the recall of host immunity, and thus easily to be achieved by administering small doses of the remedy (as higher doses could do much harm and the effect of a stimulus is not always proportional to the intensity of the stimulus) (Sahli 1912; Pottenger 1913; Vilaplana and Cardona 2010).

Koch observed he needed high doses to obtain the effects he wanted in tuberculous focus, even if this implied reactions. For after each inoculation, a reaction happened, which was followed by tolerance. Consequently, next dose had to be higher to overcome this tolerance. This dosage method, consisting in small doses gradually incremented in short intervals, was developed by Ehrlich and coworkers on 1891, and was modified by the proposals of Goetsch in 1901 (introducing long treatment) and Petrushky later (proposing interrupted treatment in selected cases) (Vilaplana and Cardona 2010).

3.2 Sir Almroth Wright and the therapeutic vaccination

Back to 1896, Sir Almroth Wright, based on the observation of the agglutination of typhoid bacilli when being in contact with serum of someone's infected but not to someone's not infected, considered this a protective process and decided to use it to distinguish the typhoid fever from the Malta fever. It was known, from about 200 years before, human are able to generate resistance to infections, something already used by Jenner (vaccination with cowpox to prevent smallpox, 1796) and Pasteur (live attenuated bacilli to protect against anthrax, in 1870s) among others. But Wright feared using alive bacteria for this purpose could generate acute disease, and advocated for using dead bacilli instead, as he considered they should generate protective immunity as well (as did Dr. Ferran)(Vilaplana and Cardona 2010). Thus with these ideas he developed the typhoid vaccine to prevent typhoid fever, and demonstrated in vaccinated subjects a higher agglutinin levels similar to the levels found in those individuals who had survived an episode of the disease. Wright

thought that if vaccination could generate protective substances in naïve subjects, it would probably boost the already existing protective substances if administered to infected individuals. From this assumption, he developed his idea of therapeutical vaccination: the vaccines wouldn't only be useful to prevent, but also to heal. With this purpose, he began to use heat-sterilized cultures of staphylococci to treat localized infections of staphylococcical nature. In 1902, he observed the agglutinin levels were decreased in the infected individuals but increased if these subjects were treated with dead bacterial cultures.

Therapeutical vaccinations also lead him to the observation of an immediate aggravation of the patient's condition, what he called the "Negative Phase"(Wright 1902).

Fig. 2. Actual image of the St.Mary's Hospital in London, UK. Wright directed the former Inoculation Department of this hospital, devoted to administer the vaccines therapeutically.

Wright believed the reason for these Negative Phases was the vaccination exhausting the existing protective substances. Once the Negative Phase was overcome, a Positive Phase happened, with an increased well-being and healing tendency, in which the protective

substances hypothetically increased and further decreased, but remaining in a certain amount of residual levels (Cope 1966). He also attributed the intensity of both phases to a problem of dosage, and established a general basis to treat localized infection with bacterial vaccines. If the dose was too low, the Negative Phase diminished, but the Positive Phase could not appear; if it was too high, the Negative Phase was too long, the Positive Phase appearing too late or not appear at all (Wright 1903). In two cases he treated of staphylococcia, he observed a considerable inflammatory swelling in the site of infection. He soon found a relationship between this reaction and the one described by the physicians when using Koch's tubercle vaccine. He also gave an explanation for this: he considered the infectious focus to be broken up, and he warned: if the patient is in a Negative Phase the tubercle bacilli are spread, being able to originate new infectious foci. He considered this to be because a bad-regulated dosage, and propose the rule for any therapeutical vaccination: to consider the resistance ability of both the invading microorganism and the host at the time of inoculation, to well-graduate the doses of the vaccine, timing the injections for any patient in a Negative Phase to be recovered (Wright 1902). Wright believed Koch's reactions were due to an accumulation of Negative Phases, while the true objective was to achieve successive Positive Phases to increase the immunity, thus introduced a new dosage method of tuberculin, based on the inoculation of small doses at spaced intervals (Gunter 1928), giving tuberculin in a 1000 times lower doses than in Koch's time (Riviere and Morland 1913).

3.3 Dosage schedules

In spite of the early discredit which tarnish the usefulness of tuberculin remedy, and even if the history has erased any trace of it, the fact is that its use was increased after Wright's contribution, all over Europe and even America. As previously pointed by Sahli, Pottenger also remarked the difference in the effect between the tuberculins was more quantitative than qualitative (Sahli 1912) (Pottenger 1913).

The administration regimens soon derived to only two (with variations). As after an inoculation tolerance came, only two things could be done: to give a higher next dose to overcome the tolerance or to wait the tolerance to pass. To give a higher next dose implied giving increasing doses at small intervals, and is linkable to Koch's idea of treatment administration. This method was widely used in Europe and America, and seems to be the best one to treat pthtisical forms of the disease.

Wright's method instead, intended to avoid tolerance, and implied the inoculation of constant small doses administered at long intervals. This method seems to have achieved more positive outcomes in disseminated tuberculosis, local tuberculosis, surface tuberculosis (typical of childhood), and especially if combined with surgery (Wright and Reid 1906; Vilaplana and Cardona 2010).

It is true that reactions continued to happen and scientists gave different explanations for this. Some believed a synergy existed between toxins contained in tuberculin and the toxins already existing in the infected body. There was a "difference theory", believing in the presence of antitoxins in the host, able to balance the disease, which effect could be overcome by the tuberculin administrated. Other theories more concrete believed in a direct effect of the remedy on the leucocytes or the fixation ability of the complement, the lysis of

tuberculin into small toxins (Wolff-Eisner theory), antibody mediated allergy (Von Pirquet) and Hypersensitiveness (Sahli 1912; Vilaplana and Cardona 2009; Vilaplana and Cardona 2010).

But it is also described in literature the most important thing to avoid serious reactions was the ability of the physician to know when and which dose to apply to every single patient, depending on his condition.

Autopsies on fatal cases after administering tuberculin revealed the disease was that bad that no hope of cure or even improvement could be expected, no matter the remedy would have been given (Ross 19--?).

Physicians developed the sense to administer the remedy empirically without having many fatal results, and they had tools to do it.

No inoculation should be repeated before the fever to pass, according to the physicians' recommendation. The worst toxic effects were the cardiac toxic effect, with increase of the blood pressure and albuminuria, thus it is understandable that cardiac complications were among the contraindications of administering the remedy. Other contraindications were great loss of strenght, amyloid or other degeneration tissue, albuminuria and urea (Ross 19--?).

As appointed by Sahli, tuberculin seemed to not have any direct healing power, but enhancing in some way the host immune response. Healthy animals tolerated large doses which would be toxic and even fatal in tuberculous animals and humans. But either in healthy and tuberculous individuals, the tolerance could be increased up to a million times by gradual increase of dose (Sahli 1912).

As explained before, any inoculation of tuberculin was characterized by a local reaction in the site of injection (painfulness, inflammation and sometimes a little uneasiness at the site of injection), a focal reaction at the site of tubercular disease (haemoptysis, pleuritic pains, swelling of tuberculous glands, cough) and a general disturbance (basically fever and pain, loss of appetite and depression); but also to be followed by an immunizing response: with an improvement of tuberculosis symptomatology, a believed increase of the antibody content in the blood and a decrease on the response to the injected tuberculin (Riviere and Morland 1913). As it was no way to predict the effect (either good or bad) of the remedy on the infectious focus, physicians had to be guided by the effects on the symptomatology to infer the amount of the effect produced by a dose of tuberculin (Riviere and Morland 1913). The optimum therapeutic dose was the maximum amount of tuberculin which could be tolerated at any particular moment without producing any severe effects, and depended of each individual, as high interindividual variability existed (Sahli 1912).

Thus physicians used dosage tables to be helped to choose the right doses. They worked with 10% dilutions, beginning at 10000, the remedy given intradermically or subcutaneously. The most common schedule implied administering tuberculin once a week (twice a week at the most), during a minimum of a fortnight. The administration was adjusted according to the tolerance appeared, but being treated in public services or private practices also influenced this point. First, the physicians themselves prepared their tuberculins, but soon some pharmacists begun to produce and sell them at every point worldwide. Some of the products were sold as syringes with already prepared dilutions,

which increased the uniformity of the preparation (Pottenger 1913) and avoided the variations in concentration of the active principle (Sahli 1912).

The physicians recommended the treatment and patients themselves bought the remedy to be administered, and even if it was cheap compared to other treatment, it still was expensive for the poor, which were the population collective more susceptible to need it (Salvat-Papasseit 2007).

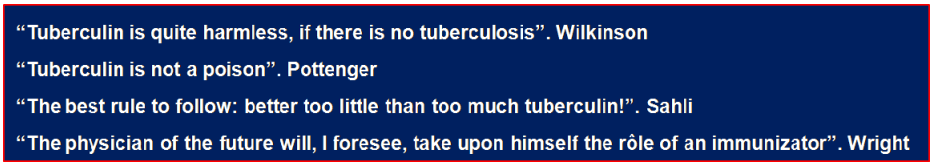

Fig. 3. Quotes on tuberculin treatment, published in the manuscripts of that time.

4. Tuberculin and its historical context

4.1 Tuberculin's use

The scientists of that time tried to stick to every treatment which seemed to give any hope to the tuberculous patients, with the basic knowledge on medicine and immunology then available. Wright invented the use of the Opsonic Index as a biomarker for predicting good responses in tuberculin treatment (Wright and Douglas 1903-1904; Wright and Reid 1906; Ogilvy 1908), which years after revealed to be non-specific (Riviere 1914; Cope 1966) and brought the idea of "Autoinoculation". Wright called the Autoinoculation to some disturbance of the site of the disease, which supposedly generated a continuous periodic escape of bacilli or bacillary toxins to the blood stream, causing bursts of clinical symptoms as any chronic disease with acute episodes would do (Wright and Reid 1906). As the balance between the host response and the virulence of the infection would be the most important fact in the way the disease would develop, while certain amount of antigen was believed to be constantly needed to immunize, its excess could fatally overpower the host response. The source of antigens needed to maintain this residual host response needed could be provided by the infectious focus itself or externally, by inoculation of tuberculin (Riviere 1926), while rest would contribute to heal the infectious foci (Canetti 1955). English sanatoria, following this idea, combined resting hours with working hours at the fresh air, as part of the therapy (Bryder 1988).

Thus tuberculin treatment was administered combined to the hygienic measures commonly prescribed at that time: rest (which would favor the healing), fresh air and an improvement of nutrition (which would favor the immune system). At that time, sanatoria had flourished all over Europe and even America, and people with means attended them to be cured of tuberculosis or at least improve their hampered health status. On 1912, more than 200 institutions in UK and the 70% of the German ones used tuberculin as a standard regimen (Riviere and Morland 1913).

But sanatoria were expensive, and even if no one was safe of suffering tuberculosis, the truth is that the poor got the worst part. They lived in small overcrowded and poor-ventilated apartments in the cities, which favored the spread of the infection, and they were

malnourished, which implied immunosupression. Charity sanatoria appeared in United Kingdom to cope with this big problem for public health, in order to both diminish the tremendous effect on the country's economy and to isolate the infectious sources (Bryder 1988). At 1921, the UK decided the remedy to be cofinanced depending on each family situation, but this measure had to be abandoned because it revealed to be non sustainable. Notes on 1937 already denounced the beneficence giving more money to cancer research to tuberculosis, as the last was considered a disease of the poor (Bryder 1988).

In 1912 it cost between 6,5 pences and 8 shillings depending on the tuberculin used (at that time, one pound was divided in 20 shillings, and each shilling into 12 pence), thus tuberculin was a cheap remedy (Riviere and Morland 1913). Receiving the treatment at non-charity sanatoria highly increased the cost of the remedy from a total of 2£ up to 32£ for the medical constant supervision, and other costs had to be added to this amount (the stay, food, etc) (Wilkinson 1909).

But the poor also had another problem: they couldn't lose their jobs, thus they didn't attend the charity sanatoria neither. With the aim of helping them considering this problem of them, Sir Robert Philip opened the Victoria Dispensary for Consumption and Diseases of the Chest in Edinburgh. Similar dispensaries appeared worldwide: in the period from 1912 and 1917 about 400 dispensaries existed in UK, 450 in America and 600 in Germany (Riviere 1926). Dispensaries were important not only because they permitted the patients to attend their jobs while being treated with tuberculin (Wilkinson 1909), but because the personnel teached the people to measure their temperature and basic guidelines on hygiene to improve their health status. Camac Wilkinson left a book devoted to these dispensaries, thoroughly describing his work at his Dispensary for the Poors in the Kennington Road of London. Besides administering tuberculin treatment and following-up the patients, the physicians in the dispensaries did screening and surveillance of contacts conducting epidemiological studies of undeniable value (Wilkinson 1909; Vilaplana and Cardona 2010). Tuberculin therapy continued to be used worldwide until the appearance of chemotherapy, when it was abandoned. Its efficacy being variable on the skills of each physician people feared the dangers of the reactions following its administration. However, a review of ancient documents provides an objective impression of the usefulness of the remedy: it was used for more than 50 years with more successes than failures, and thus even if it could be improved, it worked (Vilaplana and Cardona 2010).

The recent past years, research on tuberculosis has been focused on designing and developing new vaccines, mainly to be used prophylactically, but also therapeutic and to be given postexposure (Beresford and Sadoff 2010). The candidates are based on single antigens of Mycobacteria, obtained from cultures or by recombinant processes, or in whole organisms comminuted and/or sterilized, thus they call could be considered as tuberculins. Several candidates are in the pipeline for being used as immunotherapy, thus to be administered to a person once being exposed to the tubercle bacilli to prevent reactivation or progression to active tuberculosis, or to shorten or improve the response to chemotherapy (2009). Used as immunotherapy, all vaccines generate local reactions which intensity depends on the candidate (Johnson, Kamya et al. 2000; Sander, Pathan et al. 2009) (Vilaplana, Montané et al. 2010), and they could all be considered the local reactions described by Koch. No fatal reactions are encountered, but this could be due to the fact that nowadays infection can be easily discriminated from active disease by X ray assay, thus

patients can be carefully selected, something non even envisageable at Koch's time. Moreover, nowadays we can follow-up the patients tightly, controlling general reactions and screening serious focal reactions with imaging. So should we fear that much Koch's reaction up to the point of avoiding using vaccines therapeutically? Probably not, especially if we do consider Wright's recommendation of sterilizing as much as possible the infectious foci before administering the vaccines (Wright 1904), something now possible with the help of chemotherapy, actually an advantage that some candidates have already used as a therapeutic strategy with success (Johnson, Kamya et al. 2000; Vilaplana, Montané et al. 2010).

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21

Immunotherapy of Tuberculosis with IgA and Cytokines

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1. Introduction

Immunotherapy of tuberculosis (TB) has long been considered to be a potential adjunct to chemotherapy, by targeting 'persister' organisms which are generated during chemotherapy. In this chapter, we briefly review the current immunotherapeutic approaches in TB and then focus in more detail on a novel form of combined immunotherapy (CIT), comprising an IgA monoclonal antibody (mAb) against the α-crystallin (Acr) antigen, IFN-γ and anti-IL-4 antibodies. CIT treatment significantly reduced new pulmonary infection and also the post-chemotherapy relapse in *Mycobacterium tuberculosis* infected BALB/c mice. Translation of this approach toward application in humans has been advanced by the development and characterization of a novel human IgA1 mAb which was generated by co-transfecting the V domains of the Acr-binding 2E9 scFv clone and IgA1 constant region domains into CHO-K1 cells. The monomeric 2E9IgA1 has strong binding affinities for Acr and for the human FcαRI/CD89 receptor. Intranasal inoculation of affinity purified 2E9IgA, and mouse IFN-γ inhibited *M. tuberculosis* pulmonary infection and granuloma formation in the lungs of CD89 transgenic, but not in littermate control mice. 2E9IgA1 also inhibited infection of human whole blood and monocyte cultures. Demonstration of the mandatory role of the FcαRI/CD89 receptor for passive protection is novel and important for the elucidation of mechanisms of IgA action. Further development of the described new human mAb is required for the translation of immunotherapy for the control of TB in humans.

2. Immunotherapy of TB

TB is a major killer, causing 1.5 million deaths annually, with the majority occurring in developing countries, also bearing the brunt of the rampant HIV epidemic. Although TB chemotherapy is highly effective, it is very protracted, lasting for six months or longer. This impacts negatively on completion rates, and defaulting leads to the emergence and spread of multi-drug resistant (MDR) strains of tubercle bacilli. Although new drugs have been proposed for treatment [1], the need for new therapies is of major concern in the fight against the MDR-TB. Arresting the global TB epidemic and also reducing the incidence of

MDR-TB could be achieved by shortening the duration of the treatment. Since combined drug and immunotherapy treatments probably carry the greatest potential, several immunotherapeutic approaches have been considered, with the three described below receiving most attention.

2.1 Immunotherapeutic vaccines

One of the first immunotherapeutic applications of vaccines to show some promise in a clinical trial was the heat-killed *Mycobacterium vaccae* [2]. Its mode of action has been proposed to be an enhancement of Th1 and down-regulation of Th2 cytokine expression. Multiple doses of vaccine are required to achieve faster bacteriological conversion, improved radiological picture and recovery of body weight. However, subsequent clinical trials with *M. vaccae* produced inconclusive (reviewed in [3]), or negative results [4, 5]. Plasmid DNA expressing mycobacterial antigens have also been evaluated for their therapeutic capacity. Thus, Hsp65-based DNA vaccine prevented the post-chemotherapy relapse in mice [6], while an Ag85-expressing DNA vaccine was effective in one [7], but not in another [8] study. A detoxified extract of *M. tuberculosis* in liposome form (termed RUTI), prevented post-chemotherapy relapse in the 'Cornell model', and was proposed for the immunoprophylactic treatment of latent tuberculous infection [9]; this vaccine has recently undergone a phase 1 clinical trial in Spain (Cardona-PJ, personal communication).

2.2 Cytokine therapy

Cytokines are highly pleiotropic proteins that can promote host immune defence mechanisms. For effective treatment of mycobacterial infections, the administered cytokines must first reach their target cells, bind to the specific receptors and finally, activate an intact signal transduction pathway to elicit a cellular response. Due to their pleiotropic activities, the dose and route of administration must be carefully considered, in order to avoid the risk of toxicity and other unwanted pharmacological effects. Several cytokines have been considered for treatment of mycobacterial infections, including IFN-γ, Il-2, IL-12, GM-CSF (granulocyte-macrophage colony-stimulating factor) and G-CSF (granulocyte colony-stimulating factor). In TB patients, Th1 cytokines are produced at high levels at the site of infection, but the systemic response is characterised by high levels of Th2 and reduced levels of Th1 cytokines [10, 11]. Given the established protective role of Th1 immunity to intracellular pathogens, this provides a strong rationale for using these cytokines as immunotherapeutic adjunct treatment for TB. Two small clinical trials utilising recombinant IL-2 reported a definitive benefit in TB patients [12, 13]. However, a subsequent large-scale randomized IL-2 trial of HIV-negative TB patients yielded disappointingly negative results. Paradoxically, it even appeared that IL-2 had a detrimental effect on bacillary clearance, probably due to IL-2-mediated induction of $CD25^+$ regulatory T cells [14]. These studies show that although the cytokines carry a significant therapeutic potential, their application for treatment of TB is yet to be fully explored.

2.3 Monoclonal antibodies

Historically, the view that protective immunity against TB is imparted exclusively by T cells, but not by antibodies has been influenced by the assumption that antibodies cannot reach

the bacilli which shelter within the phagosomes of infected macrophages. However, a review of the early literature on passive 'serum therapy' indicates both positive and negative results[15], with the one consistent theme being that such treatments appeared more effective in patients with early and localised TB rather than long-standing, chronic cases. With the development of modern approaches and tools, most notably the monoclonal antibody technology, it became possible to address the role of antibodies in intracellular infections in a far more controlled, reproducible fashion. Thus, significant new evidence emerged that antibodies can play a role in suppressing intracellular infections, including those caused by *Cryptococcus neoformans* [16], *Listeria monocytgenes* [17] and *Erlichia chaffensis* [18]. This led to a reappraisal of the role of antibodies in TB, which has recently been reviewed by us [19] and others [15, 20]. However, this approach still remains contentious, and further work is clearly needed to address the role of antibodies and their potential therapeutic application in TB and other intracellular infections.

3. Evidence for a therapeutic potential of antibodies in TB

The possible protective role of antibodies in *M. tuberculosis* infection has been indicated by clinical studies, showing that antibody titres to LAM [21] or Ag85 antigens [22] were higher in patients with milder forms of active TB. Support for a protective role comes also from animal experiments showing higher level of infection in mice genetically depleted of B cells (μ-chain knock-out)[23] or defective for IgA production [24].

Recently, a significant 100-fold reduction of the postchemotherapy relapse of pulmonary infection in SCID mice was reported following intraperitoneal inoculation of mouse antisera containing predominantly IgG antibodies [25]. These antibodies were stimulated by *M. tuberculosis* infection, chemotherapy and immunization of DBA/2 mice with a detoxified *M. tuberculosis* extract. In addition, intraperitoneal administration of a standard preparation of human gamma globulin from normal donors, reduced bacterial loads in the spleen and lungs of intravenously infected mice [26]. Antibodies could have played a role, since normal human sera contain high antibody titres for LAM and mycobacterial heat shock proteins [27].

Passive inoculation of mouse monoclonal antibodies (mAb) against a number of antigens was reported to be protective in mouse models of TB infection, but the mechanisms involved differed. Thus, pre-opsonization of intratracheally administered tubercle bacilli with IgG3 against LAM antigen [28] enhanced the granulomatous infiltration and prolonged the survival of mice, without affecting the bacterial load in the lungs, while an intravenously administered IgG1 against the same antigen decreased the bacterial load, and also prolonged survival [29]. The authors of both these studies suggested that antibody action involved blocking of the LAM-mediated uptake of bacilli by macrophages.

Another study, utilising an antibody against heparin-binding hemagglutinin (HBHA) glycoprotein, showed impaired bacterial dissemination from the lungs, due to the antibody inhibiting HBHA interaction with epithelial cells [30]. In addition to the above quoted passive protection studies, *in vitro* coating of *M. tuberculosis* bacilli with monoclonal anti-lipomannan IgG3 [28] or anti-MPB83 surface glycoprotein IgG1 [31] prolonged the survival (but not the infection of lungs) of infected mice.

Taken together, these studies have clearly demonstrated that antibodies can influence *M. tuberculosis* infection, despite the intracellular location, by probably interacting with the bacilli during the extracellular phase following the initial inhalation, or the release from apoptotic macrophages. No clinical trials have been conducted as yet, but they seem justified, subject to development and evaluation of 'humanised' mAbs.

4. Immunotherapy of TB with mouse IgA mAb TBA61

IgA is the most abundant antibody class in mucosal fluids, where it plays important anti-microbial roles involving several different mechanisms of action. The majority of the IgA found in mucosal fluids is secretory IgA (sIgA), which is formed when polymeric IgA binds to the poly-immunoglobulin receptor (PIGR), expressed on the basolateral side of epithelial membranes. While retaining a portion of PIGR, the antibody is then translocated into the mucosal lumen, where it can bind to invading pathogens, leading to their neutralisation or 'exclusion' of infection. sIgA can also intercept viruses infecting epithelial cells, during the process of antibody transcytosis [32]. These important functions of sIgA, coupled with its increased stability in harsh mucosal environment, make this form of IgA antibody particularly suitable for therapeutic purposes. Unfortunately, sIgA is difficult to make in recombinant form, though advances in expression technology have been made [33]. Therefore, most of the passive protection studies have been conducted with the serum forms of monomeric IgA.

IgA can bind to a number of different cellular receptors. In addition to the already mentioned PIGR on epithelial cells, the main Fc receptor of mononuclear cells for human IgA is CD89, though its mouse equivalent has not been identified. Other known IgA receptors include the asialoglycoprotein receptor, which plays a role in IgA catabolism by hepatocytes [34], the transferrin receptor, which binds IgA1 but not IgA2 [35] and the IgA/IgM receptor (Fcα/μR), which is expressed on B cells and monocytes [36].

IgA was reported to be protective against pathogenic bacteria in a number of studies, although the mechanisms of action appear different. For example, immune exclusion was reported as the key protective mechanism against *Salmonella typhimurium* [37] and *Vibrio cholera* [38], while agglutination was shown to play a role in inhibition of *Chlamydia trachomatis* genital infection [39]. In addition, binding to a defined virulence factor and neutralisation, were the mechanisms of inhibition of *Helicobacter felis* gastric infection [40], while multiple mechanisms were suggested for IgA-mediated inhibition of *Shigella flexneri* infection [41].

Transmission of mAbs against mycobacterial antigens into the lungs following intranasal (i.n.) or parenteral administration [42] was more efficient for IgA, than for IgG mAbs. When comparing these mAbs for their protective capacity in BALB/c mouse model of *M. tuberculosis* infection, the IgA mAb TBA61, which is specific for the *a*-crystallin (Acr, 16 kDa) antigen, was superior to both an IgG1 of the same antigen and epitope specificity, and also to another IgA mAb, specific for the PstS1 (38 kDa) antigen [43]. Both monomeric and polymeric form of IgA were found to be protective, inducing an approximately 10-fold reduction of the bacterial load in infected animals. Interestingly, both pre- and post-challenge mAb inoculations were required for optimal protection and the Acr antigen specificity and IgA isotype were both important for the observed inhibitory effect [43].

Acr is a small heat shock protein of *M. tuberculosis* which is expressed at particularly high levels during conditions of anoxia and stress during growth in macrophages [44, 45]. Although the protein is largely expressed in the cytosol, an increased association with the bacterial cell wall is observed under the conditions of stress and low oxygen concentration [46]. These conditions are present during the stationary phase of growth *in vitro* and also during the intracellular phase of infection. The recent evidence suggests that *M. tuberculosis* clinical strains recovered from the sputum of TB patients have a changed phenotype consistent with stationary, rather than actively dividing organisms [47], lending further support to the importance of the Acr antigens as the antibody target. Evidence from the guinea pig model of *M. tuberculosis* infection indicates that the majority of residual 'persister' bacilli following short-term drug treatment are extracellular [48]. Most likely, such non-dividing organisms would express high levels of cell wall associated Acr, making them a suitable target for anti-Acr IgA mAbs.

The IgA-mediated inhibition of the early *M. tuberculosis* infection in mice was transient, and therefore we explored the possibilities for extending and further enhancing the observed therapeutic effect. Cytokines play crucial roles in modulating immune responses to infection, and therefore, could be harnessed to aid therapeutic treatments. We considered the immune-stimulating cytokine IFN-γ, and the suppression/removal of Th2 cytokine IL-4, that can undermine protective immunity in TB. The rationale for inclusion of IFN-γ and also the neutralising anti-IL-4 antibodies, as well as the effect of combined immunotherapy is described in the following section.

5. Combined immunotherapy for TB with IgA, IFN-γ and anti-IL-4

5.1 Rationale for IFN-γ

IFN-γ has many important activities, such as activation of phagocytes, stimulation of antigen presentation, induction of cell proliferation and cell adhesion, and regulation of apoptosis. These important roles of IFN-γ for the immune responses to pathogens are best described in the context of the so-called Th1/Th2 paradigm. IL-12, another important cytokine, directly induces IFN-γ gene transcription and secretion in antigen-stimulated naive $CD4^+$ cells [49], while in turn, IFN-γ induces IL-12 expression in macrophages and monocytes [50], thus creating a positive feedback loop. This leads against a Th1 type immune response to an intracellular infection. In contrast, Th2 cytokines IL-4, IL-13 and IL-10 suppress production of IL-12 by monocytes, and consequently also inhibit effector functions of IFN-γ, notably, the expression of inducible nitric oxide synthase [51, 52] and the respiratory burst [53].

The critical role of IFN-γ in the immunity to mycobacterial infections was confirmed in IFN-γ deficient mice, when two groups showed independently [54, 55] that mice with a disrupted IFN-γ gene were unable to control *M. tuberculosis* infection. The lack of protective immunity in IFN-γ deficient mice could be attributed exclusively to their inability to activate macrophages, since these mice otherwise developed antigen specific T cell responses, albeit more rapidly than the control mice [56]. Humans with a mutation in the IFN-γ receptor show enhanced susceptibility to TB [57] and the results of a first small scale clinical trial for treatment of MDR-TB with aerosolised IFN-γ [58] indicated a short-term treatment benefit. Therefore, IFN-γ may have a therapeutic potential for treatment of TB, although additional components may be required to achieve a more robust therapeutic effect [59].

5.2 Rationale for Th2-suppressing agents

The regulatory and potentially detrimental role of Th2 cytokines in TB has recently attracted considerable research interest, in relation to studies of both immunopathogenesis of TB and vaccine development. TB develops only in a small proportion (5-10%) of the exposed immunocompetent individuals. It is tempting to speculate that these individuals could have the normally protective innate and acquired immunity 'dis-regulated' by Th2 cell mediated inhibitory immune mechanisms. It has been proposed that IL-4 in particular, could downregulate the protective Th1 cytokine IFN-γ and lead to mediated toxicity and fibrosis [60].

However, the exact mechanism of the negative IL-4 effect on the course of mycobacterial infection is not fully understood. One possibility is that IL-4 inhibits the expression of nitric oxide synthase [61, 62] and since nitric oxide is a mandatory mediator of macrophage activation mediated killing of tubercle bacilli, its decreased levels could delay the clearance of mycobacterial infection [52].

Additional circumstantial evidence from experimental studies also points to a possible negative role of IL-4 in TB. Thus, β-glucan mediated inhibition of TGF-β, resulted in upregulated expression of IFN-γ and IL-2 and downregulated production of IL-4, leading to a significant reduction in bacterial counts in the absence of chemotherapy [63]. This was unfortunately associated with an increased risk of inflammation in the lungs, which required anti-inflammatory treatment for optimal anti-tuberculous effect.

Similarly, immunisation of *M. tuberculosis* infected mice with heat-killed *M. vaccae* resulted in decrease of bacterial burden in the lungs, which was correlated by decrease in IL-4 expression [64]. Two other immunotherapeutic vaccines have been proposed (though not tested in clinical trials), both interfering with Th2 cytokine expression levels. A DNA vaccine incorporating mycobacterial heat shock protein 65 (HSP65) was shown to be protective in mice [6] and the protection was clearly correlated with down-regulation of IL-4 production. More recently, a fragmented and detoxified *M. tuberculosis* based vaccine termed RUTI, was shown to be protective when given to chemotherapy-treated mice [9], and this effect was at least in part mediated by suppression of Th2 cytokine activity. Therefore, therapeutic approaches targeting Th2 cytokines could potentially be utilised for adjunctive treatment of TB.

6. Development and testing of CIT

IgA-mediated protection against early *M. tuberculosis* in mice could be further extended by co-inoculation with IFN-γ [65, 66]. IFN-γ was inoculated to mice i.n., 3 days before aerosol *M. tuberculosis* challenge, and then again together with IgA mAb, on the day of the infection and 2 days later. Co-administration of IgA and IFN-γ synergistically prolonged and enhanced the CFU-inhibitory effect of IgA alone and also reduced lung pathology [65].

IL-4 depleted or genetically deficient IL-4$^{-/-}$ mice are more resistant to *M. tuberculosis* infection; this could be reversed by reconstitution of mice with recombinant IL-4 [67, 68]. Combined treatment of mice with a neutralizing anti-IL-4 antibody, anti-α-crystallin IgA mAb and IFN-γ reduced lung infection with *M. tuberculosis* profoundly more than individual treatment regimens. Most importantly, however, this combined triple treatment with anti-IL-4 mAb, IgA and IFN-γ, prevented post-chemotherapy relapse of the infection in

three different strains of mice [69], suggesting that CIT has the therapeutic potential for adjunctive application with standard TB treatment.

Multiple mechanisms are likely to be involved in protection against *M. tuberculosis* conferred by CIT. Some of them may operate on a cellular level (for example, stimulation of phagocytosis by IgA and IFN-γ), while others may involve more complex interactions within the immune system, resulting in modulation of the early response to *M. tuberculosis* infection. A schematic representation of some of the potential mechanisms of CIT action is depicted in Fig.1.

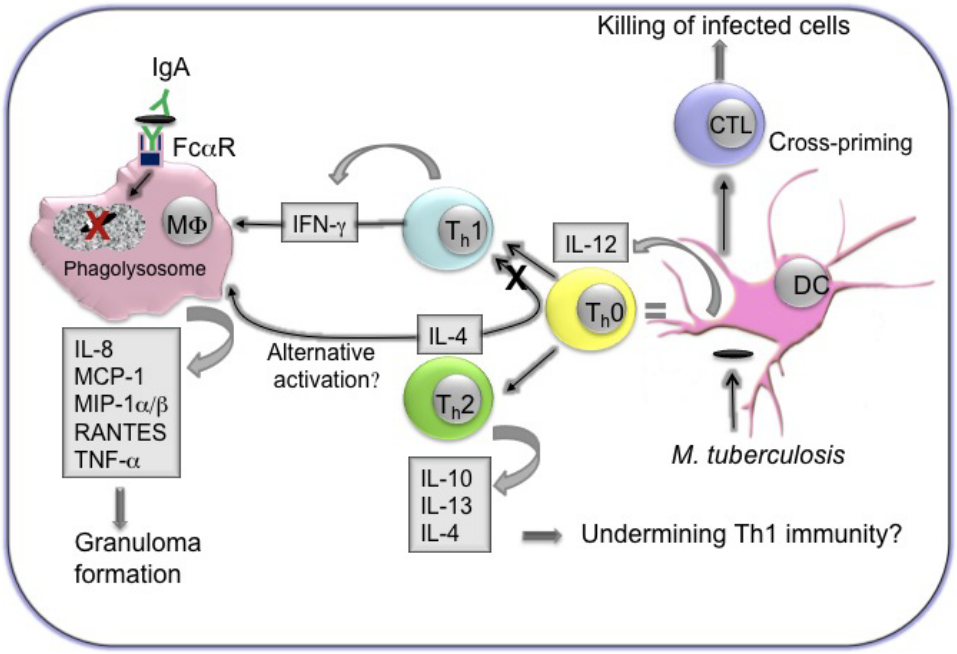

Fig. 1. Proposed mechanisms of action of combined immunotherapy (CIT) in *M. tuberculosis* infected hosts. Antibody could target extracellular bacteria and following their phagocytosis *via* IgA-receptor (indicated is human FcαR, though the mouse equivalent is not known), the bacilli are destroyed in phagolysosome. IFN-γ activates non-infected monocytes/macrophages, thus enhancing their bactericidal activity towards incipient infection. IL-4 could induce alternative activation of macrophages that does not lead to killing of intracellular organisms; in addition, it could also negatively modulate the early immune response to *M. tuberculosis*, by undermining the Th1 type response. Other, unknown mechanisms might also be involved, possibly including cytotoxicity of lymphocytes and granuloma formation.

7. Translational studies with human IgA

In order to further develop the combined TB immunotherapy for potential application in humans, a human IgA antibody specific for Acr antigen has been generated. As mentioned

earlier, the human and mouse IgA systems differ significantly, both in terms of IgA structure and also the availability of IgA Fc receptors. Thus, IgA exists in two forms in humans, IgA1 and IgA2, while the mouse IgA exists in a single form, corresponding to IgA1. The most significant difference, however, is that there is a well characterised IgA-Fc receptor on human myeloid cells, CD89, which is responsible for much of the IgA-mediated anti-microbial activity [70-72], while an equivalent receptor in mice has not be identified. Therefore, it is an important consideration that therapeutic recombinant IgA antibodies should bind efficiently not only to the target antigen, but also to CD89 on monocytes/macrophages, the target cell population for immunotherapy.

A single chain Fv fragment (scFv) specific for Acr was generated using a human phage library and then expressed in CHO cells as a human IgA1 molecule with 'grafted' scFv epitope binding site [73]. The expressed 170 kDa recombinant IgA was purified by affinity chromatography and found to be glycosylated, with both N- and O-linked sugars present. The purified human antibody, termed 2E9IgA1, bound to both Acr (7.0×10^{-8}) and CD89 (2.9×10^{-6}), with both the affinity constants being well within the range for antibody-antigen and antibody-receptor type interactions, respectively.

The effect of 2E9IgA1 on *M. tuberculosis* infection was tested in mice transgenic for the human IgA receptor. Antibody was administered at the time of infection and again, at either 1 or 21 day after infection. Separate groups of mice were inoculated with IFN-γ or with both IFN-γ and IgA. The bacterial load in the lungs and spleen, as well as the immunopathology of the lungs were analysed four weeks later. Both 2E9IgA1 and IFN-γ caused partial reduction in bacterial load, but the greatest therapeutic effect was observed when the two were co-administered together, with the difference between treated and untreated animals being statistically highly significant [73]. Early and late treatment applications following challenge of mice with *M. tuberculosis* produced a similar therapeutic effect. Importantly though, the treatment had no significant effect on the infection in non-transgenic littermate controls, suggesting a mandatory role for CD89 in the observed reduction of infection. In agreement with decreased bacterial load in the lungs, the treated animals showed also reduced granulomatous infiltration of their lungs.

Studies on whole human blood cultures infected with *M. tuberculosis* showed that 2E9IgA1 reduced the infection at least in some donors. This effect required a relatively high concentration of the antibody (100 μg/ml) and the inhibition was apparent only when the ratio of bacteria:cell was 10 or less [73]. Interestingly, IFN-γ did not enhance the bactericidal effect of 2E9IgA in whole blood cultures although it did do so in purified human monocytes infected with *M. tuberculosis*. The outcome of the *in vitro* studies is generally consistent with the finding using mouse IgA, that the therapeutic effect *in vivo* was greater than the inhibition of infection *in vitro*, hence suggesting the involvement of complex *in vivo* mechanisms of antibody action.

These studies showed that the therapeutic potentials of 2E9IgA1 human mAb for tuberculosis deserve further evaluation in the form of CIT for treatment. The history of the past advances using IgA based CIT are summarised in Table 1.

Year	Stage of development	Reference
2000	TBA61 anti-Acr mAb generated and shown to be superior to IgG for transmission to lungs	[42]
2004	TBA61 IgA induced a 10-fold inhibition of early *M. tuberculosis* infection in BALB/c mice; however, inhibition was transient	[43]
2006	Co-administration of IgA and IFN-γ extended the duration of inhibition compared to IgA alone	[65]
2007	Addition of anti-IL-4 antibody profoundly enhanced the therapeutic effect of IgA and IFN-γ	[67]
2009	CIT (IgA, IFN-γ and anti-IL4) reduced significantly postchemotherapy relapse of *M. tuberculosis* infection in mice	[69]
2011	Human 2E9IgA1 anti-Acr mAb generated and shown to be protective, when co-administered with IFN-γ, in human IgA-receptor transgenic mice	[73]
Future research	Testing of 2E9IgA1-based CIT in non-human primates and subsequently, phase I human clinical trials	-

Table 1. Key stages of development of CIT based on IgA, IFN-γ and anti-IL-4

8. Targets for future research, development and clinical evaluation

There is scope for future research on the following different aspects of the combined immunotherapy:

1. *Mechanisms of IgA action*. We proposed previously that binding of mouse IgA to the intracellular lectin galectin 3 [74], which accumulates in phagosomes [75], could 'unblock' the *M. tuberculosis* induced inhibition of phagosome maturation [19]. In principle, galectin 3 could act as a mediator of the intracellular actions of IgA, considering that it has structural homology with TRIM21, which mediates the virus neutralizing activity of IgG antibodies [76].
2. *Studies in CD89 transgenic mice*. There is a need to demonstrate: i) if there is synergy between the actions of the 2E9IgA human antibody and anti-IL-4 antibodies or IL-4 antagonists; ii) if 2E9IgA based CIT can reduce the relapse of infection following short-term chemotherapy to an extent, which had been reported for the mouse TBA61-based CIT; iii) if CIT can reduce the MDR-TB infection.
3. *Development of 2E9IgA production*. It is necessary to modify the plastic adherent CHO-K1 transfectant cell line into a suspension growing variant [77], in order to increase the yield of IgA production. This is a prerequisite for producing the GMP-grade antibody in quantities required for evaluation in clinical trials.
4. *Evaluation of 2E9IgA based CIT in non-human monkey models of TB*. Macaques are eminently suitable, since they express the IgA/CD89 receptor [78] that can bind human IgA [79]. A suitable technique for aerosol delivery of IgA would need to be developed

using the approaches for the inhaled therapy with various agents [80]. Demonstrating protection against aerosol *M. tuberculosis* infection and pathology in the macaque model of infection would justify further evaluation in human clinical trials.

5. *Evaluation in HIV-positive, low CD4⁺cell patients.* They develop active TB at a high rate and need an alternative to the current combined chemotherapy for HIV and TB [81], because it associates with drug-drug interactions and toxicity.
6. *Evaluation in patients with drug-susceptible TB, as an adjunct to chemotherapy.* The potential benefit to the widest range of patients would be to shorten the duration of treatment. This would in turn lead to higher completion rates, reduced risk of relapse and MDR-TB. The rationale of this approach has recently been strengthened by the finding, that chemotherapy generated 'persister' bacilli are extracellular [48]; this makes them a suitable target for IgA-based CIT.
7. *Evaluation in MDR-TB and XDR-TB patients*. Existing difficulties in developing effective new drugs justify evaluation of CIT as a possible alternative approach.

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22

Therapy for Tuberculosis: *M. vaccae* Inclusion into Routine Treatment

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1. Introduction

Tuberculosis (TB) – an infectious airborne disease –is a re-emerging major global health problem. Each year, there are around nine million new cases of TB, and close to two million deaths among 14 million persons with active clinical disease. All countries are affected, but 85% of cases occur in Africa (30%) and Asia (55%), of which India and China alone represent 35% (World Health Organization, 2011).

Control and cure of tuberculosis has become a very serious problem in recent years because of its association with the Acquired Immune Deficiency Syndrome (AIDS) of the Human Immunodeficiency Virus (HIV) infection and its increasing resistance to generally used antituberculosis drugs (DOTS) (Ferreira Gonçalves, M. J.; Ponce de Leon, A. C. & Fernandez Penna, M. L., 2009).

The HIV epidemic has led to an increase in the incidence of tuberculosis globally, with an important increase in the mortality rate.

Despite this, TB is in most instances, a curable disease with 85% to 90% of people with newly diagnosed drug-susceptible TB cured in six months using combinations of first-line drugs (Nunn, P.; Williams, B.; Floyd, K.; Dye, C.; Elzinga, G. & Raviglione, M., 2005). Treatment of multidrug-resistant TB (MDR-TB), of which there are around 0.5 million cases each year, is more exigent and the use of newer therapies is required. Cure rates for MDR-TB are lower, typically ranging from around 50% to 70% (World Health Organization, 2011). Extensively drug-resistant TB has been reported in 45 countries, including countries with limited resources and a high TB burden (Mitnick, C. D.; Shin, S. S.; Seung, K. J.; et. al., 2008). When tuberculosis patients (TBP) are co-infected with HIV, have drug-resistant or relapsed TB, the commonly indicated drugs are less effectives. It takes between 12-24 months to cure such patients. In these cases second line drugs are required. This involves a significant increase in the cost of therapy, particularly important in poor countries (Arjanova, O. V.; Prihoda, N. D.; Yurchenko, L. V.; Sokolenko, N. I.; Frolov, V. M.; Tarakanovskaya, M. G.; Batdelger, D.; Jirathitikal, V. & Bourinbaiar, A. S., 2011).

Considerable labors are aimed at finding new drugs and vaccines against TB and several immune-based interventions have been proposed as adjunct immunotherapy to conventional treatment.

Thus, TB is considered a re-emerging global public disease, particularly in developing countries, where its incidence has reached alarming proportions. BCG, the only vaccine available for prevention in humans has been inefficient when tested in several field trials. It is therefore an urgent need for new vaccines against tuberculosis to be developed. A better understanding of the immune response induced during infection with *Mycobacyterium tuberculosis* (*M. tuberculosis, Mtb*) could help in a relatively short time to obtain the desired vaccine against this organism (García, M. A.; Sarmiento, M. E. & Acosta, A., 2009).

TB accounted for one in four deaths among HIV-positive people. Coinfection with HIV leads to difficulties in both the diagnosis and treatment of tuberculosis. Because of the poor performance of sputum smear microscopy in HIV-infected patients, more sensitive tests—such as liquid culture systems, nucleic acid amplification assays, and detection of mycobacterial products in various body fluids—are being investigated. The treatment of coinfected patients requires a combined therapy of antituberculosis and antiretroviral drugs administered concomitantly. Difficulties include pill burden and patient conformity, drug interactions, extending beyond the toxic effects, and immune reconstitution syndrome. Both multidrug-resistant and extensively drug-resistant tuberculosis can spread rapidly among an immunocompromised population, with resulting high mortality rates. Current guidelines recommend starting antiretroviral treatment within a few weeks of antituberculosis therapy for patients with CD4 cell counts <350 cells/μL. However, important problems concerning the drug regimens and timing of antiretroviral therapy still remain unresolved. Ongoing trials may answer many of these questions (Swaminathan. S.; Padmapriyadarsini, C. &, Narendran, G., 2010).

The risk of developing tuberculosis is estimated to be between 20-37 times greater in people living with HIV than among those without HIV infection. In 2009 there were 9.4 million new cases of TB, of which 1.2 (13%) million were among people living with HIV and of the 1.7 million people who died from TB 400,000 (24%) were living with HIV. With 13% of new TB cases and 24% of TB deaths being HIV associated, TB is a leading cause of morbidity and mortality among people living with HIV and as such TB remains a serious health risk for people living with HIV. The AIDS and Rights Alliance for Southern Africa (ARASA), in collaboration with WHO hosted a workshop to develop an advocacy toolkit on the *Three I's for HIV/TB* based on WHO policy for healthcare workers, HIV/TB advocates (World Health Orgamization, 2011). Several factors including previous therapeutic failure, duration of antiretroviral therapy, low CD4+ T-cell count at the initiation of HAART, severe manifestations of disease, low adherence to HAART, and previous treatment interruption are contributory of defective immune reconstitution. It was not definitively demonstrated that age, viral strain/clade, or host genetic factors play a role in these different responses to HAART (Aiuti, F. & Mezzaroma, I., 2006).

The roles of different T-cell subsets which participate in the protector mechanisms against *M. tuberculosis*, thymic function, and cytokines involved in immune response against the bacilli have been investigated. The increased T-cell activation or apoptosis has been associated with a deficiency of effective immunologic response. The continuous virologic

replication in lymphoid tissues, regardless of the undetectable plasma viral load, has been proposed as the fundamental mechanism of cellular activation. This incoherent response probably can be associated with other procedures. Insufficient CD4+ T-cell repopulation of lymphoid tissues may be due to a thymus failure or a defect in bone marrow function. Permanent infection, the toxic effect of antiviral drugs on T- and B-cell precursors, the severity of disease, and the low number of CD4+ T-cells before HAART could also prime for thymus exhaustion and deficient T-cell renewal. Finally, an imbalance in the production of cytokines such as TNF-α, IL-2 and IL-7 may also be crucial for the induction of immune system failure. In patients in which CD4+ T-cells are not increased by HAART, therapeutic tactics aimed at increasing these cells and reducing the risk of infections are needed. IL-2 and/or other cytokines may be of benefit in this scene. Some antiviral drugs may be better than others in immunologic reconstitution. Protease inhibitors may have additional, independent positive effects on the immune system.

There may be little justification for using immunosuppressive agents such as cyclosporine or hydroxyurea in this subgroup of immunologic non responder patients, as these molecules may increase T-cell decline and/or favor susceptibility to infections

Different mechanisms are involved in the control of the tuberculosis dissemination such as granuloma.

Granulomas, the hallmark of the host response to mycobacterial infection, represent a strategy to physically contain infections that cannot otherwise be eradicated by host defenses. The successive recruitment of cells to the site of *M. tuberculosis* infection forms a physical barrier to mycobacterial propagation and creates a hostile microenvironment in which oxygen tension, pH, and micronutrient supply may all be reduced. In this environment, mycobacteria go through profound alterations in metabolism, biosynthesis, and replication.

This adaptation creates the basis of clinical latency in tuberculosis. Although these sequestered, semidormant bacilli have been much investigated, their paucity makes direct studies *in vivo* problematic, and multiple researches on this question have been performed such as *in vitro* oxygen deprivation or intracellular growth in macrophages (Wallis, R. S., 2005).

M. tuberculosis is an atypical member of its genus (Stanford, J. L.; Bahr, G. M.; Rook, G. A. W.; Shaaban, M. A.; Chugh, T.D.; Gabriel, M.; Al-Shimali, B.; Siddiqui, Z.; Ghardanis, F.; Shahin, A. & Behbehani, K., 1990). Apparently the capacity of *M. tuberculosis* to cause illness is due not only to the severity of the damage it causes to the host tissue but also to its aptitude to alter the immune response, to one that is inappropriate. It is evident that new alternative and improved treatment options are needed. In consequence, more efficient resources were considered crucial to improve the employed chemotherapy. Significant efforts have been directed at finding new drugs and vaccines against TB. (Small, P. M., 2009). Thus, the immunomodulatory effects of a heat killed *Mycobacterium vaccae* (*M. vaccae, Mv*) preparation have been investigated by Stanford, J. et. al. during the 1970´s.

It has been stated that the variation of disease expressions and severity was entirely inherent in the host and his surroundings, disease depending on human genetic control of the immunological response in interaction with environmental factors rather than to bacterial

features. In the environment a free-living mycobacterium, the potentially beneficial *M. vaccae* was recognized as an important source for influencing the human immune response (Stanford, J.L. & Paul, R. C., 1973; Stanford, J. L. & Rook, G. A.W., 1983).

Several studies using an optional new therapy, which involved the addition of a preparation of inactivated *M. vaccae*, were carried out over the last twenty-five years with successful results. In those investigations it has been shown that the killed bacterium or its components are enhancers of the immune responses in opposition to different infectious agents. A number of pre-clinical studies of tuberculosis, bronchospasm, *Trypanosoma cruzi* infection, Leishmaniasis, autoimmune conditions and cancer have been also carried out in mice, demonstrating protection induced by this treatment. (Hernandez-Pando, R.; Pavon, L.; Arriaga, K.; Orozco, H.; Madrid-Marina, V. & Rook, G., 1997; Zuany-Amorim, C.; Sawicka, E.; Manlius, C.; Le Moine, A.; Brunet, L. R.; Kemeny, D. M.; Bowen, G.; Rook, G. & Walker, C., 2002; Valian, H. K.; Kenedy, L.K.A.; Rostami, M.N.; Mohammadi, A. M. & Khamesipour, A., 2008).

Some promising results have been reported of its immune stimulative action against *M. tuberculosis* infection, tumors such as melanoma and adenocarcinoma, and pollen-induced asthma (Hopkin, J.M.; Shaldon, S.; Ferry, B.; Coull, P. P. A.; Enomoto, T.; Yamashita, T.; Kurimoto, F.; Stanford, J.; Shirakawa, T. & Rook, G. A. W., 1998; Maraveyas, A.; Baban, B.; Kennard, D.; Rook, G. A.; Westby, M.; Grange, J. M.; Lydyard, P.; Stanford, J. L.; Jones, M.; Selby, P. & Dalgleish, A. G., 1999; Stanford, J. L.; Stanford, C. A.; O'Brien, M.; Grange, J. M., 2008; Hrouda, D.; Souberbielle, B. E.; Kayaga, J.; Corbishley, C. M.; Kirby, R. S. & Dalgleish, G., 1998).

2. Clinical trials of adjunctive immunotherapy

The concept of immunotherapy in tuberculosis is not new and many immune based interventions have been investigated as adjuncts to convenional chemotherapy. It is evident that the modulation of immune reactivity can be of great therapeutic value.

IFN-γ: As IFN-γ is central to antimycobacterial host defenses; it has been used in several clinical trials of adjunctive immunotherapy. In mice, IFN-γ enhances the mycobactericidal capacity of macrophages by increasing the production of reactive nitrogen intermediates, such as nitric oxide. Condos et al. reported in 1997 the first study of therapeutic IFN-γ in patients with tuberculosis without evident defects on IFN-γ production or responsiveness. In this investigation 500 μg of IFN-γ was administered 3 times per week by aerosol to 5 patients with MDR tuberculosis together with their previous therapy. The study found that sputum smear results became negative and the number of colony-forming units (CFU) tended to fall. Three similar successive studies performed by other investigators showed that differed in IFN-γ type, dose, and route of administration were not successful in inducing any hopeful results. The only randomized, placebo-controlled, multicenter trial of inhaled adjunctive IFN-γ for MDR tuberculosis was done by InterMune in 2000, and the trial was stopped because of a lack of efficacy and the data obtained have never been published. Subsequent investigations have indicated that IFN-γ–induced genes, such as *IP-10* and *iNOS,* are already upregulated in the lung in patients with tuberculosis and that therapeutic aerosol IFN-γ has a relatively minor additional effect. These findings indicate that the fairly

small mycobactericidal capacity of lung macrophages cannot effectively be increased by therapeutic IFN-γ (Wallis, R. S., 2005).

IL-2 Considering that IL-2 is able to induce T cell replication and is essential for cellular immune function and granuloma formation, a small, unblinded study of 2 low-dose IL-2 regimens (daily or in 5-day "pulses") in patients with MDR tuberculosis demonstrated that the daily treatment produce a decrease of sputum counts of acid-fast bacilli (Johnson, B. J.; Bekker, L. G.; Rickman, R.; Brown, S.; Lesser, M.; Ress, S.; Willcox, P.; Steyn, L. & Kaplan, G., 1997; Wallis, R. S., 2005).

Taking into account this observation, a randomized, double-blind, placebo-controlled study of the effect of IL-2 on conversion of sputum culture was conducted by the Case Western Reserve University Tuberculosis Research Unit (Cleveland, OH) with 110 Ugandan, HIV-uninfected patients with drug-susceptible tuberculosis (Johnson, J. L.; Ssekasanvu, E.; Okwera, A.; Mayanja, H.; Hirsch, C. S.; Nakibali, J. G.; Drzayich Jankus, D.; Eisenach, K. D.; Boom, W. H.; Ellner, J. J. & Mugerwa, R. D., 2003; Wallis, R. S., 2005).

IL-2 or placebo was administered twice daily for the first month of standard therapy. Contrary to expectations, the study found significant delays in clearance of viable *M. tuberculosis* CFU and conversion of sputum culture results in the IL-2 treatment arm. This report suggested a possible antagonism during combined chemotherapy and immunotherapy for tuberculosis.

TNF-α: TNF-α, like IFN-γ, is crucial for host defenses against tuberculosis. TNF-α is a potent proinflammatory cytokine, expressed by macrophages and T cells, (Wallis, R.S.; Amir Tahmasseb, M. & Ellner, J. J., 1990; Black, R. A.; Rauch, C.T.; Kozlosky, C.J.; Peschon, J. J.; Slack, J. L.; Wolfson, M. F.; Castner, B.J.; Stocking, K. L.; Reddy, P.; Srinivasan, S.; Nelson, N.; Boiani, N.; Schooley, K. A.; Gerhart, M.; Davis, R.; Fitzner, J. N.; Johnson, R. S.; Paxton, R. J.; March, C. J. & Cerretti, D. P., 1997; Wallis, R. S., 2005). TNF-α stimulates the release of inflammatory cytokines, endothelial adhesion molecules, and chemokines, and is considered essential for the formation and conservation of granulomas.

Monocytes express TNF-α after phagocytosis of mycobacteria or after stimulation by mycobacterial proteins or glycolipids (Wallis, R.S.; Amir Tahmasseb, M. & Ellner, J. J., 1990; Wallis, R. S.; Paranjape, R. & Phillips, M., 1993; Valone, S.E.; Rich, E. A.; Wallis, R. S. & Ellner, J. J., 1988; Barnes, P. F.; Chatterjee, D.; Abrams, J. S.; Lu, S.; Wang, E.; Yamamura, M.; Brennan, P. J. & Modlin, R. L., 1992; Wallis, R. S., 2005). TNF-α is produced at the site of disease in patients with newly diagnosed tuberculosis (Ribeiro-Rodrigues, R.; Resende Co, T.; Johnson, J. L.; Ribeiro, F.; Palaci, M.; Sá, R. T.; Maciel, E. L.; Pereira Lima, F. E.; Dettoni, V.; Toossi, Z.; Boom, W. H.; Dietze, R.; Ellner, J. J. & Hirsch, C. S., 2002; Barnes, P. F.; Fong, S. J.; Brennan, P. J.; Twomey, P. E.; Mazumder, A. & Modlin, R. L., 1990; Bekker, L. G.; Maartens, G.; Steyn, L. & Kaplan, G., 1998; Wallis, R. S., 2005). It have been shown a small increase of TNF-α level occurs after initiation of antituberculosis therapy (Bekker, L. G.; Maartens, G.; Steyn, L. & Kaplan, G., 1998; Wallis, R. S., 2005), possibly attributed to microbial constituents that stimulate TNF-α production (Wallis, R. S.; Perkins, M.; Phillips, M.; Joloba, M.; Demchuk, B.; Namale, A.; Johnson, J. L.; Williams, D.; Wolski, K.; Teixeira, L.; Dietze, R.; Mugerwa, R. D.; Eisenach, K. & Ellner, J. J., 1998; Wallis, R. S.; Phillips, M.; Johnson, J. L.; Teixeira, L.; Rocha, L. M.; Maciel, E.; Rose, L.; Wells, C.; Palaci, M.; Dietze, R.; Eisenach, K. & Ellner, J. J., 2001; Aung, H.; Toossi, Z.; Wisnieski, J. J.; Wallis, R. S.; Culp, L.

A.; Phillips, N. B.; Phillips, M.; Averill, L. E.; Daniel, T. M. & Ellner, J. J., 1996; Wallis, R. S., 2005). Levels subsequently decrease as the bacillary burden is diminished by treatment (Ribeiro-Rodrigues, R.; Resende Co, T.; Johnson, J. L.; Ribeiro, F.; Palaci, M.; Sá, R. T.; Maciel, E. L.; Pereira Lima, F. E.; Dettoni, V.; Toossi, Z.; Boom, W. H.; Dietze, R.; Ellner, J. J. & Hirsch, C. S., 2002; Wallis, R. S., 2005). It was shown in experimental animals that neutralization of TNF-α interferes with the early recruitment of inflammatory cells to the site of *M. tuberculosis* infection and inhibits granulomas formation (Kindler, V.; Sappino, A. P.; Grau, G. E.; Piguet, P. F. & Vassalli, P., 1989; Algood, H. M.; Lin, P. L.; Yankura, D.; Jones, A.; Chan, J. & Flynn, J. L., 2004; Wallis, R. S., 2005), and TNF-α blockade also reduces the microbicidal activity of macrophages and natural killer (NK) cells (Roach, D. R.; Bean, A. G.; Demangel, C.; France, M. P.; Briscoe, H. & Britton, W. J., 2002; Hirsch, C. S.; Ellner, J. J., Russell, D. G. & Rich, E. A., 1994; Wallis, R. S., 2005).

The effects of potent immunosuppressive and/or anti-TNF-α therapies on microbiologic outcomes in tuberculosis have been investigated in two controlled clinical trials. Both were conducted with HIV-1–infected patients who had relatively well -preserved tuberculosis immune responses (based on the presence of high CD4 cell sum and cavitary lung disease). The studies shared a single placebo control arm (for tuberculosis therapy only). Their major aim was to examine the role of TNF-α in the HIV disease progression due to tuberculosis; as such, their main end points were CD4 cell count and plasma HIV RNA load. Nevertheless, both studies prospectively accrue clinical and microbiologic data as indicators of safety.

High-dose methylprednisolone: In a comparative study was reported (Mayanja-Kizza, H.; Jones-Lopez, E.; Okwera, A.; Wallis, R. S.; Ellner, J. J.; Mugerwa, R. D.; Whalen, C. C. & Uganda-Case Western Research Collaboration, 2005; Wallis, R. S., 2005) in which 189 subjects received either prednisolone (2.75 mg/kg/day) or placebo during the first month of conventional anti-TB therapy. The prednisolone dosage was selected on the basis of a phase I study indicating that it reduced the rate of tuberculosis-stimulated TNF-α production *ex vivo* by one-half. During the second month, the daily dose was reduced to 0 mg/kg; the average subject received a cumulative dose of 16500 mg. Though there is extensive experience with the use of corticosteroids to diminish tuberculosis symptoms, no previous studies have examined the microbiologic effects of doses of this magnitude. Unexpectedly, one-half of prednisolone-treated subjects had conversion of sputum culture results to negative after 1 month of treatment, compared with 10% of subjects in the placebo arm (P<0.001). This effect was bigger than that observed in the landmark study in which the addition of rifampin to a 6-month regimen of streptomycin and isoniazid reduced the relapse rate from 29% to 2% and increased the 2-month sputum culture conversion rate from 49% to 69% (East African-British Medical Research Councils, 1974; Wallis, R. S., 2005). The effect of prednisolone therapy was not due to reduced sputum production, which decreased similarly during treatment in both study arms. There were no serious opportunistic infections. However, prednisolone-treated subjects were more likely to experience other early serious adverse events, including edema, hyperglycemia, electrolyte disturbances, and severe hypertension.

Two other prospective, randomized trials of adjunctive corticosteroids administered at lower doses have observed similar, albeit smaller, effects on the kinetics of conversion of sputum culture results (Bilaceroglu, S.; Perim, K.; Buyuksirin, M. & Celikten, E., 1999; Wallis, Horne, N.W., 1960; R. S., 2005), but a third trial found no effect (Tripathy, S.P.;

Ramakrishnan, C.V.; Nazareth, O.; Parthasarathy, R.; Santha Devi, T.; Arumainayagam, D.C.; Balasubramaniam, R.; Rathasabapathy, S.V. & Manjula Datta, S., 1983; Wallis, R. S., 2005).

There have been no reports of deleterious effects of corticosteroids on microbiologic outcomes in patients withTB.

Early studies of immunotherapy for TB were those of Robert Koch who used injections of "old tuberculin" during the last 10 years of the 19th century (Koch, R., (a) 1890; Koch, R., (b) 1890).

In the early 20th century, Charles Stevens developed "Stevens cure" based on a root called Umckaloabo from South Africa (Sechehaye, A., 1920), recently shown to have potent anti-mycobacterial activity (Seidel, V. & Taylor, P. W., 2004; Kim, C. E.; Griffiths, W. J. & Taylor, P. W., 2009) and particularly to act as a TNF-α antagonist. In 1904 Friedrich Friedmann developed a turtle tubercle suspension of live *Mycobacterium chelonae*, which he later called "Anningzochin" which was available until recently from Laves-Arzneimittel GmbH, Barbarastr. 14, A-30952, Ronnenberg, Germany (Friedmann, F., 1904; Hart, C. A.; Beeching, N. J. & Duerden, B. I., 1996; Rosenau, M. J.& Anderson, J., 1915). Although Friedman investigated this mycobacterium species and showed that it was able of confer immunity against tuberculosis, he never considered that it might cause a limited tuberculous process. In the 1920s and 30s, Henry Spahlinger developed a serum from horses immunized with various extracts of tubercle bacilli (Spahlinger, H.; Macassey, L. & Saleeby, C. W., 1934). Even though many investigations supported the success of these different preparations in the treatment of tuberculosis, until very recently immunotherapy has not contributed significantly to its treatment (Sechehaye, A., 1920).

2.1 Immunomodulatory therapy in tuberculosis

Two problems confronted the early attempts of immunotherapy for tuberculosis. First, in the absence of drugs, the immunotherapy was directed towards the total destruction of the tubercle bacillus in the host. Secondly it was then thought that the triggering of immune reactivity in tuberculosis was synonymous with protection. The concept of immune reactivity in mycobacterial infections embraces both protective immunity and also tissue destruction. Distinguishing between them has been a controversial topic for many years (Stanford, J. L. & Rook, G. A.W., 1983). During the last decades it was resolved by the demonstration of two functional subpopulations of helper T cells - TH1 and TH2 (Flynn, J. L. & Ernst, J. D., 2000).

Immunotherapy, is directed to replace an inadequate immune reaction by an appropriate one. The keys to reaching success for immunotherapy arise from the evidence of the considerable variation in the efficacy of vaccination with BCG from one country to another. This is due to prior contact with environmental mycobacteria, which, depending on species, could provide some degree of protection or the antagonistic reaction of tissue necrosis.

Although the search for new vaccines and immunotherapies should continue, investigation of those already available to us is important and is the purpose of our investigations.

For many years it has been accepted that variation in clinical presentation and severity entirely rested in the host and his environment, disease depending on an interaction

between human genetic control of the immunological response influenced by environmental factors. In the environment are the free-living mycobacteria and it was from amongst them that the potentially beneficial *M. vaccae* and the deleterious *M. scrofulaceum* were identified as important factors influencing the human immune response (Stanford, J. L. & Paul, R. C., 1973; Stanford, J. L. & Rook, G. A.W., 1983). It is now established that genetic diversity within *Mtb,* expressing significant phenotypic differences between clinical isolates, may also be important (Flynn, J. L. & Ernst, J. D., 2000).

BCG is commonly referred to as a vaccine but its effects are very different from those of other vaccines and it is better designated as an immune modulator influencing susceptibility to leprosy (Truoc, L. V.; Ly, H. M.; Thuy, N. K.; Trach, D. D.; Stanford, C. A. & Stanford, J. L., 2001) and malignant melanoma (Grange, J. M.; Stanford, J. L.; Stanford, C. A. & Kölmel, K. F., 2003) as well as tuberculosis. Indeed the concept of a vaccine in its commonly used sense against tuberculosis is a difficult one as illustrated by the difficulty in interpreting the Tuberculin test. A positive Tuberculin test can signify protection, susceptibility and the presence of disease (Stanford, J. L. & Lemma, E., 1983), thus attempting to vaccinate using the species-specific, group iv antigens of *Mtb* (Stanford, J.; Stanford, C.; Stansby, G.; Bottasso, O.; Bahr, G. & Grange, J., 2009) is unlikely to be successful.

3. Immunotherapy with *Mycobacterium vaccae* in the treatment of respiratory disease

3.1 *Mycobacterium vaccae* - a part of our environment

The idea of using a saprophytic mycobacterium that causes no harm, has few side effects and is unable to induce adverse reactions in patients, as a potential immunotherapeutic or vaccine has only been considered during the last few years. *Mycobacterium vaccae* (NCTC 11,659), is a rapidly growing scotocromogenic organism.

First isolated in Germany from the surroundings of cattle, the potential and the importance of the species was first appreciated from field studies in Uganda. A killed suspension of this strain was first added to BCG and investigated as a combined vaccine. Later it was recognized as an immunotherapeutic agent. Immunotherapy with *M. vaccae* improves immune recognition of common mycobacterial antigens and also regulates immune reactions away from necrotic processes. The re-introduction of cellular responsivness to common mycobacterial antigens indicates that *M. vaccae* should induce protective immunity and suppress antagonostic responses. Looked at in the opposite way, failure to make a response to common mycobacterial antigens is an attribute of diseases that should be responsive to treatment with heat-killed *M. vaccae*.

3.2 *M. vaccae*, its adjuvants

The cell walls of all mycobacteria possess potent adjuvant activity attributed to structural lipids and glyco-lipids.

The actions of these adjuvants vary between species. Thus BCG and most species of mycobacteria enhance the type of immune response for which the recipient is already primed, whereas *M. vaccae* and probably a small number of other Actinomycetales enhance the most beneficial cellular immune responses.

3.3 *M. vaccae*, its antigens

M. vaccae possesses the group i antigens shared by all mycobacteria and most other aerobic genera of the Actinomycetales.

Some of these antigens are partially cross-reactive with those expressed by mitochondria, when stressed, in animal tissues.

M. vaccae lacks the groups ii and iii antigens, and the group iv antigens of pathogenic mycobacterial species.

All the information obtained from several studies performed in countries around the world, from minor investigations to those made using a placebo control and a properly randomized trial, show that increased cure rates in newly diagnosed TB patients receiving *M. vaccae* is only associated with minimal side effects. Studies of immunotherapy with *M. vaccae* in drug-resitant, relapsed and chronic TB Patients have shown that it is also favorable under these conditions. The effects are more readily seen when specific chemotherapy is difficult to establish or ineffective because of low patient compliance, or resistance to multiple drugs.

Progress was suggested from the early work with irradiation-killed organisms in leprosy to the study in London of modulation of tuberculin skin-test responses, and the first comparative trials in The Gambia and Kuwait. In these successive investigations the dose of 10^9 heat-killed organisms, equivalent to 1 mg wet-weight of bacilli, has been used as a standard dose. A series of small trials in Argentina, India, Nigeria, Romania, South Africa, Uganda and Vietnam have shown that the method can be effective across wide-ranging geographic variability, with South Africa as the only country where almost no effects were recorded (Dlugovitzky, D.; Stanford, C. & Stanford, J., 2011).

Despite this wide geographical efficacy, it is likely that the schedule of treatment with *M. vaccae* should change with different environments. Thus single doses were effective in the Gambia, Nigeria, Kuwait, Romania and the UK, but further South in Africa the environment may necessitate multiple doses, just as some diseases such as cancer require repeated doses to overcome the drive towards Th2 exerted by the tumour.

Numerous studies have shown that certain patterns of cellular immunity are associated with active disease and others are associated with health and recovery from disease. Modulating the immune response from the one to the other is now possible with *M. vaccae* and this chapter records its successful achievement (Ottenhoff, T.H.; Verreck, F. A. & Lichtenauer-Kaligis, E. G., 2002; Dlugovitzky, D.; Torres-Morales, A.; Rateni, L.; Farroni, M.A.; Largacha, C.; Molteni, O. & Bottasso, O.A., 1997).

3.4 Our initial studies on immune response against *M. tuberculosis*

The purpose of the early series of studies that we have carried out to investigate the immune response of patients with pulmonary tuberculosis has been to make steps towards immunotherapy as an effective addition to standard short-course chemotherapy and to identify proper *in vitro* alternative markers of successful treatment for its evaluation. A good deal of the immunological work on TB has been done on murine models – animals that have

short lives and do not normally suffer from this disease. And in consequence we wanted to make use of appropriate methods for and related to human patients.

Initial studies in our laboratory in Rosario, Argentina, have shown that the changes in cellular immune response in pulmonary tuberculosis patients are related to the severity of disease and to the administration of tuberculosis chemotherapy. We showed that increased levels of IL-8 in the pleural exudates of patients with pulmonary tuberculosis, in comparison with those patients with pneumonia-associated pleural effusions, was associated with different levels of expression of CD3, CD4, CD19, CD25 and CD68 markers on their cells (Dlugovitzky, D.; Rateni, L.; Torres-Morales, A.; Ruiz-Silva, J.; Piñesky, R.; Canosa, B.; Molteni, O. & Bottasso, O., 1997; Caruso, A. M.; Serbina, N.; Klein, E.; Triebold, K.; Bloom, B. R. & Flynn, J. L., 1999). This data suggested that increased IL-8 levels in pleural effusions plays a key role in initiation and maintenance of inflammatory reactions.

Patients with moderate to severe pulmonary tuberculosis showed a marked and significant decrease in their circulating levels of cells bearing these phenotypes when compared with those of healthy persons, with patients with pneumonia-associated pleural effusions or with patients with mild pulmonary tuberculosis. Differences between the levels of these cell markers on pleural and peripheral T-cells from pulmonary tuberculosis patients may be the consequence of an incursion of T-lymphocytes from the circulatory system to the pleural cavity, probably linked to the presence of chemokines within the pleural fluid including IL-8 (Fulton, S.A.; Reba, S. M.; Martin, T.D. & Boom, W. H., 2002).

In other assays in pulmonary tuberculosis, circulating immune complexes and the main peripheral blood T-cell subsets were evaluated (Dlugovitzky, D.; Luchesi, S.; Torres-Morales, A.; Ruiz-Silva, J.; Canosa, B.; Valentini, E. & Bottasso, O., 1995). This showed that immune complex levels in cases with severe disease are significantly higher, and expression of CD4 on T lymphocytes significantly lower than in cases of mild disease (Fiorenza, G.; Farroni, M. A.; Bogué, C.; Selenscig, D.; Martinel Lamas, D. & Dlugovitzky, D., 2007). Diverse studies of our group helped to explain the effective cellular immune response detected in less severe tuberculosis cases and simultaneously, the impaired cell-mediated immune response in severe cases. Several immune mechanisms within cell-mediated immunity generate a multifaceted response involving activated macrophages, T cells, and cytokines directed to manage mycobacterial infection. Other cell populations also take part in the immune response against mycobacteria and may be important in the development of the disease (Dlugovitzky, D.; Torres-Morales, A.; Rateni, L.; Farroni, M.A.; Largacha, C.; Molteni, O. & Bottasso, O.A., 1997; Dlugovitzky, D.; Bay, M. L.; Rateni, L.; Urízar, L.; Rondelli, C. F.; Largacha, C.; Farroni, M. A.; Molteni, O. & Bottasso, O. A., 1999; Dlugovitzky, D.; Bay, M. L.; Rateni, L.; Fiorenza, G.; Vietti, L.; Farroni, M. A. ; Bottasso, O. A., 2000).

Polymorphonuclear neutrophils (PMN) are the professional phagocytes first at the site of bacterial invasion and are able to play a protective role in opposition to *M. tuberculosis* in the early phase of infection controlled by T lymphocytes. Although recruitment of neutrophils to bronchoalveolar spaces has been described during active human tuberculosis and associated with local chemokine expression, it has not been clarified whether neutrophils have direct bactericidal or immunologic functions. *In vitro* studies suggest that human neutrophils are mycobacteriocidal and are activated by soluble mycobacterial antigens (Fiorenza, G.; Bottasso, O. A.; Rateni, L.; Farroni, M. A. & Dlugovitzky, D., 2003).

Several mechanisms including phagocytosis of bacteria and the subsequent generation of reactive oxygen intermediates during oxidative bursts are considered important instruments for destruction of mycobacteria (Jones, G. S.; Amirault, H. J. & Andersen, B. R., 1990). Several findings demonstrated a significant alteration in PMN functions in pulmonary tuberculosis. Production of reactive oxygen intermediates was reduced in severe disease and was significantly increased by antituberculosis chemotherapy (Denis, M. J., 1991). Recognition of *Mtb* by phagocytic cells leads to cell activation and production of cytokines, which in itself leads to further activation and cytokine production in a complex process of regulation and cross-regulation (Denis, M. J., 1991). Thus phagocytic cells are thought to contribute to the control of infection through the production of chemokines (Appelberg, R.; Castro, A. G.; Gomes, S.; Pedrosa, J. & Silva, M. T., 1995), the induction of granuloma formation (Riedel, D. D. & Kaufmann, S. H., 1997) and the transference of their own microbicidal molecules to infected macrophages (Ehlers, S., 2003). Levels of circulating cytokines correlate significantly with the severity of the disease, antibody concentration and the reduction of Th1 activities. We evaluated plasma cytokines of type-1 and type-2 in relation to humoral and cell-mediated responses in patients with different amounts of lung damage and with different clinical symptoms of tuberculosis. We found that patients with pulmonary tuberculosis of different levels of severity have higher serum levels of IFN-γ, IL-2, IL-4 and IL-10 when compared with those of healthy controls. Mean titers of IFN-γ, and IL-2, in mild and moderate patients were found to be greater than in those with severe disease, whereas moderate and advanced patients showed higher levels of IL-4 in comparison with mild cases. Raised levels of interleukin-10 were more prevalent in advanced disease, and statistically different from those in patients with mild disease. This cytokine pattern would explain the effective cellular immune responses found in patients with less severe tuberculosis in comparison with those of patients with advanced disease in whom cellular immunity is seriously damaged (Dlugovitzky, D.; Luchesi, S.; Torres-Morales, A.; Ruiz-Silva, J.; Canosa, B.; Valentini, E. & Bottasso, O., 1995).

We investigated the relationship between the competence of lymphocytes to proliferate and induce cytokine synthesis *in vitro*, in response to stimulation with antigens, and the amount of pulmonary involvement in tuberculosis patients. Higher levels of IFN-γ compared with IL-4 in culture supernatants of Peripheral Blood Monuclear Cells (PBMC) stimulated with *Mtb* antigens were observed in patients with mild tuberculosis (Bay, M. L.; Dlugovitzky, D.; Urízar, L., 1997). To amplify these results we assessed *in vitro* the synthesis of the cytokines - transforming growth factor beta (TGF-β) and IL-1β. Reduced concentrations of IFN-γ and IL-4 and an increased synthesis of TGF-β were observed in patients with moderate tuberculosis in comparison with those with mild disease.

In patients with severe disease, PBMC synthesize the highest levels of IL-4 and TGF-β, with low levels of IFN-γ synthesis, suggesting that in these cases an expressed Th2-type response suppresses the Th1 reaction *in vitro* (Dlugovitzky, D.; Bay, M. L.; Rateni, L.; Urízar, L.; Rondelli, C. F.; Largacha, C.; Farroni, M. A.; Molteni, O. & Bottasso, O. A., 1999).

Rook *et al* confirmed this type of response and demonstrated strong links between IL-4 and TGF-β. In their studies PBMC from patients with the most advanced TB showed the highest release of both IL-4 and TGF-β (Rook, G. A. W.; Lowrie, D. B. & Hernandez-Pando, R., 2007; Hernández-Pando, R.; Aguilar, D.; Orozco, H.; Cortez, Y.; Brunet, L. R. & Rook, G. A., 2008).

The immune system generally responds in a regulated way to microbes and eliminates them, but it does not respond to self-antigens unless regulatory mechanisms are impaired and unresponsiveness or tolerance to self-antigens is not maintained (Van Parijs, L. & Abbas, A. K., 1998). Such a disharmonic immune response may result in several autoimmune diseases. The altered Th1 and Th2 expression found in severe tuberculosis patients may lay them open to such diseases. To investigate this we inquired into the incidence of arthritic manifestations (Poncet's disease) in such patients. The kinds and distribution of T cell subsets in these cases and the presence of several auto-antibodies were also investigated. In the detected arthritic cases an augmented number of CD4+ Tcells was observed in comparison with CD8+ T cells and autoantibodies were detected. However, we could not rule out the presence of unknown factors that might be partly responsible for the reactive arthritis. (Dlugovitzky, D.; Torres, A.; Hourquescos, M. C.; Svetaz, M. J.; Quagliato, N.; Valentini, E.; Amigot, B.; Molteni, O. & Bottasso, O., 1995; Kroot, E. J.; Hazes, J. M.; Colin, E. M. & Dolhain, R. J., 2006)

In addition to these results it has been demonstrated that CD8+ cells also synthesize IL-4, and this cytokine profile correlates with cavitation (van Crevel, R.; Karyadi, E.; Preyers, F.; Leenders, M.; Kullberg, B. J.; Nelwan, R. H. & van der Meer, J. W., 2000).

Several studies have established that continuous IL-12 production is necessary for maintenance of the pulmonary Th1 cells required for host control of persistent *Mtb* infection and suggest that breakdown of this mechanism could be a contributing factor in the reactivation of disease (Feng, C. G.; Jankovic, D.; Kullberg, M.; Cheever, A.; Scanga, C. A.; Hieny, S.; Caspar, P.; Yap, G. S. & Sher, A., 2005).

The capacity of IL-12 to induce the differentiation of naive CD4+ T cells into Th1 cells and stimulate production of IFN-γ was investigated by studying the capacity of PBMNC from patients with different severities of tuberculosis to produce IFN-γ, IL-4 and IL-12. The production of IFN-γ is increased in patients with less severe tuberculosis rather than in those with severe disease (Dlugovitzky, D.; Bay, M. L.; Rateni, L.; Fiorenza, G.; Vietti, L.; Farroni, M. A. & Bottasso, O. A., 2000). In this study we also demonstrated that Tumour Necrosis Factor-alpha (TNF-α) production is increased in moderate and advanced tuberculosis patients and nitrite levels are augmented in severe tuberculosis cases, significantly different from those of healthy controls (Feng, C. G.; Jankovic, D.; Kullberg, M.; Cheever, A.; Scanga, C. A.; Hieny, S.; Caspar, P.; Yap, G. S. & Sher, A., 2005; Trinchieri, G., 2003; Casanova, J. L. & Abel, L., 2002; Fieschi, C. & Casanova, J. L., 2003). Several mycobactericidal and immunoregulatory mechanisms are developed by host cells including the production of NO and inflammatory cytokines, through extra- and intra-cellular mediated cytotoxicity, or cytostatic activity, which restrain a variety of pathogens including *Mtb* (Vouldoukis, I.; Riveros-Moreno, V.; Dugas, B.; Ouaaz, F.; Bécherel, P.; Debré, P.; Moncada, S. & Mossalayi, M. D., 1995; Kitabatake, A; Sakuma, I., 1999).

Our results suggest that the synthesis of nitric oxide by the host is not always associated with a favourable evolution since higher levels are synthesized in cases with severe tuberculosis. Other authors propose that this event may be related to the interaction of several cytokines and/or eicosanoids through disease related induction of immune reactions (Tunçtan, B.; Okur, H.; Calişir, C. H.; Abacioğlu, H.; Cakici, I.; Kanzik, I. & Abacioğlu, N., 1998). It has also been shown that an inverse correlation exists between TNF-

α, TGF-β and NO concentrations in serum, behavior that could be a predominantly TGF-β effect (Fiorenza, G.; Rateni, L.; Farroni, M A.; Bogué, C. & Dlugovitzky, D. G., 2005).

The production of NO, TNF-α and IL-12 by the peripheral blood monocytes of patients suffering from MDR-TB has been investigated by others and NO production was found to be significantly depressed. A sub-cellular fraction of *Mtb* whole cell lysate, culture filtrate protein or lipoarabinomannan induced higher concentrations of NO to be released by peripheral blood monocytes from newly diagnosed tuberculosis patients in comparison with those from MDR-TB patients (Sharma, S.; Sharma, M.; Roy, S.; Kumar, P. & Bose, M., 2004).

Respiratory diseases treated with *M. vaccae* to date have been: Pulmonary tuberculosis, Bronchial aspects of hay-fever, Bronchial asthma, Lung cancer

Related conditions under investigation Chronic obstructive pulmonary disease (COPD) in man and recurrent airway obstruction (RAO) in horses.

Arterial disease.
Myocarditis.
(These are being investigated with related bacterial immuno-modulators)

4. Salient results of immunotherapy studies in treatment of tuberculosis

In a preliminary study conducted some years ago in Carrasco Hospital, 14 pulmonary tuberculosis patients receiving heat-killed, borate-buffered *M. vaccae* (SRL172) had a better outcome than did 7 patients who received placebo (Vacirca, A.; Dominino, J. C.; Valentín, E; Bottasso, O. & Stanford, J., 1993. Subsequently we have carried out three small studies of this immunotherapy. All were performed in newly diagnosed, moderate to severe, pulmonary tuberculosis patients. In the first of these, the effects of a single dose given by intradermal injection was monitored to evaluate the potential of the approach and assess the value of the selected investigations. Levels of IFN-γ rose and TNF-α fell, with decreases also in levels of IL-4, IL-10 and anti-hsp 70 kDa (Dlugovitzky, D.; Bottasso, O.; Dominino, J. C.; Valentini, E.; Hartopp, R.; Singh, M.; Stanford, C. & Stanford, J., 1999) (Table 1). From subsequent researches performed by our group, we concluded that immunotherapy with *M. vaccae* promotes changes in the immune response and improves patient recovery.

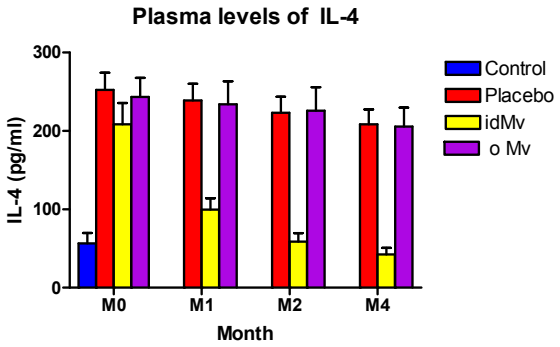

Fig. 1. Plasma levels of interleukin-4 for patients treated with placebo, intradermal or oral *Mycobacterium vaccae*. id: Intradermal, o: oral.

	Immunotherapy		Placebo	Controls
Hsp 65 kD	n=13		n=11	n=12
On admission	0.30±0.03		0.23±0.04	0.20±0.04[a]
	P<0.001		P<0.05	
After 1 month	0.19±0.02		0.2±0.02	
% decrease	32±5.5	P<0.05	15.6±5.4	
Hsp 70 kD				
On admission	0.59±0.05		0.62±0.06	0.25±0.06[b]
	P<0.001		n.s.	
After 1 month	0.31±0.03	P<0.001	0.53±0.06	
% decrease	48±3.6	P<0.0001	17±2.6	
IL-4				
On admission	685±77		586±63	69±9[b]
After 1 month	342±36	P<0.02	495±58	
% decrease	47±4.7	P<0.001	15±4.9	
IL-10				
On admission	3800±302		3863±270	35±6[b]
After 1 month	2292±187	P<0.002	3663±286	
% decrease	38±5.3	P<0.007	16.5±5.8	
IFN-γ				
On admission	524±76		553±57	157±7[b]
After 1 month	1172±173	P<0.05	700±99	
% increase	124±21	P<0.005	41±20	
TNF-α				
On admission	86±6		85.5±3.3	None detectable[b]
After 1 month	52±5	P<0.001	74±3.7	
% decrease	38±3.6	P<0.01	14±4.1	

[a] Different form immunotherapy group. P<0.02
[b] Different from immunotherapy and placebo groups. P<0.001 n.s., not significant

Table 1. Result of ELISA absorption measurements. ± SE of IgG antibodies to heat shock proteins 65 kD and 70 kD and of the serum cytokines interlukin-4 (IL-4), interleukin-10 (IL-10), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) in pg/ml

Salient results of serology and cell culture supernatant immunology of data drawn from all three studies of *M. vaccae*, injected or oral are shown. With the rise in serum IFN-γ and fall in serum IL-4 (Fig. 1), is seen a reduction in production of IgG antibodies to stress proteins and a reduction in circulating TNF-α (Fig. 2). Culture supernatants of both PBMC and PMN cells showed steady increases with time of IFN-γ and IL-2, and as the disease regressed production of TNF-α fell steeply.

In addition to these results it has been demonstrated that CD8+ cells also synthesize IL-4, and this cytokine profile correlates with cavitation (van Crevel, R.; Karyadi, E.; Preyers, F.; Leenders, M.; Kullberg, B. J.; Nelwan, R. H. & van der Meer, J. W., 2000).

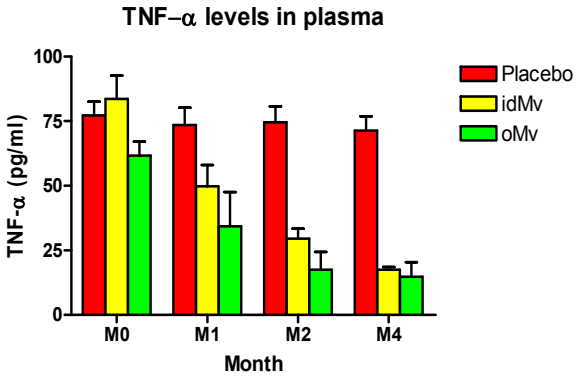

Fig. 2. Plasma TNF-α values for patients treated with placebo, intradermal or oral *Mycobacterium vaccae*. id: Intradermal, o: oral.

Respiratory Burst expression (Fig. 3) increased in the successives samples of intradermal and oral *M. vaccae* treated TBP, and it was higher in those patients receiving oral *M. vaccae* then in those receiving intradermal *Mv* in relation to placebo recipients.

IFN-γ (Fig. 4), TNF-α (Fig. 5), IL-6 and IL-10 (Fig. 6) levels in PBMC and PMN culture supernatants. and IL-6, IL-10 (Fig. 7) and TNF-α values in plasma (Fig. 2) also increased more in those receiving oral *M. vaccae* than in intradermal *M. vaccae* recipients.The immunomodulatory effect of both oral *M. vaccae* and intradermal. *M. vaccae* treatments was shown both by Respiratory Burst expression and cytokine increase in culture supernatants and plasma, with oral therapy the more effective.

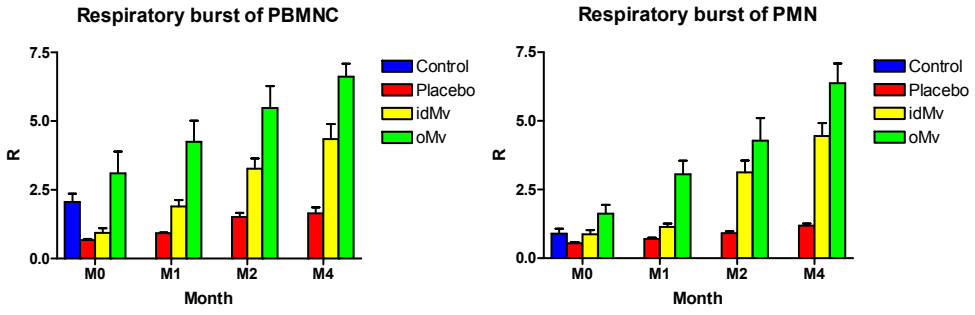

Fig. 3. Respiratory index for polymorphonuclear cells and for mononuclear cells, calculated by dividing the mean fluorescence value for H37Rv-stimulated cells by the mean fluorescence value for unstimulated cells.

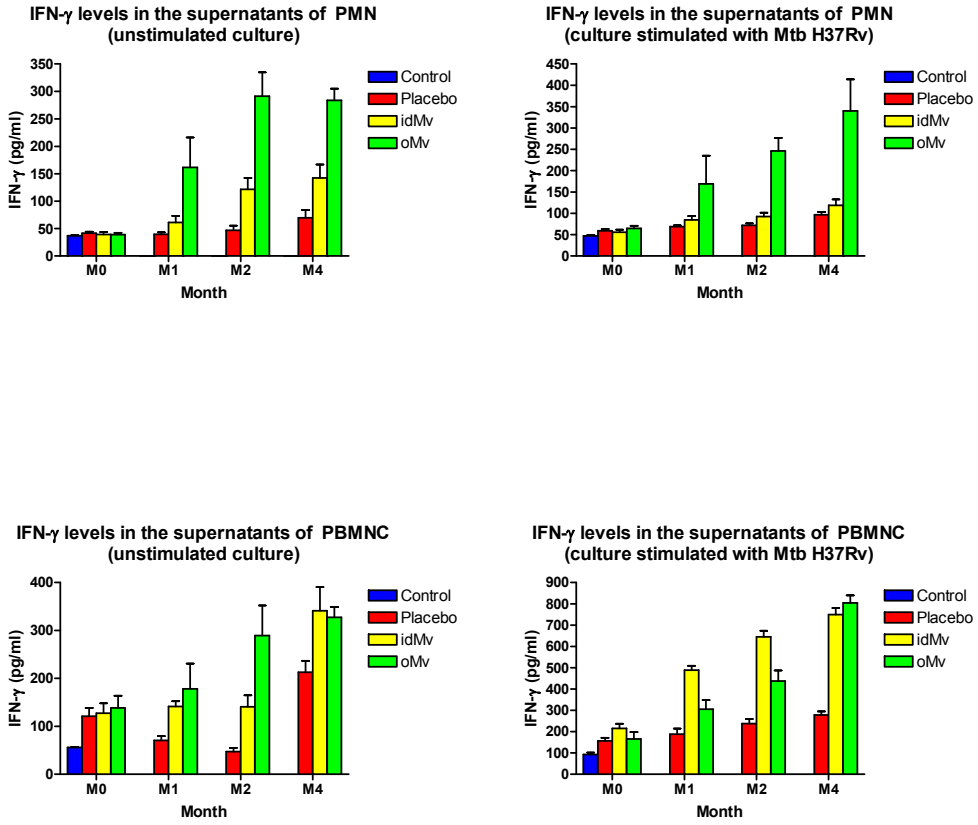

Fig. 4. IFN-γ levels in the supernatants of cultured cells

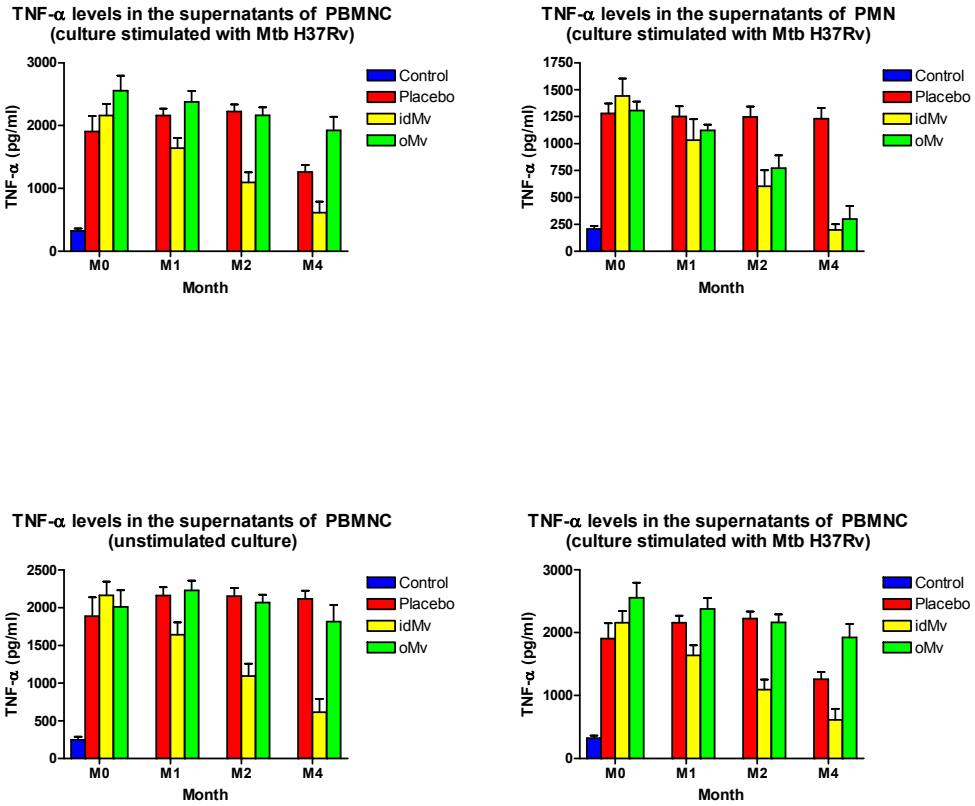

Fig. 5. TNF-α levels in the supernatants of cultured cells

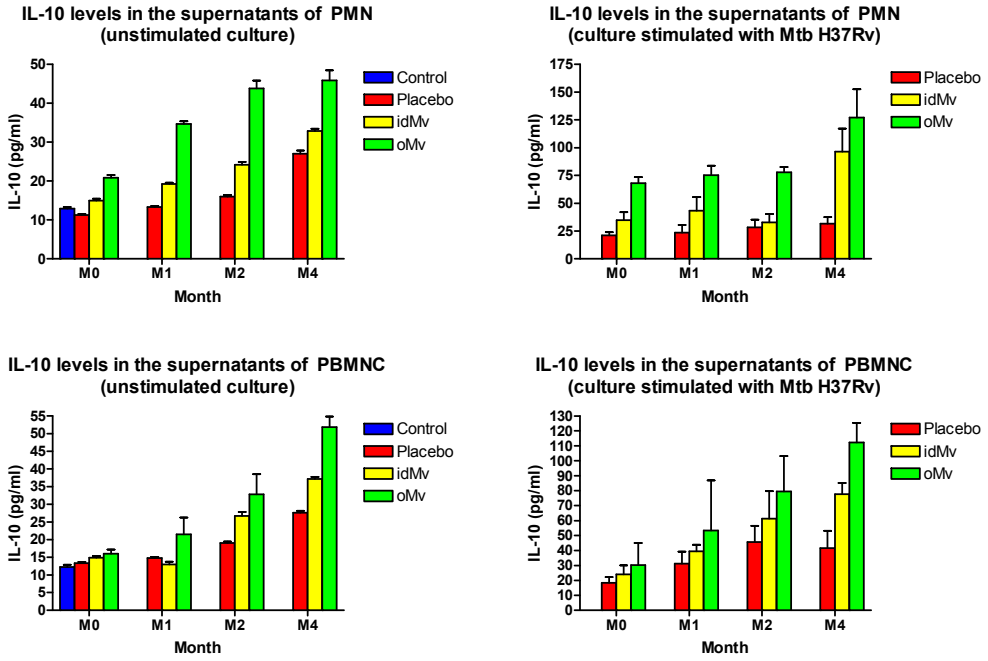

Fig. 6. IL-10 levels in the supernatants of cultured cells.

The data obtained with all 3 immunotherapy regimens produced significantly better results than those achieved with chemotherapy alone.

The data show that the addition of oral capsules of *M. vaccae* to a DOTS program in the treatment of drug-sensitive tuberculosis would have the clinical advantages of hastening sputum negativity and recovery from the disease. Such a strategy would reduce new infections, both among contacts and in the community at large and might allow shortening of the treatment period.

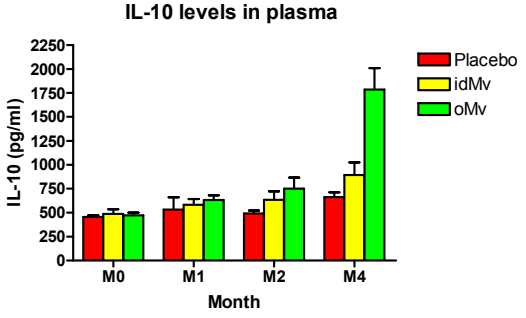

Fig. 7. Plasma IL-10 values for patients treated with placebo, intradermal or oral *Mycobacterium vaccae*. id: Intradermal, o: oral.

The mechanism of action of injected *M. vaccae* is thought to be via immunomodulating adjuvant activities of the mycobacterial cell envelope. The short amino acid-chain lengths of common mycobacterial antigens and sugars preserved by the borate buffer on dermal dendritic cells (Stanford, J. L. & Grange, J. M., 1974; Stanford, J.; Stanford, C.; Stansby, G.; Bottasso, O.; Bahr, G. & Grange, J., 2009; Hernández-Pando, R.; Aguilar, D.; Orozco, H.; Cortez, Y.; Brunet, L. R. & Rook, G. A., 2008) act to direct the modulated response to the sites of expression of host-cell stress proteins (Matzinger, P., 1994). This may be especially to those stress proteins of mitochondrial origin showing homologies with the common antigens of mycobacteria (Cohen, I. R. & Young, D. B., 1991) and the bacteriomimetic sugars expressed by rapidly replicating cells. The results reported in animals (Stainsby, K. J., 1989; Zuany-Amorim, C.; Sawicka, E.; Manlius, C.; Le Moine, A.; Brunet, L. R.; Kemeny, D. M.; Bowen, G.; Rook, G. & Walker, C., 2002; Hernandez-Pando, R.; Pavon, L.; Arriaga, K.; Orozco, H.; Madrid-Marina, V. & Rook, G., 1997) and our observations in man suggest that the same, or similar, beneficial immunomodulation can be stimulated via the mucosal immune system, where the multifold (M) cells (Gebert, A.; Rothkotter, H. J. & Pabst, R., 1996) of the intestine play a part analogous to that of the dermal dendritic cells in the skin.

In addition to the reported immunological results, the bacteriological findings indicated that the conversion to negative of both sputum smear and culture was significantly enhanced by injected or oral immunotherapy with *M. vaccae* above that achieved by chemotherapyalone.

Although this study deals with drug-sensitive tuberculosis, the reported immunological changes, which are paralleled in both injected and oral studies, allow confidence that the oral formulation will prove of similar efficacy in patients infected with drug-resistant bacilli (Stanford, J. L.; Stanford, C. A.; Grange, J. M.; Lan, N. N. & Etemadi, A., 2001). This would accord with our earlier experience of intradermal injection of *M. vaccae* in patients with a variety of drug resistance (Farid, R.; Etemadi, A.; Mehvar, M.; Stanford, J. L.; Dowlati, Y. & Velayati, A. A., 1994; Corlan, E.; Marica, C.; Macavei, C.; Stanford, J. L. & Stanford, C. A., 1997) in many countries (Stanford, J. L.; Stanford, C. A.; Grange, J. M.; Lan, N. N. & Etemadi, A., 2001), where excellent clinical results have already been obtained in the treatment of MDR-TB. As the immunological data obtained in the oral study, albeit with a more intensive schedule, paralleled that of the intradermal study it is logical to suppose that MDR-TB could also be treated successfully with oral *M. vaccae*. The properly functioning immune system recognizes and regulates the appropriate response to disease and would be capable of destroying both drug-sensitive and drug-resistant organisms quite impartially.

This approach to treatment at the outset would allow initial resistance to be treated early and at the same time discourage secondary resistance due to treatment inadequacy. As an example, at the chest hospital in Ho Chi Minh City, Vietnam, 12 patients accepted for immigration into the USA were subsequently found to be infected with highly drug-resistant organisms. They failed to be cured with the latest drugs provided from the USA, but following up to twelve injections of *M. vaccae* (administered on the initiative of the staff of the Chest Hospital) all were cured and allowed into the USA. Similar results have been obtained in several countries (Stanford, J. L.; Stanford, C. A.; Grange, J. M.; Lan, N. N. & Etemadi, A., 2001).

5. Conclusions of the 3 studies

The inclusion of immunotherapy with SRL-172 improved the results of DOTS chemotherapy and it led us to the conclusion that this therapy might allow a reduced period of chemotherapy without loss of efficacy and help to prevent the development of multi-drug-resistance. The three small studies of immunotherapy with heat-killed, borate-buffered, *M. vaccae* for drug-susceptible pulmonary TB developed in the department in Medicine Faculty of Rosario have produced successful results. It was demonstrated that the transformation of a Th2 response, towards Th1, is accompanied by clinical, bacteriological and radiological improvement in the immunotherapy recipients.

The results showed that three injected doses of *M. vaccae* were more effective than a single dose, and that ten oral doses scattered throughout the period of chemotherapy, were as effective, or more so, than was the injected preparation. The reagent deserves formal field trials, particularly in patients infected with highly drug-resistant strains of tubercle bacilli.

In conclusion, we have found that immunotherapy with *M. vaccae* in TB, whether by injection or by the oral route, hastens recovery, bacteriologically, clinically and radiologically, as well as returning immune responses towards those of healthy persons.

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Adjuvant Interferon Gamma in the Management of Multidrug - Resistant Tuberculosis

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1. Introduction

Tuberculosis is an opportunistic infection, the minute it finds an immunocompromised host, it flourishes. The risk of tuberculosis is much higher in patients who are human immunodeficiency virus (HIV) positive. Drug resistance among microbes is testimony to their adaptive skills. In *Mycobacterium Tuberculosis* the resistance occurs due to random, single step, spontaneous mutation and is invariably induced by inadequate or incomplete therapy. This resistance was termed as Multidrug Resistant (MDR) tuberculosis when the organism was resistant to more than one anti-tuberculosis drug. The presence of MDR tuberculosis, in general population, exposes the immunodeficient patients to an MDR strain of tuberculosis, which has very serious consequences for them. The risk of tuberculosis is also higher in non-HIV immunocompromised patients such as those with genetic absence of Interferon (IFN) gamma receptors, or acquired immune defect in the elderly. In either these situations IFN gamma or its absence seems to play a major role. In addition to the pulmonary infection, immunocompromised patients (with or without HIV) fall victim to extrapulmonary tuberculosis.

IFN gamma belongs to a family of endogenously produced immunoregulators that induces an array of receptors for binding to pathogens and endothelia, degradative enzymes, transcription factors and cytokines involved in host defense. These agents have antibacterial activity against host of pathogens including *Mycobacteria* (*avium* complex, tuberculosis and *bovis*). Interferon gamma has also a potent antifibrotic effect and suggests that it can lead to pulmonary lesions improvement. Exogenously administered IFN gamma has demonstrated therapeutic effect against MDR Tuberculosis, atypical mycobacterial infections, and leprosy.

Attempts to control MDR tuberculosis, is a part of the overall strategy to finally eradicate the disease. Had it not been for the emergence of drug resistance, tuberculosis would in all

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probability been wiped off by this time. The advent of acquired immunodeficiency syndrome (AIDS) has provided new fodder for the *Mycobacterium*, which thrives in the immunocompromised and protects itself by acquired resistance. A consistent strategy to control MDR by using agents such as IFN gamma can control further spread of the disease and protect individuals at risk.

In this chapter we will focus on the role of IFN gamma as the principal macrophage-activating cytokine as well as their antifibrotic properties. Later on we will showed the results of several clinical trials which recombinant IFN gamma was used as immunoadjuvant to standard chemotherapy in patients with drug-resistant tuberculosis and other mycobacterial diseases.

2. Why can Interferon gamma be used for the treatment of Multidrug Resistant (MDR) tuberculosis?

Interferon (IFN) gamma, a dimeric protein composed of 146 amino acids and variable molecular weight depending of their glycosylation patterns, was discovered in 1965. The recombinant monomeric non glycosylated form has a molecular weight of 16-17 Kd, but it is twice when the active dimeric form is formed (Schreiber & Farrar, 1993). This cytokine is secreted by CD4+, CD8+ and Natural Killers (NK) cells. Nevertheless CD4+ Th1 lymphocytes, in response to an antigenic stimulus, are the main producers (Wang et al., 1999). IFN gamma is different to other interferons regarding its physiology, activation/modulation system and genetic regulation. The most striking differences between IFN gamma and other classes of interferons concern the immunomodulatory properties of this molecule. While gamma, alpha and beta interferons share certain biological properties (e.g. antiviral, antitumoral), IFN gamma, also known as immune IFN, has potent phagocyte-activating effects not seen with other IFN preparations. IFN gamma function has been strongly conserved throughout evolution and across multiple species. The biological response to IFN gamma is mediated by a cascade of complex cytoplasm and nuclear events that presuppose as first condition the binding of the ligand (IFN gamma) to their specific surface receptor (IFNGR). This receptor is a heterodimer, with IFNGR1 and IFNGR2 chains, and is present on the surface of many inflammatory cells. Binding of IFN gamma to IFNGR leads to modulation of nuclear gene expression via the Janus kinase (JAK)-STAT signaling pathway as follows. JAK associated with IFNGR phosphorylates STAT1. This enters the nucleus, where it binds to promoter regions of IFNG-inducible genes [Schroder et al., 2004]. The rationale of the use of exogenous IFN gamma for the treatment of MDR tuberculosis is based on:

2.1 Adjunctive immunotherapy may be particularly useful in the management of difficult-to treat tuberculosis or tuberculosis in the immunodeficient host

Tuberculosis (TB) is not yet a defeated affection. Although it is a controllable infection at community level and curable in an individual manner, its eradication seems distant. TB is an endemic disease in many parts of the world steadily decimating the population. At present, at least one third of the world population is infected with the *Mycobacterium tuberculosis*. The emergency of multidrug-resistant (MDR) strains has increased this world problem, leading to a high morbidity and mortality. Global estimates showed 9.27 million

new cases of TB and 1.77 million deaths from TB in 2007 (WHO, 2009), which is the highest number of deaths attributable to a single infectious agent and corresponds to the 7th cause of death in the world. The World Health Organization (WHO) has estimated that in 2008 there were 440 000 people had MDR-TB worldwide and that a third of them died. Almost 50% of MDR-TB cases worldwide are estimated to occur in China and India (WHO, 2010). The mean survival of MDR-TB affected patients ranges from 2 to 14 months. The importance of treating MDR-TB can therefore not be overemphasized.

Directly Observed Treatment, Short-course chemotherapy (DOTS) strategy has helped prevent non-compliance and treatment failure. However drug resistance has been reduced though not eliminated, and what is been particularly worrisome is the MDR-TB. Multi drug resistance has different definitions in different countries. In the United States it is defined as resistance to rifampicin and isoniazid, while in South America it is resistance to one reserve drug in addition to rifampicin and isoniazid. It is therefore recommended that patients be classified as those resistant to the essential drugs, and those resistant to essential and reserve drugs (Mishin et al., 2002). A subset of MDR-TB strains has been identified as extensively (or extremely) drug-resistant (XDR-TB). These are now defined as being resistant not only to isoniazid and rifampin, but also to fluoroquinolones and to at least one of three injectable drugs usually employed in second-line therapy of MDR-TB: capreomycin, kanamycin and amikacin (Ginsberg, & Spigelman, 2007).

The increase in MDR-TB represents a serious set back to efforts in gaining control of tuberculosis. Resistance to drugs means a greater chance for an infected person remaining infectious and spreading the disease. It is thus imperative to control MDR-TB if we are to ever eradicate this disease. Patients of MDR-TB are difficult to treat, and mortality is significantly higher than in TB caused by susceptible organisms, as is the rate of re-infection. MDR tuberculosis poses a threat to both, the patient and the society. The patient is at risk of losing if not the life, a part of the lung permanently, while the society is at a risk of an MDR-TB epidemic (Yew, 2011). Such an epidemic is a serious threat to the life of individuals with a compromised immune system. The number of people with co-infection of HIV and TB is rising by leaps and bounds. This is a population at very high risk since, TB is the largest single cause of death in HIV infected persons (Daikos et al., 2003).

The infection is mainly transmitted by inhalation of the bacilli coming from infected secretions of the respiratory airways. Once inhaled, the bacilli are subjected to phagocytosis within the alveolar macrophages, where they can be destroyed. Nevertheless, *Mycobacterium tuberculosis* has developed mechanisms to adapt to the noxious intracellular environment of macrophages and escapes the host's innate immunity. It uses several strategies to avoid their destruction, including inhibition of the acidification/maturation of the phagosomes and phagosomal-lysosomal fusion (Pietersen et al., 2004) or by a directly inhibition of the human T cell IFN gamma production and proliferation in response to stimulation (Peng et al., 2011). Thus the *mycobacteria* can persist, replicate and disseminate, leading to new infectious foci. The emergence of resistance depends on several factors such as bacillar initial load, inadequate or incomplete chemotherapy administration, and the patient's immune condition.

Chemotherapy is successful in most cases given that they follow thoroughly the treatment schedule, which is prolonged, costly, and needs to be directly observed. Otherwise it is inadequate to kill all the bacilli and drug resistance emerges. Toxicities are frequent as well.

Treatment for MDR-TB typically requires 18–24 months of combination therapy with second-line drugs that are less efficacious, more toxic and much more expensive than the four first-line drugs. TB treatment in HIV-positive patients is further complicated by drug-drug interactions between some of the antiretroviral agents and key antituberculous drugs, especially rifampin. As *Mycobacterium tuberculosis* drug resistance is increasing worldwide, there is an urgent need for novel interventions in the fight against tuberculosis. The main goal consist in improving capacity to treat existing drug-resistant cases effectively, in order to provide patients with the greatest opportunity for a successful outcome (Ginsberg, & Spigelman, 2007). At the global level, the rational use of existing compounds must be urgently promoted to preserve their utility in treating the most difficult tuberculosis cases and intensify efforts to develop novel interventions (including new drugs and vaccines) to fight tuberculosis more effectively.

The immunologic approach to TB treatment can be promising since only 10 - 20% of infected people develop the disease and many of them have spontaneous remission. Therefore, an alternative therapeutic target can be directed to the manipulation of the host's defenses. In patients with active tuberculosis, *M. tuberculosis*-specific T-cell responses are low, and tissue-destructive and macrophage-deactiviting cytokines are upregulated. These patients have a relative weakness of production of the Th1-like cytokines Interleukin (IL-2) and IFN gamma. By contrast, the production of the immunosuppressive/macrophage-deactiviting cytokines Transforming Growth Factor (TGF) beta and IL-10 is upregulated (Tomioka, 2004). These immune dysfunctions correlate with the extent of pulmonary tuberculosis, more markedly in HIV-infected patients (Zhang et al., 1994). TGF beta is produced in excess by monocytes of patients with tuberculosis, and is present at sites of tuberculous granulomas (Aung et al., 2000).

Therapies that would upregulate the host immune response and/or attenuate the effects of tissue-damaging, macrophage-deactivating and/or T-cell-suppressive cytokines may prove to be helpful in the treatment of tuberculosis, particularly MDR-TB and tuberculosis among patients with HIV infection. Enhancing host immune responses by adjunctive immunotherapy may truncate the duration of chemotherapy, and thereby abolish the need for administration of and compliance with complex drug regimens. In that sense, T helper 1 cytokines, such as IFN gamma, IL-2, and IL-12 through increment of T-cell function and macrophage activation may prove to be potent immunotherapeutic agents.

Interferons are endogenous immunomodulators that play an active role in protecting the individuals from opportunistic infections. They were first used for the treatment of hard to treat virus and fungal infections, now with the availability of recombinant IFN it is possible to use this agent for the treatment of infections caused by drug resistant organisms. IFN gamma activates macrophages and also promotes a range of host immune responses. It helps in decreasing the bacterial load by a number of intermediate messengers such as the superoxide moiety, hydrogen peroxide, etc (Mata-Espinosa & Hernández-Pando, 2008).

2.2 IFN gamma plays a key role in the modulation of immune response and is responsible for the defense against intracellular *mycobateria*

Due to their pleiotrophic effects on the immune system, IFN gamma was thought to have great promise as an immunomodulatory drug. IFN gamma has been shown to be important

for the function and maturation of multiple immune cells. It is essential for Th1 immune responses and regulates T cell differentiation, activation, expansion, homeostasis, and survival. Killing of intracellular pathogens requires IFN gamma production by T cells showing to be a critical cytokine in the resistance of infected macrophages. T regulatory cell (Treg) generation and activation requires IFN gamma. This cytokine stimulates dendritic cells and macrophages to upregulate the immune response. NK cells secrete IFN gamma early in host infection, facilitating immune cell recruitment and activation. IFN gamma also activates NK cells and enhances the antibody-dependent cellular cytotoxicity (ADCC). It recruits neutrophils, stimulates them to upregulate chemokines and adhesion molecules, and triggers rapid superoxide production and respiratory burst (Miller et al., 2009).

As most of the intracellular infections, immunity to tuberculosis depends on the development of CD4+ T cells- and macrophages-mediated Th1 response. The proper formation and function of granulomas at sites of *Mycobacterium tuberculosis* infection depends on the collective activity of several cytokines. Enough evidences exist related to the action of IFN gamma on the immunoregulatory activity of macrophages, including alveolar macrophages, which are important in host immunity against *M. tuberculosis* (Tomioka, 2004). There is present certain heterogeneity in human IFN gamma responses to *M. tuberculosis* according to specific strain sensibility (Cabral et al., 2010).

The role of IFN gamma as the main macrophage–activator Th1 cytokine has been clearly established in animal models infected with *M. tuberculosis* since it was able to produce bacilli destruction. Mice rendered incapable of IFN gamma production by gene targeting develop widespread mycobacterial infection with very poor granulomatous response and succumb rapidly. Exogenously supplied IFN gamma has not able to restore normal mycobacterial resistance in these mice, suggesting that IFN gamma plays a critical development role as well (Flynn et al., 1993).

IFN gamma action on the macrophages leads to kill intracellular *Mycobacteria*. Their broad range of biological activities include stimulation of macrophages to produce Tumor Necrosis Factor (TNF) alpha, oxygen free radicals (superoxide anion and H_2O_2) and nitric oxide, increases MHC surface antigens and Fc receptors display, increases expression of costimulatory molecules and decreases lysosomal pH. IFN gamma and TNF alpha cooperate in the induction of phagocytic activity in the mononuclear cells and are also involved in the regulation of the inflammatory response. IFN gamma downregulates the production of the macrophage-inhibitory cytokines IL-4 and IL-10. Additionally, IFN gamma increases the intracellular concentration of certain antibiotics among then macrolides and quinolones (Herbst, 2011; Holland, 2001; Tomioka, 2004). Therefore, its use as adjuvant is justified since existent multidrug therapy, despite its limited efficacy, must be offered to the patients.

The general involved pathway is the following: IL-12 and the pro-inflammatory cytokines (IL-1, TNF alpha and IL-6) are produced early after the interaction of *Mycobacterium tuberculosis*-infected macrophages and CD4+ T cells, and upregulate CD4+ T-cell production of IFN gamma and IL-2. IFN gamma upregulates macrophage ability to contain the growth of *M. tuberculosis*, and IL-2 is key in the clonal expansion of specific CD4+ T cells. IL-10 and TGF beta are later products of these macrophages, and both inhibit the CD4+ T cell cytokine (IL-2, IFN gamma) response and interfere with the effects of IFN gamma. TGF beta is also auto-induced (Figure 1).

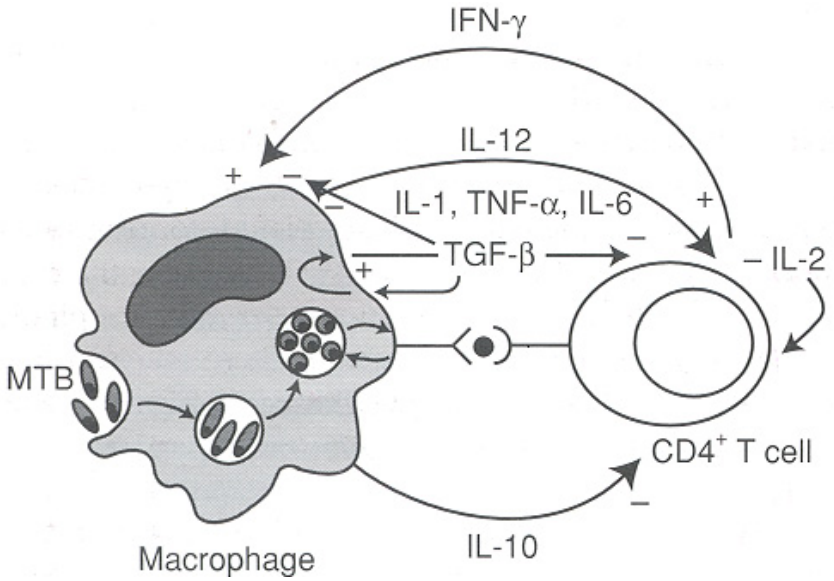

Fig. 1. Schematic representation of the known cytokine network produced by the interaction between CD4+ T cells and *Mycobacterium tuberculosis* (MTB)-infected macrophages.

IFN gamma and TNF alpha are present *in situ* in the paucibacillary pleural form of tuberculosis, in which the host successfully contains the replication of *M. tuberculosis*. In contrast, TGF beta increases the intracellular growth of *M. tuberculosis*. Also, neutralizing antibody to TGF beta reduce the intracellular growth of *M. tuberculosis* in monocites. TGF beta interferes with the production of TNF alpha and IFN gamma and it also downmodulates the bactericidal effect of both cytokines in *M. tuberculosis*-infected monocytes (Hirsch et al., 1994).

The Th1 cells-mediated generation of toxic oxygen metabolites within phagocytes *in vitro* is also capable of mediating the intracellular killing of other selected bacterial or parasites microorganisms such as *Staphylococcus aureus*, *Toxoplasma gondii*, *Leishmania donovani*, *Listeria monocytogenes*, *Mycobacterium avium intracellulare*, *Mycobacterium Leprae*, *Mycobacterium ulcerans* and *Trypanosoma cruzi* (Billiau et al., 1998; El Ridi et al., 2006; Silva et al., 2009).

Mice models confirm the requirement of T CD4+ cells for immunity to *M. avium* strains with low or intermediate virulence. Addition of IL-4 or IL-10 to macrophages culture tried with IFN gamma inhibited the generation of oxygen free radicals (Holland, 2001; Tomioka, 2004). On the other hand, IFN gamma plays an important role in the resistance to *M. leprae* infection (Lima et al., 2000). Those individuals who present absence of IFN gamma and live in endemic areas of visceral leishmaniasis have disease progression (Carvalho et al., 1992).

The concept that IFN gamma can be useful in mycobacterial infections is supported by individuals with impaired IFN gamma action. Lack of production of this cytokine or expression of its receptor increase susceptibility to develop the disease or is associated to the infection's most lethal forms or disease progression. Recurrences or development of the

serious forms of infections with atypical *mycobacteria* have been detected in certain families that present mutations in the gene encoding for the IFN gamma receptor binding chain (IFNGR1) (Sexton & Harrison, 2008). Patients with defects in the production of IFN gamma or partial deficiencies of IFN gamma receptor can obtain benefits with IFN gamma treatment (Hallstrand et al., 2004). Similar outcome could be obtained in patients with dysfunctions related to other Th1 cytokines and their receptors (Alangari, et al., 2011). Additionally, patients without genetic disorders but with serum anti-IFN gamma autoantibodies have a higher susceptibility to develop Mycobacteriosis (Kampitak et al., 2011). IFN gamma production appears to decline with age, and this may contribute to the increased susceptibility of the elderly to mycobacterial infection (Rink et al., 1998).

Although for many years IFN gamma have been considered as a pro-inflammatory cytokine, sometimes associated with the pathogenesis of inflammatory and autoimmune diseases, more and more evidences of their anti-inflammatory actions appeared nowadays, supposing a dual effect. It unregulated several pro-inflammatory parameters such as IL-12, TNF alpha, IFN-inducible protein 10 (IP-10), among others, but it also induces anti-inflammatory molecules as IL-1 receptor antagonist (IL-1Ra) or IL-18 binding protein (IL-18BP), modulates the production of pro-inflammatory cytokines, and induces suppressive pathways of the inflammation (Mühl & Pfeilschifter, 2003).

2.3 Interferon gamma has also a potent antifibrotic effect

Extensive tissue destruction, formation of cavities, and fibrosis are characteristic of the pathology of human tuberculosis. Although some components of the *mycobacteria* may be directly associated in activating cellular proteases, most of the affection induced by the organism is probably cytokine-mediated.

The molecular biology of the fibrosis is characterized by a shift to increased production of Th2 cytokines and decreased production of Th1 cytokines. Th1 cytokines promote cell-mediated immunity and remove cellular antigens; decrease fibroblast procollagen mRNA, fibroblast proliferation, and fibroblast-mediated angiogenesis; and downregulate the growth mediator TGF beta. Contrarily, Th2 cytokines promote humoral immunity and produce antibody responses that can lead to fibroblast activation and fibrosis. The Th1 response is characterized by increased expression of IFN gamma, IL-2, IL-12, and IL-18. The net effect of a predominantly Th1 response is tissue restoration. The Th2 response is characterized by increased expression of IL-4, IL-5, IL-10, and IL-13. The net effect of a predominantly Th2 response is fibroblast activation and matrix deposition, leading to fibrosis (Figure 2). IFN gamma appears to restore the balance between Th1 and Th2 responses.

Enough evidences demonstrate the relevant role of IFN gamma to control the disease, since its antifibrotic properties. IFN gamma inhibits lung fibroblast proliferation and chemotaxis in a dose dependent manner. In the bleomycin-induced model of lung fibrosis, IFN gamma downregulates the transcription of the gene for TGF beta but production of IFN gamma may be decreased in patients with Idiopathic Pulmonary Fibrosis (IPF). IFN gamma reduces collagen synthesis and increases the activity of the collagenase (Tredget et al., 2000; Williams et al., 2008). Furthermore, IFN gamma contributes to the tissue repair and their remodeling (Pilette et al., 1997). This antifibrotic action agrees with that obtained with IFN gamma in IPF patients (see last paragraph on this section) and suggests that FN gamma may have a

potential therapeutic role in the management of pulmonary fibrotic diseases, including tuberculosis (Williams & Wilson, 2008; Zhang & Phan, 1996).

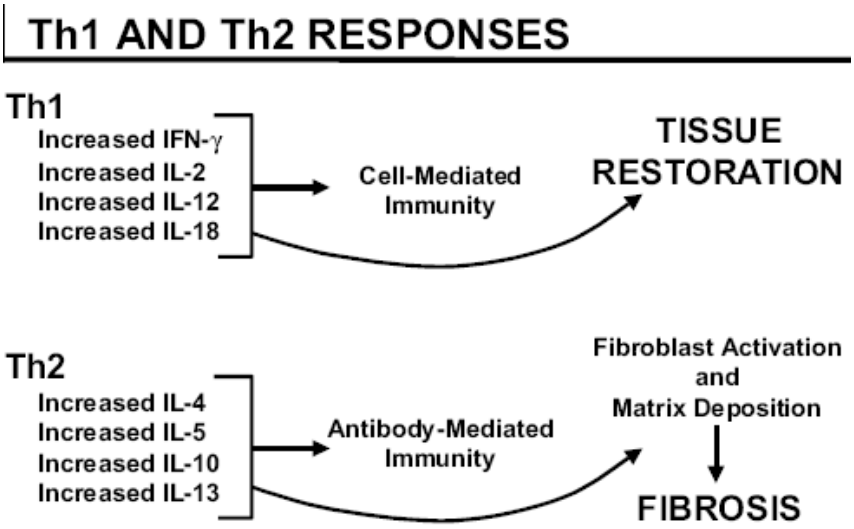

Fig. 2. Th1 and Th2 Responses and pulmonary fibrosis.

TGF beta and IFN gamma have opposite effects on diverse cellular functions and the fibrotic events are not an exception. The excessive production of TGF beta is associated with extensive fibrosis and tissue damage. TGF beta is a strong inhibitor of epithelial and endothelial cell growth, and while it promotes the production and deposition of collagen matrix, it has also shown to increase the production of macrophage collagenases. Mice injected intraperitoneally with TGF beta develop generalized fibrosis (Xu et al. 2003). IFN gamma is a potent antagonist of TGF beta (Tredget et al., 2000), involved directly in the pathogenesis of many fibrotic lung diseases (e.g. IPF, bleomycin-induced fibrosis and sarcoidosis) (Zhang & Phan, 1996).

TGF beta signals through a receptor serine kinase that phosphorylates and activates the transcription factors Smad2 and Smad3, whereas the IFN gamma receptor and its associated protein tyrosine kinase Jak1 mediate phosphorylation and activation of the transcription factor Stat1. IFN gamma inhibits the TGF beta–induced phosphorylation of Smad3 and its attendant events: the association of Smad3 with Smad4, the accumulation of Smad3 in the cell nucleus, and the activation of TGF beta–responsive genes. IFN gamma, acting through Jak1 and Stat1, induces the expression of Smad7, an antagonistic SMAD, which prevents Smad3 from interacting with the TGF beta receptor. The results indicate a mechanism of transmodulation between the STAT and SMAD signal-transduction pathways and suggest a role for IFN gamma in the treatment of pulmonary fibrosis (Ulloa et al., 1999).

The first report about the use of IFN gamma in IPF demonstrated a considerable clinical improvement in these patients treated during one year compared to those that received placebo (Ziesche et al., 1999). Afterward, a phase III study was carried out, but no significant advantages in progression-free survival, pulmonary functionality or quality of life were

observed. Nevertheless, patients with an initial less deteriorate pulmonary function impairment showed better survival (Raghu et al., 2004). Other authors indicate that IFN gamma can slow or arrested the loss of lung function, increase longevity and make possible lung transplantation (Nathan et al., 2004). Long-term treatment with this cytokine may improve survival and outcome in patients with mild-to-moderate IPF (Antoniou et al., 2006). However, the members of the recent INSPIRE trial declared that they cannot recommend one-year treatment with IFN gamma-1b since the drug did not improve survival in this disease (King et al., 2009). Our group found that in IPF a rapid clinical response could be obtained with a therapeutic schedule with IFN gamma combined with decreasing-dose prednisone (Cayón et al., 2010).

3. Clinical application of recombinant IFN gamma in multidrug – resistant tuberculosis and other mycobacterial diseases

There are reported several clinical trials where IFN gamma was used in combination with anti-TB drugs for the treatment of pulmonary TB. Some of these trials were conducted in drug-susceptible patients. Therefore, in our opinion these last studies have lower relevance or clinical impact than MDR-TB cases; despite in some of them combined treatment yielded better results than chemotherapy alone. In this review we include, in chronological order, several uncontrolled or controlled trials in patients with MDR-TB. Available communications of case report will be also included. Different routes of administration, subcutaneous, intramuscular, aerosol, have been evaluated for this immunoadjuvant cytokine. The aerosol route of administration has been proposed as organ specific delivery method, obtaining a high release to infected alveoli (Condos et al., 2004).

In spite of their high heterogeneity most of the studies refer as primary outcome the sputum negative conversion (sputum smear and/or *M. tuberculosis* culture) at a specific number of months after therapy. The secondary outcomes included chest radiographic improvement and severe adverse events. Chest radiographic improvement was defined as a decrease in the extent of lesions in the lungs, and some cases as a >50% decrease in the cavity size at a specific number of months after treatment. Other outcomes included biochemical variables reflecting immune function, and bacteriological relapse after completion of treatment. Nevertheless all trials did not have remarkably large sample sizes, which made it difficult to obtain definitive evidences.

Systemic or aerosolized IFN gammas have been reported as satisfactory in other similar intracellular infections, including other mycobacterial infections (e.g. intrinsically resistant *Mycobacterium avium*). At the end of this chapter we also show the most relevant reports regarding these species. The majority of the clinical trials here presented have been performed using Actimmune® (InterMune) or IFN gamma-1b, a genetically engineered form of human IFN gamma.

3.1 Clinical trials and case report of aerosolized or systemically administered IFN gamma in patients with drug-resistant tuberculosis

In a first report (Condos et al., 1997) safety and tolerability of aerosolized IFN gamma was investigated in patients with MDR-TB in an open-label trial. In addition, its efficacy in terms of sputum-smear grades was assessed. Aerosolized IFN gamma was given to five patients

with smears and cultures positive for pulmonary MDR-TB, despite documented adherence to therapy. The patients received 500 micrograms three times a week for 1 month. IFN gamma was well tolerated by all patients. In all five, bodyweight stabilized or increased. Sputum acid-fast-bacillus smears became negative in all patients, and the time to positive culture increased (from 17 to 24 days, not significant), which suggested that the mycobacterial burden had decreased. The size of cavitary lesions was reduced in all patients, 2 months after treatment had ended. These preliminary, encourage data suggested that IFN gamma may be useful as adjunctive therapy in patients with MDR-TB who are otherwise not responding well to therapy.

Later on, a randomized, placebo-controlled, multicenter trial of inhaled adjunctive IFN gamma for MDR-TB was initiated by InterMune in 2000 (InterMune, 2000). The trial was halted prematurely because of a lack of efficacy, but its findings have never been published.

We carried out an open-label, non-randomized, non-controlled, pilot trial with the aim to evaluate IFN gamma effect on drug resistant pulmonary TB patients regarding their clinical, bacteriological and radiological evolutions (Suárez-Méndez et al., 2004). The study population was constituted by Cuban patients, both sexes, more than eighteen years old, with diagnosis of TB without a favorable response to the usual therapy, who gave their written, informed consent to participate. Patients received 1 x 10^6 IU of human recombinant IFN gamma (Heberon Gamma R®, Heber Biotec, Havana, produced in *Escherichia coli*, specific activity of 10^7 IU/mg protein), intramuscularly, daily during 4 weeks and then 3 times per week for the next 20 weeks. They received anti-TB drugs (WHO schemes) (Crofton et al., 1997), according to the resistance detected in each case by the antibiogram. After the end of the 6-months IFN gamma treatment period, chemotherapy continued up to 9 months if the scheme included rifampin and 18 months otherwise. Complete response was defined as total disappearance of all signs and symptoms, negative sputum acid-fast-bacilli smear and culture, and pulmonary lesions improvement at X-ray. Partial response included signs and symptoms decrease, negative sputum smear and culture and stable X-ray lesions. No response consisted in signs and symptoms persistence, positive bacteriological examinations, and lesions stabilization or progression.

Five of the eight included patients were men, six of them non-white. The age ranged between 23 and 54 years old, and Body Mass Index (BMI) between 13.2 and 22.0 Kg/m^2. Their main symptoms were cough, expectorations, dyspnea, stertors, distal cyanosis, and finger clubbing. Bacteriological tests codification was mostly high and all patients showed active lesions at thorax radiography. A rapid favorable evolution was obtained after treatment with IFN gamma (Table 1).

Clinical improvement was evident since the first month of treatment, when all signs and symptoms (except for finger clubbing) had disappeared in all patients and BMI increased in all but one of them. Sputum acid-fast-bacilli smears and cultures were negative since the 1 - 3 months of treatment. The eight patients had radiological improvement, with lesions size reduction (total disappearance in one case) (Figure 3). This radiological effect cannot be attributable to the antibiotics, since it is well known that DR-TB patients only develop radiological improvement long time after sputum smears and culture become negative. In many cases extensive fibrotic lesions never improve, and stay stable for life. Globular sedimentation rates decreased (2 of them normalized) in five out of 6 patients who had

abnormal values at inclusion. At the end of the IFN gamma treatment all the patients were evaluated as complete responders (Suárez-Méndez et al., 2004).

Patient	1	2	3	4	5	6	7	8
Drug regimen	ETB ETN PRZ CPF KAN	RIF ETB PRZ KAN	ETB ETN PRZ CPF KAN	ETB ETN PRZ CPF AMK	RIF ETB PRZ KAN	ETB ETN PRZ CPF KAN	RIF ETB PRZ KAN	RIF ETB PRZ KAN
Gain BMI (Kg/m^2)	1.8	0.4	0.4	0.4	0.3	2.2	1.8	- 2.1
Sputum smear status	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Sputum culture status	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Conversion time	2 mo.	3 mo.	3 mo.	1 mo.	3 mo.	2 mo.	2 mo.	3 mo.
Thorax X-ray	Residual fibrosis	Reab-sorption and residual fibrosis	Residual bilateral fibrosis.	Lesions resolution	Residual fibrosis	Lesions size reduction	Residual fibrosis	Lesions size reduction
GSR (mm/h)	5	48	26	40	42	20	23	77

BMI: Body Mass Index, GSR: Globular sedimentation rate. RIF: Rifampin; ETB: Ethambutol; ETN: Ethionamide; PRZ: Pyrazinamide; CPF: Ciprofloxacin; KAN: Kanamycin; AMK: Amikacin.

Table 1. Six months follow-up data of DR-TB patients treated with IFN gamma.

The treatment with Heberon Gamma R® was safe and well tolerated. The adverse events were arthralgias, fever, headache and asthenia. All adverse events were mild, except for one moderate fever, which was efficiently controlled with acetaminophen. Significant differences were not detected in other clinical laboratory tests. Seven of the eight patients remained bacteriologically, clinically and radiologically negative at least twelve months after the treatment with IFN gamma concluded. Clinical practice demonstrates that these results are very difficult to obtain in such a short period of time with the chemotherapy alone. None of previous historical controls at the same hospital reached culture conversion at three months of treatment with chemotherapy and less than half had converted at six months. Their clinical outcome was also worse (Suárez-Méndez et al., 2004).

The same IFN gamma was evaluated with a similar trial design in a MDR-TB Indian outpatient setting (unpublished data). Ten patients were included, 60% were men, with a mean age of 29 years. Previous treatment all the isolations were resistant to rifampicin and isoniazid. A reduction in the number of patients with positive sputum was recorded. A significant increment (1.6 g/dL) in hemoglobin values took place. The percent of damaged left lung decreased significantly (twice). Right lung and total fibrosis were also reduced but not significantly. At the end of treatment a complete clinical response and radiological

improvement was obtained in most of the cases. All the patients presented adverse events, headache prevailed (50%). All the events were mild or moderate, and no case stop the treatment because intolerability.

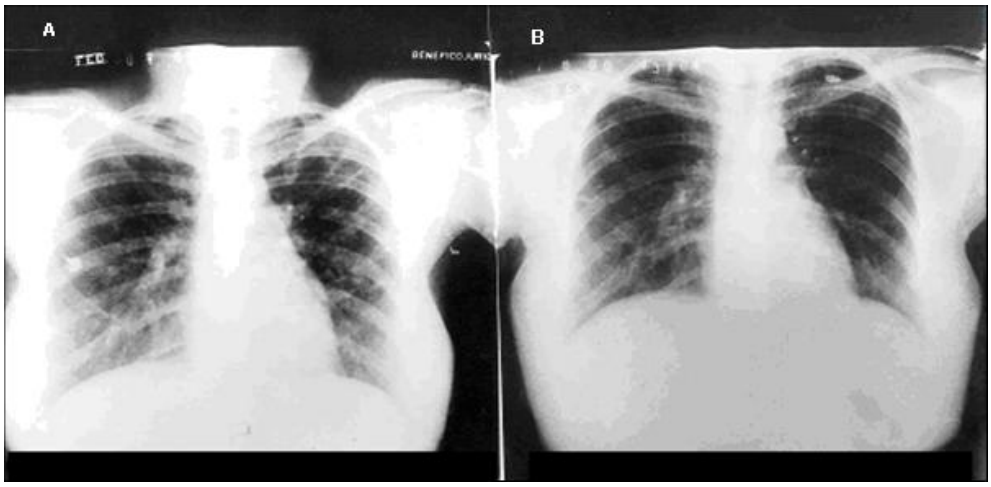

Fig. 3. Radiological improvement with IFN gamma treatment (ray-x of one patients are shown). Legend: (A), left-lung fibroexudative lesions, and (B) complete resolution after IFN gamma treatment. (Picture taken from Suárez-Méndez et al., 2004)

Aerosolized IFN gamma was given to six MDR-TB Korean patients with persistent positive smears and cultures despite long-term medical treatment (Koh et al., 2004). The patients received aerosolized 2 x 10^6 IU of IFN gamma three times a week for 6 months while they continued on identical antituberculous chemotherapy. Before IFN gamma inhalation therapy, the patients received a median of 6.5 (range, 4 to 7) antituberculous drugs for median duration of 29 months (range, 7 to 76). After IFN gamma inhalation therapy, sputum smears remained persistently positive in all patients throughout the study period. Sputum cultures were transiently negative at the 4th month in two patients, but became positive again at the end of 6 months of IFN gamma therapy. Five patients had radiological improvement including three patients who showed a decrease in the size of the cavitary lesions. Resectional surgery could be performed in one patient in whom substantial clinical and radiological improvement was noted after IFN gamma inhalation therapy (Figure 4).

In contrast, adjunctive subcutaneous therapy not improved the sputum culture conversion of refractory or advanced MDR-TB (Park et al., 2007). The authors evaluated the clinical and laboratory effects of subcutaneously administered IFN gamma in this class of patients. Eight patients with sputum smear and culture persistently positive MDR-TB were subcutaneously administered 2 x 10^6 IU of recombinant human IFN gamma three times a week for 24 weeks (72 doses total). Subjects also received a customized drug regimen containing second- and third-line antituberculosis agents based upon drug susceptibility testing and previous treatment history. Body weight remained stable or slightly decreased in all subjects during the study period, and none displayed radiographic improvement on serial chest computed

tomography scanning. Sputum smears and cultures remained positive for all patients, and there was no increase in the mean time to yield a positive culture (from 16.5 to 11.8 days). There was no enhancement of cell-mediated immune responses in terms of production of IFN gamma or IL-10, or of composition of lymphocytes among peripheral blood mononuclear cells. In four patients, therapy was discontinued because of adverse reactions. In conclusion they did not obtain improvement in clinical, radiologic, microbiologic, or immunologic parameters.

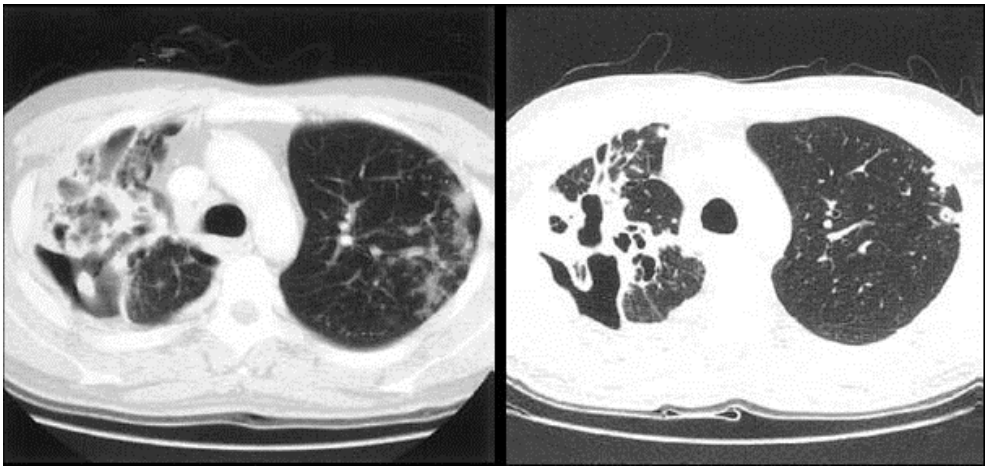

Fig. 4. 27-yr-old male patient with MDR-TB. (Left image) Computed tomographic scan of the chest showed multiple cavities in the right upper lobe and big nodular infiltrations in the left upper lobe. (Right image) After 6 months of IFN gamma inhalation therapy, computed tomographic scan showed reduction in the size of cavitary lesions and improvement of nodular infiltrates. (Picture taken from Koh et al., 2004).

In another protocol, four MDR-TB patients were treated with aerosolized recombinant IFN gamma twice weekly for 8 weeks and anti-tuberculosis drugs. Patients were monitored clinically and T-cell subpopulations were analyzed. The treatment was well tolerated. All sputum smears cleared within 6–8 weeks, and radiological signs of recovery lasted in all patients for 73–106 months (the entire follow-up period). Before treatment, a patient with a 20+ year history of TB showed no γδ T-cells; these cells appeared during treatment. The proportion of natural killer (NK) cells was enhanced during treatment and remained elevated. The proportion of CD4+/CD25+ T-cells in the blood rose after treatment and remained elevated at 2 and 10 months afterwards. No significant change in T-cell levels appeared in patients with a shorter history of TB, except for a tendency toward a slight increase in γδ T-cells during treatment (Grahmann & Braun, 2008).

At least two controlled clinical studies were carried out in Chinese MDR-TB patients (Yang et al., 2009; Yao, & Liu, 2003). These trials directly compared aerosolized IFN gamma plus anti-TB drugs with the same anti-TB drugs. The sample size was around 30 patients per group. Human recombinant IFN gamma was administered by aerosol at 1 x 10^6 IU per dose, three times weekly for 2 - 3 months. Anti-TB drug regimens varied in the trials. The follow-

up time ranged from 9 to 12 months. Both trials were open-labeled. One trial (Yang et al., 2009) was randomized and the other (Yao, & Liu, 2003) was unclear. Both studies reported higher smear conversion rates in the IFN gamma-treated group compared with the control group after 3 months of treatment or at the completion of chemotherapy, although there were no statistically significant differences. Chest radiographs demonstrated cavitary lesion reduction after 2 months of treatment.

Gamma interferon therapy in patients co-infected with HIV and tuberculosis receiving TB medications is safe, improves clinical outcome and enhances host defense mechanism (Yola et al., 2006). Recombinant IFN gamma-1b adjuvant therapy plus DOTS in cavitary pulmonary tuberculosis can reduce inflammatory cytokines at the site of disease, improve clearance of bacilli from the sputum, and improve constitutional symptoms (Dawson et al., 2009).

The results of all these trials need to be viewed from both the individual patient's and the society's perspective. A patient of MDR-TB continues downhill, even in the presence of therapy, to finally lose a lung or even his life. From the point of view of the society, the conversion of patients from infective to non-infective is a major achievement (Noeske & Nguennko, 2002; Subhash et al., 2003).

In the literature can be also found the adjunctive treatment with IFN gamma of an immunocompromised patient who had refractory MDR-TB of the brain and spinal cord (Raad et al., 1996). Despite treatment with six antituberculous drugs for 11 months, there was no appreciable clinical or radiological improvement in the patient's condition. Within 5 months of initiating adjunctive therapy with IFN gamma and granulocyte colony stimulating factors, substantial neurological and radiological improvement was noted. Therapy with IFN gamma was continued for 12 months, resulting in complete resolution of the lesions in the brain and spinal cord.

3.2 Results from different trials and case report in other mycobacterial diseases or similar

IFN gamma has been effective as adjuvant in AIDS patients co-infected with *Mycobacterium avium* complex (MAC), where a clear decrement in the bacteremia was verified. These results were obtained in patients with low CD4+ lymphocytes counts, suggesting a non T cell-mediated effect (Squires et al., 1992). Holland and colleagues treated non-HIV patients with refractory disseminated nontuberculous mycobacterial infections. Three patients were from a family predisposed to the development of MAC infections; four patients had idiopathic CD4+ T-lymphocytopenia. Their infections were culture- or biopsy-proved, involved at least two organ systems, and had been treated with the maximal tolerated medical therapy. IFN gamma was administered subcutaneously two or three times weekly in a dose of 25 to 50 $\mu g/m^2$ in addition to antimycobacterial medications. In response to phytohemagglutinin, the production of IFN gamma by mononuclear cells from the patients was lower than in normal subjects ($P<0.001$). Within eight weeks of the start of IFN gamma therapy, all seven patients had marked clinical improvement, with abatement of fever, clearing of many lesions and quiescence of others, radiographic improvement, and a reduction in the need for paracentesis (Holland et al., 1994).

Around one year later was reported a 38-yr-old man negative for HIV, with silicosis and advanced cavitary lung disease due to *Mycobacterium avium intracellulare*, who failed to improve despite 3 yr of continuous medical therapy with three or more drugs. He received three courses of aerosolized IFN gamma (500 micrograms 3 d per week for 5 wk in two courses and 200 micrograms 3 d a week for 5 wk after a short single trial of subcutaneous IFN gamma). The numbers of bacilli decreased in the sputum during therapy, but cultures of the organism remained positive at the same level for the first two treatment periods. The patient's sputum became smear negative and the number of colonies decreased significantly after the third course of IFN gamma therapy. Cessation of IFN gamma was associated with a rapid increase in the numbers of bacilli (Chatte et al., 1995).

Fifteen patients with disseminated MAC and other nontuberculous mycobacteria infections were treated with subcutaneous IFN gamma during one year or more, 13 of them had clinical improved and 7 had even apparent disease eradication (Holland, 1996). Two human immunodeficiency virus–infected patients with refractory disseminated MAC infection were treated with recombinant IFN gamma given subcutaneously for 3 and 4 months, respectively. Although both patients demonstrated some clinical improvement initially, IFN gamma therapy did not produce sustained benefit (Lauw et al, 2001). It has been reported that a randomized trial testing this option was stopped early due to lack of efficacy (Lam et al, 2006).

A randomized, double-blind, placebo-controlled trial was done with the objective to assess the immunoadjuvant IFN gamma effect in patients with pulmonary atypical Mycobacteriosis regarding their clinical, bacteriological and radiological evolutions. Additionally, several immune response and oxidative stress markers were measured. The diagnosis comprised isolation and classification of any of the atypical Mycobacteria species three or more times in sputum-culture samples, symptoms such as cough and expectoration, and tuberculosis-like pulmonary lesions at thorax radiography. Patients were distributed to receive intramuscular IFN gamma as adjuvant to oral chemotherapy (IFN group) or chemotherapy plus placebo (placebo group) during 6 months (Milanés-Virelles et al., 2008). Patients received 1 x 10^6 IU of Heberon Gamma R® or placebo intramuscularly. The schedule of administration and the response criteria were similar to the referred TB study (see Suárez-Méndez et al., 2004). All the patients received the same conventional daily antibiotic schedule, as follows: azithromycin 500 mg, ciprofloxacin 1000 mg, rifampin 600 mg, and ethambutol 2000 mg.

Thirty-two patients were enrolled. Eighteen patients were included in the IFN group and 14 received placebo. Groups were homogeneous at entry; average age was 60 years, 75% men, 84% white; MAC infection prevailed (94%). At the end of treatment, 72% of patients treated with IFN gamma were evaluated as complete responders, but only 36% in the placebo group (Table 2). The difference was maintained during follow-up. A more rapid complete response was obtained in the IFN group (5 months before), with a significantly earlier improvement in respiratory symptoms and pulmonary lesions reduction. Disease-related deaths were 35.7% of the patients in the placebo group and only 11.1% in the IFN group. Three patients in the IFN group normalized their globular sedimentation rate values. Although differences in bacteriology were not significant during the treatment period, some patients in the placebo group converted again to positive during a one-year follow-up. Significant increments in serum TGF beta and advanced oxidation protein products were

Evaluation	Month	IFN gamma	Placebo	P (test)
		Overall response		
Responders [a]	6	13/18 (72.2%)	5/14 (35.7%)	0.037 (χ^2)
(intention-to- treat)	18	12/18 (66.7%)	4/14 (28.6%)	0.030 (χ^2)
Responders (last evaluation)		15/18 (83.3%)	5/14 (35.7%)	0.005 (χ^2)
		Clinical		
Dyspnea	0	15/18 (83.3%)	13/14 (92.9%)	
	6	1/15 (6.7%)	3/9 (33.3%)	0.27 (FE)
	18	1/13 (7.7%)	3/8 (37.5%)	0.25 (FE)
Good general status (intention-to-treat)	0	3/18 (16.7%)	4/14 (28.6%)	
	6	13/18 (72.2%)	5/14 (35.7%)	0.037 (χ^2)
	18	12/18 (66.7%)	4/14 (28.6%)	0.03 (χ^2)
Improvement [b]	6	13/18 (72.2%)	5/14 (35.7%)	0.037 (χ^2)
(intention-to-treat)	18	12/18 (66.7%)	4/14 (28.6%)	0.03 (χ^2)
		Radiological		
Lesion extension	0 Adv	12 (66.7%)	11(78.6%)	
	0 Mod	5 (27.8%)	3 (21.4%)	
	0 Min	1 (5.6%)	0	
	6 Adv	2 (13.3%)	5 (55.6%)	
	6 Mod	12 (80.0%)	3 (33.3%)	1.00 [c] (FE)
	6 Min	1 (6.7%)	1 (11.1%)	
	18 Adv	1 (7.7%)	2 (25.0%)	
	18 Mod	5 (38.5%)	5 (62.5%)	0.085 [c] (FE)
	18 Min	7 (53.8%) [d]	1 (12.5%)	
Improvement	6	12/18 (66.7%)	6/14 (42.8%)	0.32 (χ^2)
(intention to treat)	18	13/18 (72.2%)	4/14 (28.6%)	0.036 (χ^2)
Cavitary lesions disappearance		5/12 (41.7%)	1/12 (8.3%)	0.15 (FE)
		Bacteriological		
Sputum- Direct (+)	0	14/18 (77.8%)	10/14 (71.4%)	
	Cod.	7 ± 4	8 ± 8	
	6	1/15 (6.7%)	2/10 (20.0%)	0.54 (FE)
	Cod.	0 ± 0	0 ± 2	0.28 (MW)
	18	1/13 (7.7%)	3/8 (37.5%)	0.253 (FE)
	Cod.	0 ± 0	0 ± 7	0.112 (MW)
	Relapse	1/13 (7.7%)	3/8 (37.5%)	0.25 (FE)
Sputum- Culture (+)	0	18 (100%)	14 (100%)	
	Cod.	8 ± 2	9 ± 4	
	6	2/15 (13.3%)	2/10 (20.0%)	1.00 (FE)
	Cod.	0 ± 0	0 ± 2	0.60 (MW)
	18	1/13 (7.7%)	4/8 (50.0%)	0.11 (FE)
	Cod.	0 ± 0	0 ± 8	0.042 (MW)
	Relapse	1/13 (7.7%)	3/8 (37.5%)	0.25 (FE)

(St): Student's t test; (MW): Mann-Whitney's U test; all binary variable comparisons were with the Fisher's exact test. [a] All overall responses were complete except for one IFN group case at month 6 with partial response. [b] General clinical status improvement if the patient passed from "bad" to "moderate" or from "moderate" to "good". Adv: Advanced; Mod: Moderate; Min: Minimum; [c] Combining advanced-moderate; [d] One of them had lesions disappearance at this time.

Table 2. Clinical, radiological, bacteriological and overall outcomes during the trial.

observed in the placebo group but not among IFN receiving patients. Treatments were well tolerated. Flu-like symptoms predominated in the IFN gamma group. No severe events were recorded. This report constituted the first and largest randomized, controlled clinical study, using an immunomodulating agent systemically in pulmonary or disseminated atypical Mycobacteriosis (Milanés-Virelles et al., 2008).

Use of a combination of IFN gamma and IL-2 resulted in a remarkable improvement in a 5-year-old girl presented with disseminated *Mycobacterium avium* complex infection during advanced HIV infection, together with an increase in circulating CD4+ T cells (Sekiguchi et al., 2005). A highly unusual case suggests that IFN gamma may be effective in patients with *M. chelonae* infection that fails to respond adequately to antimicrobials (Jousse-Joulin et al., 2007). Short-term IFN gamma-1b and IL-2 might be considered as therapeutic options in refractory mycobacterial infections in patients with idiopathic CD4 lymphopenia (Sternfeld et al., 2010).

The disseminated mycobacterial infection after *Bacillus Calmette-Guerin* (BCG) vaccination is a very rare disorder that appears mainly in immunocompromised patients. Two pediatric patients with adverse reactions induced by the BCG vaccine, both expressed by suppurative and abscessed regional lymphadenitis, one month after birth, were successfully treated with recombinant IFN gamma (6 months as minimum) after failed courses of chemotherapy (Abreu-Suárez et al., 2008). They showed a marked improvement of lesions after IFN gamma treatment. The evolution of the lesions in the case No.1 is showed in Figure 5. She had imperceptible lesions after 6 months of treatment.

During IFN gamma treatment, only few febrile episodes occurred, well-controlled with antipyretic medication. Both children conserved good general status, normal bodyweight, and no other adenopathies or visceromegaly appeared. During or after IFN gamma treatment no other infections were detected. The first case had a familiar history of tuberculosis (maternal great-grandfather, maternal grandmother and mother), which clearly increases susceptibility to mycobacterial infections by inherited recessive genetic defects. However, the second case did not present those antecedents and an IFNGR1 deficiency was not perceived (Abreu-Suárez et al., 2008).

IFN gamma has been shown efficacy (decrease in acid-fast bacilli) and safety in the treatment of patients with *Mycobaterium leprae*, where immunological pathways for killing intracellular pathogen are similar (Gallin et al., 1995; Nathan et al., 1986). In the 90s IFN gamma was administered to Cuban patients with lepromatous leprosy. Five patients received 1 $x10^6$ IU of IFN gamma three times per week during six months and other five received placebo solution with the same schedule. Those patients treated with IFN gamma showed better clinical and histological evolution. These patients remained with sensibility damage but all the infiltrated cutaneous lesions were clarified. They had less granuloma and reduced greatly the number of bacilli, which look mostly fragmented. Lepromin skin test and lymphoblastic proliferation test didn't have changes in these patients (unpublished data).

In visceral leishmaniasis, patients treated with short courses of recombinant IFN gamma and pentavalent antimony exhibit favorable results such as decrease of the splenic parasitic load, improvement of the symptoms, gain of body weight and reduction of the spleen size, without relapses after several months of follow-up. Doses up to 8 x 10^6 UI/m^2 of body surface has been used for 20 days without important toxic effects (Badaro et al., 1990; Squires et al., 1993; Sundar et al., 1994).

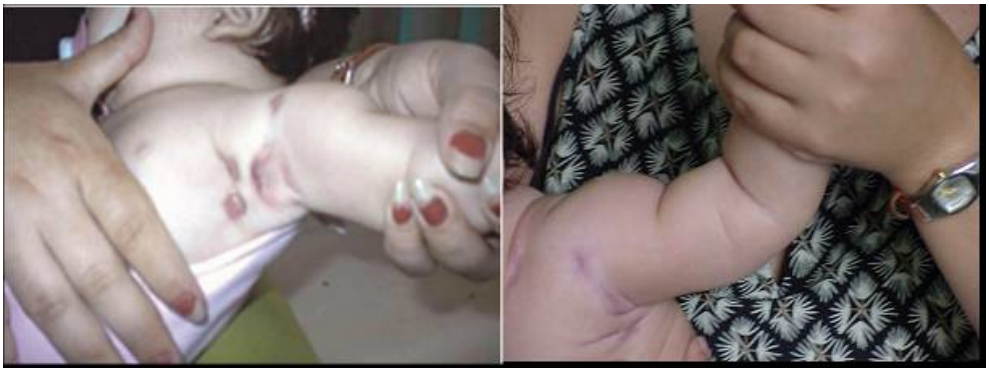

Fig. 5. BCGitis Case No.1: Suppurative axillary and supraclavicular adenopathies after BCG vaccination in a nursing girl. (Left photo): before IFN gamma treatment, (Right photo): complete healing after only 3 months of treatment. (Picture taken from Abreu-Suárez et al., 2008).

4. Conclusions

There is a scientific rationale for the use of recombinant IFN gamma in difficult-to treat cases of tuberculosis and other mycobacterial diseases. By activating macrophages and promoting a range of host immune and antifibrotic responses, IFN gamma may provide an effective adjunct to antimycobacterials in patients not responding to conventional courses of therapy.

Clinical and laboratory experience suggest that adding IFN gamma to established treatment regimens may upregulate macrophage function and decrease mycobacterial load in pulmonary, disseminated and cutaneous infections. Prospective, randomized, more extensive, controlled clinical trials are necessary to confirm previous clinical reports.

Combination with second-line drugs can reduce the time of treatment, diminishing toxicities and possible relapses; in many cases could reduce the application of recessional surgery. Adjunctive immunotherapies, including IFN gamma, will likely play a role in the treatment of mycobacterial disease in the years ahead.

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Biochemical and Immunological Characterization of the *Mycobacterium tuberculosis* 28 kD Protein

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1. Introduction

Tuberculosis is currently a worldwide public health problem [Tiruviluamala, LB., 2002. World Health Organization, 2005]. Up to 33% of the world population may be infected due to the accelerated resurgence of the disease showing high resistance to drugs, which has been favored by the HIV epidemic [De Cock, KM., 2002], and to individual levels of susceptibility [Cooke, GS., 2001] and predisposition [Ogus, AC., 2004]. The unequal protection conferred by the BCG vaccine, which may range from 0% to 80% [Kipnis, A., 2005] depending on the individual, has led to the search for new vaccination strategies. Recent studies have focused on the identification of immunoprotective bacterial antigens [Zhang, Y., 2004].

Evidence has been forwarded which suggests that protective immunity against TB is closely related to the presence of CD4+ cells [Zhang, M. 1995]. Certain antigenic fractions of MW less than 10 kD with immunoprotective activity have been described [Boesen, H., 1995]. Other fractions of MW 45-64 and 61-80 kD [Harris, D.P., 1993] as well as a heat shock protein of 65 kD transfected to macrophages have been found to induce protective immunity [Cooper, A., 1995]. Moreover, CD8+ cells are known to lyse macrophages which express TB bacillus peptides on their surface [Flynn, JL., 1992]. Some bacillus proteins which induce protection are those of 30-31 and 19 kD [Méndez-Samperio, P., 1995], as well as the 38 kD fraction [Elinos, C.M., 2009]. Such observations suggest that the living microorganism releases certain protective and other potent immunosuppressive molecules. The specific activity of these molecules must therefore be determined before they can be suggested as vaccines.

The present study focused on a 28kD protein (Gp28). This fraction had not been characterized yet but is known to be immunogenic since it stimulates the production of

CD4+, CD8+ and TCRαβ, which in turn produce IL-2 and INFγ in healthy individuals [Elinos, C.M., 2009]. The present work determined certain biochemical and immunological characteristics of Gp28 obtained from the cultured strain H37Rv of *Mycobacterium tuberculosis.* A total protein extract (TPE) was obtained from which Gp28 was subsequently isolated. Anti-TPE hyper-immune serum (HIS) and monospecific polyclonal anti-p28 serum were prepared and used to determine p28 purity; as well as the number of peptides that form the protein; to identify the dominant immunoglobulin (IgG2a); to search for mannose-free radicals and classify it as a glycoprotein (Gp28); to determine the percentage of sugars that recognize the anti-Gp28 antibody, and the N-terminal amino acids, thus establishing the predictive nucleotides which form part of the gene. An *M. tuberculosis* strain H37Rv library was used to isolate the gene and confirm DNA purity. Studies were performed by analyzing phenotypic changes in peripheral mononuclear blood cells (PMBC) induced by Gp28 protein obtained from healthy subject involving the production of CD4+ helper T cells [van Crevel, R., et al. 2000] and CD8+ cytotoxic/suppressor cells [Lazarevic, J., 2002] in high risk healthy staff from the National Institute of Respiratory Diseases (INER) in Mexico, and from four TB patients.

2. Materials and methods

2.1 *M. tuberculosis* culture

Bacteria were sown in the Proskawer and Beck synthetic culture medium modified by Youmans (PBY) [Youmans, G.P., 1949], which was incubated for 6 weeks at 37°C to obtain a colony monolayer that completely covered the surface of the liquid culture medium.

2.2 *M. tuberculosis* Total Protein Extract (TPE)

The culture medium was sterilized by filtering through: 1) Whatman sterile #40 filter paper; 2) 1.2 μm micropore filter; 3) 0.45 μm micropore filter; 4) 0.22 μm micropore filter. A sterile filtrate was obtained and added with solid $(NH_4)_2SO_4$

to precipitate proteins and thus obtain the total protein extract (TPE) of *M. tuberculosis*. The TPE was then dialized with water.

2.3 *M. tuberculosis* 28 kD protein purification

The TPE was treated with the method described by Seibert [Seibert, F. B., 1949]. The acid-alcoholic fraction 2 was obtained enriched with 28 kD glicoprotein (Gp28) from which it was purified by SDS-PAGE. The gel was immersed for 15 min in 6M urea and was subsequently placed in a dark chamber. P28 was found to consist of 2 bands (peptids), which were then separated from the gel and dialized against water, thus obtaining the pure Gp28.

2.4 Anti-*M. tuberculosis* hyperimmune serum

One New Zealand rabbit was immunized with the TPE. Pre-immune serum was obtained, then 100 μl of EPT with 100 μl of complete Freund's adjuvant were administered i.d. on days 1, 10 and 20 thus obtaining the anti-*M. tuberculosis* hyperimmune serum (HIS).

2.5 Polyclonal monospecific anti-p28 serum

One New Zealand rabbit was immunized with p28 following the same protocol as described above. Polyclonal monospecific anti-Gp28 serum was thus obtained.

2.6 TPE and Gp28 purity determination by Western blot

Three SDS-PAGE gels were prepared. Gel 1 was stained with Coomassie blue and contained the following samples in the corresponding wells: a) MW control; b) TPE; c) Seibert fraction 2 enriched with p28; d) pure p28. Gel 2 was used for Western blot incubated with HIS, and contained the following samples in the corresponding wells: e) TPE; f) pure p28. Gel 3 was used for Western blot incubated with anti-p28 monospecific polyclonal serum, and contained the following samples in the corresponding wells: g) TPE and h) pure p28. The Western blots were incubated with alkaline phosphatase labeled anti-rabbit antibody for 1 h at room temperature and were developed with NBT/BCIP.

2.7 Electrofocusing assay for p28

Two dimensional gel electrophoresis would indicate if Gp28 consist of 2 peptides or is formed by more peptides. [Rosenkrands, I., 2000]. It is realized in two parts:

Part 1. The electrofocusing gel contained: 1.1 g urea; 30 µl 10% 30%-acrylamide-(0.8% bis-acrylamide); 400 µl 10% NP-40; 80 µl pH 5-7 ampholyte; 20 µl pH 3.5-10 ampholyte; 5 µl 10% ammonium persulfate; 2 µl Temed; water q.s.p. 400 µl. The sample buffer contained: 2.85 g urea; 1ml 10% NP-40; 80 µl pH 5-7 ampholyte; 20 µl pH 3.5-10 ampholyte; 250 µl β-mercaptoethanol; water q.s.p. 5ml.

Gel pre run: a capillary test tube was filled with recently prepared gel, placed vertically, and when the gel had polymerized the sample was added at the top together with 5 mg of urea per each 10 µl of sample. The gel was run at 400 V, for 12 h, with the cathode buffer (20 mM NaOH) at the top and the anode buffer (10 mM H_3PO_4) at the bottom.

Part 2. A 10% acrylamide larger gel (30%-acrylamide-(0.8% bis-acrylamide)) was prepared. The gel was extracted from the capillary test tube and placed on the surface of larger previously polymerized gel. The gel was run at 100 V for 1 h. The gel was transferred to nitrocellulose paper and incubated with anti-Gp28 serum for 3 h. It was subsequently incubated with alkaline peroxidase-labeled anti-rabbit antibody raised in mouse for 1 h at room temperature, and subsequently developed with ortho-chloronaphthol.

2.8 IgG1 and IgG2 in immun anti-p28 sera by ELISA

Pre immune serum was obtained from 6 month-old Balb/c mice immunized i.p. with Gp28 diluted 1:1 with Freund's complete adjuvant. Immune sera were obtained at 1, 4, 8, 12 and 16 weeks after immunization. Immunoglobulin expression times were determined for Gp28 using ELISA.

Microtitration plates were sensitized by overnight incubation with Gp28 protein antigen in carbonate buffer. Thereafter, the plate was blocked with PBS.BSA for 1 h. The first antibody (mouse anti-Gp28 of 1, 4, 8, 12 or 16 weeks) was added to the wells, and incubated for 3 h.

This was followed by the second antibody (rabbit anti-IgG1 or anti-IgG2) to which alkaline phosphatase-labeled anti-rabbit serum was added. This was later developed with a solution of p-nitrophenyl phosphate disodium in diethanolamine buffer and the reaction blocked with 2N NaOH. The plate was read with an ELISA reader using a 405 nm filter.

2.9 Free mannose radicals in p28

A 10% acrylamide gel (30%-acrylamide-(0.8% bis-acrylamide)) with Gp28 protein in a well was run and transferred to nitrocellulose paper. The nitrocellulose paper was first incubated for 1 h with Concanavaline A-biotin diluted 5:1000 in PBS.BSA at room temperature and then for 1 h with streptavidin peroxidase at room temperarure It was developed with ortho-chloronaphthol.

2.10 p28 amino acid sequence

The Gp28 amino acid sequence was determined by Joe Gray of the Molecular Biology Unit, and Catherine Cookson of the Medical School at the University of Newcastle Upon Tyne. Results showed that Gp28 is a doublet consisting of two Gp28 kD bands containing amino acids A(M) – P(C) – K(Y) – V(E) –A (Y or L).

The predictive nucleotides for the 2 peptides were determined as:

A	P	K	V	A		M	C	Y	E	Y	L
GCC	CCC	AAG	GUC	GCC		AUG	UGC	UAC	GAG	UAC	CUG

These nucleotide sequences were not found in registered nucleotide banks and had not been previously identified.

2.11 Sodium periodate determination of sugar percentage in the anti-p28 antibody-recognizing epitope

ELISA method [Woodward, M. P., 1985]. The microtitration plate was sensitized with 5 μg Gp28 antigen per ml of PBS-BSA, placing 100 μl per well and incubating the plate overnight at 37°C. The plate was subsequently washed with 50 mM acetate buffer, pH 4.5. Recently prepared sodium periodate of 0.1, 1, 5 and 10 mM concentration in 50 mM acetate buffer, pH 4.5 was then added, each to a different well, and incubated in the darkness for 1 h at 37 °C. To block the aldehyde groups, the plate was incubated for 30 min at 37 °C with a recently prepared 1% glycine solution in PBS. The plate was washed with PBS-Tween 20 and immediately added 100 μl/well, with anti-Gp28 antibody and incubated for 1 h to 37°C, after which alkaline phosphate labeled anti-rabbit antibody was added and incubated for 1 h at 37°C. It was then developed with p-nitrophenyl phosphate disodium in diethanolamine buffer. The reaction was blocked with 2N NaOH and the plate was read in an ELISA reader at 405 nm.

2.12 Gene p28 from an *M. tuberculosis* library

The phage λgt11 was used to transfect lysogenic *E. coli*, strain Y1090hsdR, and construct a DNA expression library. Competent *E. coli* cell for λgt11 phage were incubated with 0.01 M

$MgSO_4$ for 1 h at 37°C. The phage carries the pMC9 plasmid that codes for the lac repressor, which, in turn, prevents the synthesis of fusion proteins potentially toxic for the β-galactosidase promoter. This plasmid carries the selective marker (amp^r). The HIS used was recognized by the strain H37Rv *Mycobacterium tuberculosis* library. The anti-p28 monospecific polyclonal serum was used to clone gene p28.

Titration of λgt11 phage. A 10 µl sample of the phage under study was added to a tube containing 1 ml dilution buffer, and a serial dilution was prepared in 10 tubes. The contens of each tube were next added to a tube containing Top agar a 50°C, and then transferred to a Petri dish containing Luria Bertani culture broth with ampicillin. Petri dishes were incubated at 37 °C for 24 h. Colonies were counted and those with 100 colonies were considered to contain $1x10^8$/ml λgt11 phage titre.

Titration of the M. tuberculosis library. A tube containing 1 ml of dilution buffer was added with 10 µl of λgt11 phage at $1x10^8$/ml titre and a serial dilution was prepared in 10 tubes. Each tube was added with 10 µl of the *M. tuberculosis* library. The contents of each tube were next added to a tube containing Top-agar at 50 °C and then transferred to a Petri dish containing Luria Bertani broth with ampicillin. They were then incubated at 37 °C for 24 h. Petri dishes with separate colonies were chosen and a nitrocellulose paper disc moistened with Isopropil-β-D-Thiogalactoside (IPTG) was placed in the dish marking its position and other nitrocellulose paper disc moistened with IPTG was placed in other dish marking its position These were then incubated for 1 h at 37 °C. The marked paper discs were then separated and one was incubated with HIS while the other was incubated with monoespecífic polyclonal anti-Gp28 for 3 h at 37°C, the two paper discs were incubated with alkaline peroxidase-labeled anti-rabbit anti-rabbit antibody for 1 h at room temperature.

Control. One tube containing 1 ml of dilution buffer was added with 10 µl of the studied phage. The contents of this tube were then added to a tube containing Top agar at 50°C and then transferred to a Petri dish containing Luria Bertini broth with amppicillin the dish was incubated for 24 h at 37°C. A nitrocellulose paper disk moistened with IPTG was placed in the dish marking its position, and it was then incubated for 1 h at 37°C. The disk was then separated from the Petri dish and incubated with HIS for 3 h at room temperature. Subsequently, it was incubated with alkaline peroxidase-labeled anti-rabbit serum for 1 h at room temperature and developed with NBT/BCIP. This served to prove that no protein from the *M. tuberculosis* library was recognized.

The Gp28 colonies were identified in the Petri dish and sown in Luria Bertani broth and ampicillin, incubated at 37 °C, 240 cycles/min for 20 h. Samples were then centrifuged, resuspended in lysis buffer centrifuged again and the supernatant was added with isopropanol to precipitate DNA, and incubated at 4 °C for 20 h. The samples were then centrifuged and the pellet was dissolved in 50 µl of Milli Q sterile water. A 2292 □g/µl concentration was determined by O.D. reading in a nanospectrophotometer; purity was established by the ratio of the values obtained at 260 nm = 52.697 and at 280 nm = 27.88 nm, which gave a value of 1.89 corresponding to DNA and of 0.11 corresponding to the protein. A 1% agarose gel with 1 µl/well of DNA diluted 1:10 was stained with ethidium bromide (10 µl/100 ml gel; obtained of 10mg/ml dilution) and run at 80 V for 50 min. Results were photographed.

2.13 Human Peripheral Blood Mononuclear Cells (PBMC)

PMBC were obtained from healthy subjects (PPD+, PPD-, with and without BCG vaccination), high risk subjects (staff from the Respiratory Disease Institute (Instituto de Enfermedades Respiratorias, INER)), and from tuberculosis patients (provided by INER) with the following characteristics:

Patient 1. Male, age 23, with untreated progressive tuberculosis; chest X-rays showing fibrosis in left lung and cavitary lesions in right lung.

Patient 2. Female, age 60, diabetic; chest X-rays showed unilateral and apical affliction .

Patient 3. Male, age 64, resistant to treatment with 6 years evolution.

Patient 4. Male, age 25, early infection; one brother died of tuberculosis at age 34, two sisters showed no infection.

Peripheral blood was obtained by venous puncture, subsequently it was treated with heparin (SIGMA, St. Louis Missouri, MO) at concentration of 10 U/ml of blood. An equal volume of RPMI medium was added. Samples of 8 ml of diluted blood were stratified with 4 ml of Ficoll-Hypaque. Tubes were centrifuged at 1500 rpm for 10 min to obtain PMBC and viability was determined with trypan blue.

2.14 T lymphocyte phenotype and antigen receptors

T lymphocytes were characterized by flow cytometry (FACScan, Becton Dickinson, San José, CA). PBMC were washed with RPMI medium and adjusted to $1x10^7$ cells/ml per patient. Next, 100 µl/tube were used to determine the following surface molecules: CD3, CD4, CD8, TCR αβ. The corresponding tube was added with 200 µl of first antibody OKT3, OKT4, OKT8, anti TCR αβ. All antibodies were raised in mouse. Tubes were incubated for 40 min at 4 ^oC in the darkness. Then they were washed 3 times with PBS for FACS, centrifuged, and the pellet resuspended in 500 µl 1% paraformaldehyde. Finally they were read by flow cytometry

3. Results

Purification of Gp28 carried out from the Seibert fraction was sucessful as illustrated by the single band obtained in a PAGE gel eluted with urea 6M and stained with Coomasie Blue (Figure 1). Molecular weight standards (band a) confirm the presence of Gp28 in TPE (band b), and in the enriched Seibert fraction (c). Purified Gp28 is contained in only one band, as shown in (d). TPE immuneblot is shown in (e) and when purified Gp28 is incubated with HIS or with anti-Gp28 serum (in bands f and g and h, respectively) only one band of 28 kD can be observed.

Electrofocusing of Gp28 indicated that it contains two peptides (see Figure 2). This was also confirmed by the N-terminal aminoacid sequences observed by Joe Gray from the Unit of Molecular Biology at Newcastle University.

ELISA analysis indicated that after 1, 4, 8, 12 and 16 weeks after Gp28 treatment, IgG2a is the predominant immunoglobulin detected in mice sera (see Figure 3).

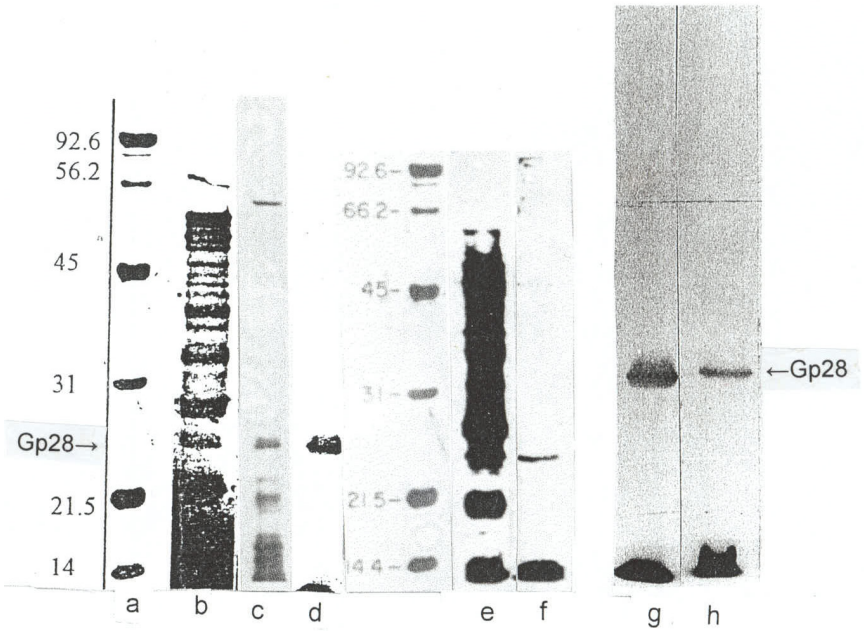


Fig. 1. Gels and immunoblots showing purification of the Gp28 protein isolated of *Mycobacterium tuberculosis* using TPE and Gp28 proteins. Gel stained with Coomassie blue contained the following sample, in **a**)MW; **b**) TPE; **C**) Seibert fraction 2; **d**) pure Gp28. Blot **e**) TPE; **f**) pure Gp28, In other blot **g**) TPE; **h**) pureGp28. Incubated with HIS or with monoespecific polyclonal anti-Gp28, showing pure Gp28

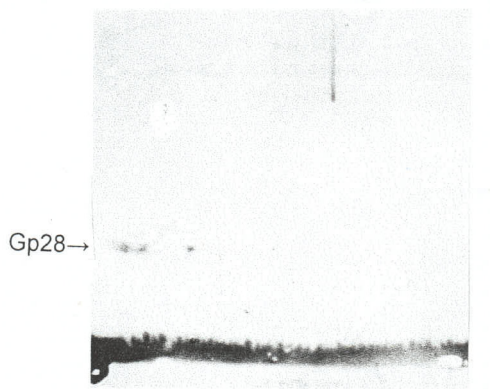


Fig. 2. Show than the protein is formed by only two peptides, which sequences was identified at the University of Newcastle Upon Tyne

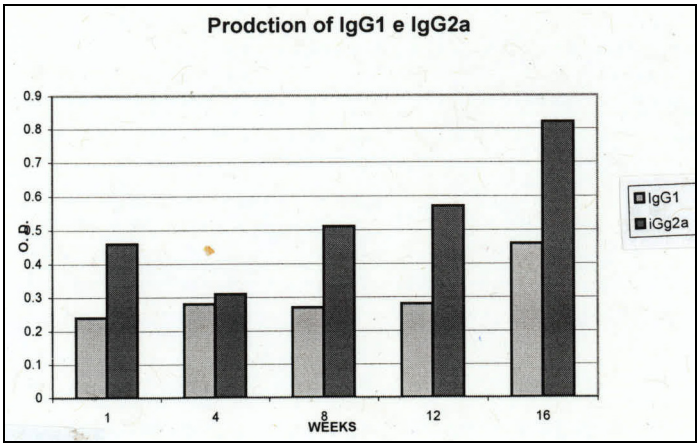


Fig. 3. Mice challenged with Gp28 showed that at 1, 4, 8, 12 and 16 weeks IgG2a predominate even after 16 weeks

The presence of free mannose residues is shown in the PAGE gel of Figure 4. Titration with NaIO3 indicates that Gp28 contains 43 % sugar residues, in the epitope that recognizes the antibody anti Gp28, and 57% peptide residues as shown in the ELISA assay in Figure 5.

Isolation of DNA coding for Gp28 was carried out using Luria Bertini plates blotted with nitrocellulose paper (Figure 6). Disc a presents staining attained when HIS prepared in our laboratory was incubated with a M. tuberculosis library. Disc b shows that HIS does not recognize any peptide when incubated only with λgt11 phage. When the policlonal monospecific anti-Gp28 is incubated with M. tuberculosis library only scattered staning is observed (Disc c). Figure 6d illustrates the electrophoresis of purified Gp28 DNA in an agarose gel that indicates the presence of 900 bp.

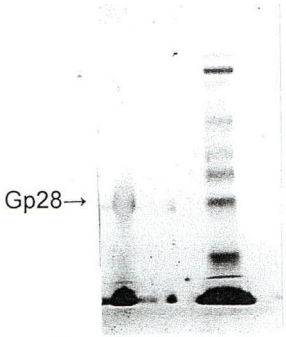


Fig. 4. 10 % acrilamida gel with Gp28 antigen in a well was run and transferred to nitrocellulose paper. This paper was incubated with Concanavaline A marked with Biotin and after it was incubated with streptavidin peroxidase and it is developed, showing that Gp28 is a glycoprotein, by the presence of free mannose radicals.

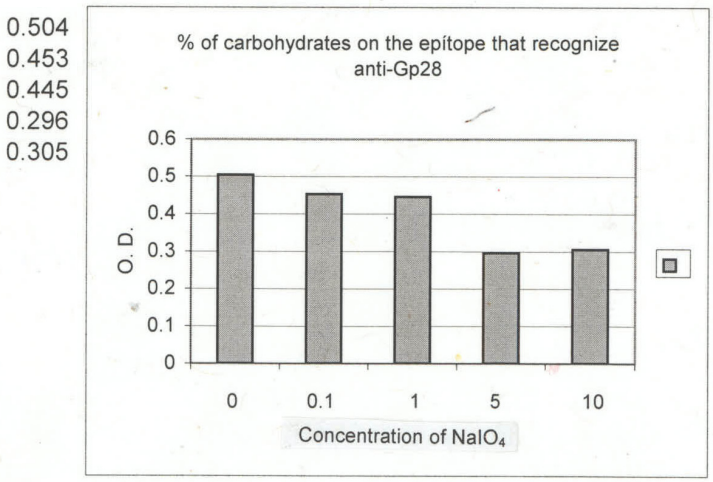


Fig. 5. The microtitration plate was sensitized with Gp28 antigens and incubating the plate overnight, The percentage of sugar in the epitope that recognize the anti-Gp28 was determined using sodium periodate of 0.1, 1, 5 and 10 Mm concentration in 50 mM acetate buffer pH 4.5. To block the aldehide groups with 1% glycine solution. Added with anti-Gp28. the Gp28 recognized the antigen a total of 43% of carbohydrates, leaving 57% of peptidic nature.

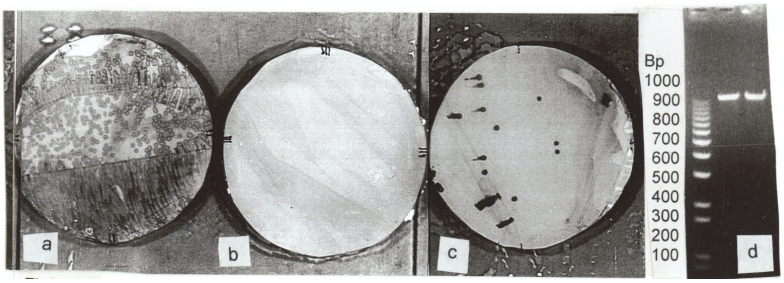


Fig. 6. The Gp28 DNA isolation process. **a**) the nitrocellulose paper incubated with HIS proved that *M. tuberculosis is recognized* in the library; **b**) no peptide of phago λgt11 was recognized in the nitrocellulose paper incubated with HIS; **c**) displays the nitrocellulose paper incubates with anti-Gp28 serum showing recognition of the Gp28; **d**) shows pure Gp28 DNA.

Incubation with pure Gp28 to induce specific cell lines in mononuclear cells obtained from venous human blood from non – vaccinated healthy subjects induced a significant increase of CD3+, CD4+ and TCRαβ+ (see Table 1). Vaccination of healthy subjects does not modify the CD3+, CD4+ nor CD8+ responses to Gp28, excepting for TCRαβ+ that is significantly higher when compared with the response observed in untreated patients with active TB: 92.5 – 94.7 versus 83.8 – 92.1 (IC 95%, $P<0.05$); this observation indicate which suggests that in active TB the response toGp28 appears to be significantly weakened. Otherwise the response to Gp28 of healthy subjects is not different from the one observed in untreated

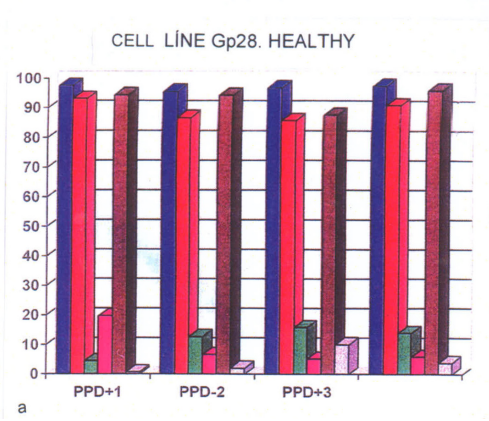
patients with active TB. The last line in Table 1 includes data from the literature for CD3+ (60 – 85), CD4+ (24 – 59) and CD8+ (18 – 48) that are consistent with the baseline values obtained in our laboratory in healthy subjects (Immunology Today, 1992). Considering the reduced number of patients analyzed, in Figure 7 are shown the individual responses to Gp28 observed in healthy subjects and untreated patients with active TB.

TABLE 1

Individual	CD3	CD4	CD8	CD4/CD8	TCRαβ
PPD+1	97.5	93.1	4.7	19.8	93.1
PPD-2	95.5	86.7	12.8	6.7	94.2
PPD+3	96.7	85.7	16.1	5.3	87.6
PPD-4	97.4	90.8	14.1	6.0	96.0

TABLE BASAL, NORMAL and CLASS

Individual	CD3	CD4	CD8	CD4/CD8	TCRαβ
PPD+3	51.1	34.6	23.0	1.5	52.3
PPD-4	59.5	50.4	18.3	2.7	62.7
Normal	73.0	44.0	33.0	1.2	95.0
Class	60-85	24-59	18-48		



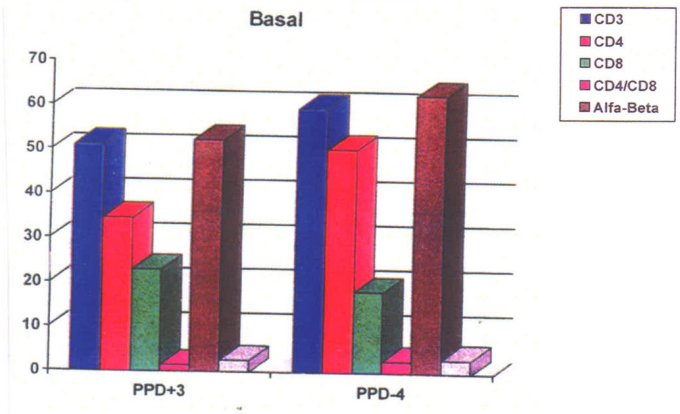


Fig. 7. and Table 1. *In vitro* production of CD3, CD4+, CD8+ and TCRαβ by the antigen specific cell lines induced with Gp28 protein obtained from healthy subjects high risk not vaccinated with BCG (PPD+1 and PPD-2) and vaccinated (PPD+3 and PPD-4), the phenotypic deviation were determined by FACS results confirmed that Gp28 induces proliferation of T helper (CD4+) by more than 90% in healthy.Table 1. bis. These results were compared with the same T cell type not treated with Gp28 of the same individuals, where they constituted approximately 50% of the total T lymphocytes.

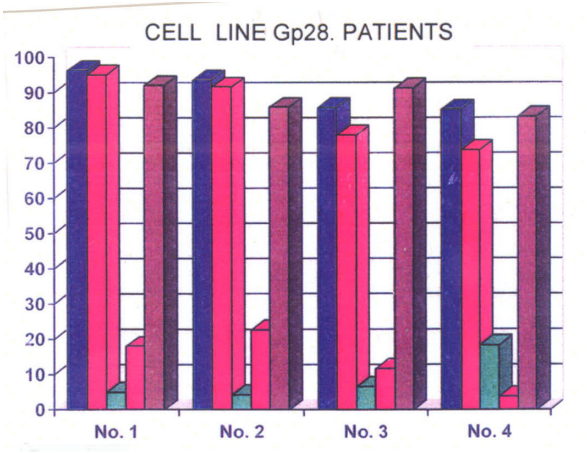


TABLE 2

Patient	CD3	CD4	CD8	CD4/CD8	TCRαβ
No. 1	96.3	94.9	5.1	18.0	92.0
No. 2	93.5	91.5	4.4	22.7	85.8
No. 3	85.7	77.8	6.7	11.6	91.1
No. 4	85.1	73.5	18.4	3.9	83.1

Fig. 8. and Table 2. In the studied tuberculosis patients CD4+ percentage increased by 90% in patients 1 and 2 and 73% in patients 3 and 4, these results are probably related with the severity of TB in these patients.

	HS		TB
Value for cell cluster density	Baseline values of non-vaccinated subjects (n=2)	Challenged with Gp28 (n=4)	Challenged with Gp28 (n=4)
CD3+	55.3 (47.1 – 63.5)*	96.8 (95.8 – 97.6)	90.1 (84.6 – 95.6)
CD4+	42.5 (27.1 – 57.9)*	89.1 (85.6 – 92.4)	84.4 (74.2 – 94.5)
CD8+	20.7 (16.0 – 25.2)	11.9 (7.0 – 16.8)	8.7 (2.2 – 15.0)
TCRαβ+	57.5 (47.3 - 67.6)*	92.7 (89.1 – 96.2)	88.0 (83.8 – 92.1)
CD4+/CD8+	2.1 (0.9 – 3.2)	9.4 (2.6 – 16.2)	14.1 (6.0 – 22.0)

Reference baseline values from (Immunology Today, 1992) for CD3+ (60 – 85), CD4+ (24 – 59), and CD8+ (18 – 48) are consistent with our findings. Data shown are mean value and confidence intervals estimated for a P value of 0.05 (IC 95%). *Indicates significant difference (P<0.05) when comparing baseline values of non-vaccinated HS with vaccinated or non-vaccinated HS challenged with Gp28. There is no significant difference between HS and TB when challenged with Gp28.

Statistic Values: confidence intervals P value of 0.05 (IC 95%).*significant diference P<0.05

Table 3. Effect of Gp28 on phenotype deviation of human peripheral monouclear cells obtained from venous blood of healthy subjects (HS) and from untreated patients with active tuberculosis (TB).

4. Conclusions

In view of the dissimilar protection conferred by the BCG vaccine, research has focused on the identification of immuneprotective antigens. This necessity has been recently magnified the increase in rates of TB, the appearance of bacilli resistant to multiple antituberculous drugs and the rise in the frequency of immunosupressor diseases [Olobo, JO., 2001]. The strategy followed in this study was first to purify the Gp28 antigen of a virulent strain of *Mycobacterium tuberculosis* from the culture medium and to examine its biochemical characteristics. The presence of the mannose radicals [Ehlers, MR., 1998. Heldwein, KA., 2002] was determined, which are identified by the complement receptors CR1, CR3 and CR5 of macrophages, inducing non-opsonic TB bacillus phagocytosis. Sugar and peptide porcentages in the epitope recognized by anti-Gp28 serum were investigated, and IgG2a was identified as the predominant immunoglobulin. The protein was found to consist of a peptide doublet. The N-terminal amino acids of the two peptides were determined as well as the predictive nucleotides, which had not been identified before. The interest in Gp28 was stimulated by its recognized capacity to induce IL2 and IFNγ and are know to be immunocompetent. Evidence has been obtained that protective immunity in tuberculosis is related to CD4+, CD8+ and TCRαβ [Zhan, M., 1995. Lazarevic, V., 2002] In the present experiment peripheral mononuclear blood cells (PMBC)from healthy individuals and TB patients were incubated with Gp28 to obtain cell lines and their phenotype and antigen receptors were determined by FACS. Results confirmed that Gp28 induces proliferation of T

helper cells (CD4+) by more than 90% in healthy individuals, and TCRαβ also increased by more than 90%, with the single exception of one individual (PPD+3) who showed an 87% increase. These results were compared with the same T cell type not treated with Gp28 of the same individuals, where they constituted approximately 50% of the total T lymphocytes, In the studied tuberculosis patients CD4+ percentage increased by 90% in patients 1 and 2 and by 73% in patients 3 and 4. As to TCRαβ, these increased by 90% in patients 1 and 3 and by 83% in patients 2 and 4, these results are probably related with the severity of TB in these patients. Protein Gp28 exhibits an epitope capable of inducing the T response more intensely in healthy PPD+ than in PPD- individuals the application of BCG surely stimulated the adaptive immune response against the TB bacillus in PPD+ individuals than in PPD- individuals, the application of BCG surely stimulated the adaptative immune response against the TB bacillus in PPD+ individuals.

5. Acknowledgment

I want to thank Ing. M. en C. Mario Farías Elinos for excellent adviser in computation

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25

P27-PPE36 (Rv2108) *Mycobacterium tuberculosis* Antigen – Member of PPE Protein Family with Surface Localization and Immunological Activities

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France

1. Introduction

The largest and most distinctive class of mycobacteria-specific genes encode a group of 167 proteins of repetitive sequence belonging to the *pe* and *ppe* families. The uniqueness of the *ppe* genes is illustrated by the fact that these genes are restricted to mycobacteria (Cole et al., 1998; Voskuil et al., 2004 (b)). The *Rv2108* gene belongs to this family and furthermore is highly specific for the *Mycobacterium tuberculosis* (*Mtb*) complex group of mycobacterium (containing notably *Mycobacterium africanum, Mycobacterium canettii, Mycobacterium microti, Mycobacterium pinnipedi, Mycobacterium bovis* and *M. bovis* BCG strain). This gene was described by Chevrier et al., (2000) and used as a molecular probe to develop a rapid test for the detection and identification of this group of mycobacteria. *Rv2108* is a gene coding for the protein P27-PPE36, member of the PPE protein family of *Mycobacterium tuberculosis,* a group of protein thought to be of immunological significance despite the fact that the exact role of the PPE proteins stills unknown.

The P27-PPE36 protein was produced as a recombinant protein in *Escherichia coli*. The expressed protein is immunologicaly active and recognized by sera from infected patients. It was used to generate specific polyclonal and monoclonal anti-P27-PPE 36 antibodies. These antibodies were used to study the immunochemical characterization of P27-PPE36, to verify its presence in *Mycobacterium bovis* BCG and clinical *Mtb* isolates**,** and to characterize and localize it in a parietal position in *M. tuberculosis* cells.

Using an ELISA test we found that the antibody immune response to P27-PPE36 in the sera of patients was dominated by an IgA antibody response accompanied by the absence of IgG response.

The immune response against the P27-PPE36 protein was investigated in mice. It was studied in the context of different pathogen associated molecular patterns (PAMPs). BALB/c mice were immunized either with the P27-PPE36 recombinant protein in Freund's adjuvant or in phosphate saline buffer (PBS), with a pcDNA3 plasmid containing the gene encoding the P27-PPE36 protein, or with the *Escherichia coli* bacteria expressing the P27-

PPE36 protein genetically fused into the flagellin. We found that P27-PPE36 expressed into the flagellin led to the strongest cellular responses, where we obtained the highest production of IFN-γ and cell proliferation, an indication of specific Th1-like orientation of the immune response.

2. Early works on Rv2108 and genetic analysis

2.1 *Mtb* PCR-based assay detection test

The *Rv2108* gene belongs to the *pe* and *ppe* families and furthermore is highly specific for the *Mycobacterium tuberculosis* (*Mtb*) complex group of mycobacterium. This gene was described by Chevrier et al., (2000) and was used as a molecular probe to develop a rapid test for the detection and identification of this group of mycobacteria. PCR targeting the insertion sequence IS 6110 has been considered specific for identification of *M. tuberculosis* and mycobacteria belonging to the *M. tuberculosis* complex and is frequently applied in numerous laboratories to confirm the presence of this organism directly in biological specimens (Thierry et al., 1990). However, several authors found that some *M. tuberculosis* strains failed to hybridize with the IS 6110 probe (Yuen et al., 1993; Thierry et al., 1995) and other authors found that false-positive results may be obtained for clinical samples when some methods based on IS 6110 are used (Lee et al., 1994: Kent et al., 1995). Conversely, the *Rv2108* gene was found to be highly specific for *M. tuberculosis* complex strains. In the PCR-based assay for rapid detection and identification of this mycobacterium (Chevrier et al., 2000), one pair of primers and two oligonucleotide probes were successfully used to amplify and to detect the DNA of strains belonging to the *M. tuberculosis* complex. These primers and probes did not hybridize with DNA from any of the 21 other mycobacterial species tested (*M. avium, M. intracellulare, M. gordonae, M. chelonae, M. xenopi, M. kansasii, M. peregrinum, M. fortuitum, M. marinum, M. flavescens, M. celatum, M. asiaticum, M. malmoense, M. fallax, M. simiae, M. terrae, M. interjectum, M. genavense, M. paratuberculosis, M. szulgaï* and *M. scrofulaceum*) . It is worth noting that the chosen primers and probes hybridize with DNA from the *M. tuberculosis* strain with no IS 6110, furthermore no strain without p27 was found among the 410 strains tested in the study (Chevrier et al., 2000).

Now that many mycobacterium genome have been completely sequenced, the results that *Rv2108* is specific to *Mycobacterium tuberculosis* compex have been confirmed. This name *Rv2108* is those of the gene in the *M. tuberculosis* strain H37Rv. In the *M. tuberculosis* strain CDC1551, the gene number is *MT2167* and in *Mycobacterium bovis,* this gene is called *Mb2132*. No ortholog has been identified in the genome of the closely related *Mycobacterium marinum, Mycobacterium segmatis, Mycobacterium ulcerans or Mycobacterium avium subs. paratuberculosis* (Stinear et al., 2008) and those despite some bacteria like *M. marinum* have an higher number of PPE genes than *M. tuberculosis* (106 vs. 69) **(**Stinear et al., 2008**).**

However an other analysis found a *Rv2108* ortholog in the same strain (Agy99) of *Mycobacterium ulcerans* (Riley et al., 2008). This work presents also that *Rv2108* gene is deleted in the strain C of *Mycobacterium tuberculosis* while it is present in the strains CDC1551, F11, H37Rv and Harlem as in the two strains of *Mycobacterium bovis* tested (BCG stain Pasteur 1173 and AF2122/97*).* According another study (Gey van Pittius et al., 2006), *Rv2108* have no orthologues in *M. smegmatis, M avium paratuberculosis, M. leprae, M. ulcerans* or *M. marinum*. These results confirm the interest of this gene in terms of diagnostic tool.

M. tuberculosis has become highly specialized for intracellular survival in a very restricted range of mammalian hosts, and several recent studies have shown that lateral gene transfer (LGT) has been a major force in the evolution of the *M. tuberculosis* complex from an environmental *Mycobacterium* (Kinsella et al. 2003; Gutierrez et al., 2005; Rosas-Magallanes et al., 2006; Becq et al., 2007). In fact, *Rv2108* appears to belong to one of the 80 regions (minimal number identified containing 360 Protein coding sequences (CDS)) that have probably been acquired by LGT in *Mtb* (Stinear et al., 2008). Whether acquired by LGT or other means, some of these *M. tuberculosis*-specific regions contain known virulence genes or code for adaptation factors making them pottentially important for bacteria belonging to *Mtb*-complex.

2.2 Genomic organizations

Analysis of the genomic environment of the *Rv2108* gene reveals that it is situated downstream a member of the *pe* gene family, *Rv2107,* coding for the PE22 protein (Fig. 1). These adjacent *Rv2107* and *Rv2108* genes lie in the same orientation. Occasionally, it can be noted that an insertion site IS*6110* is localized between this two genes in the strains H37Rv and CDC1551 (Beggs et al., 2000; Sampson et al., 2001). Genome analysis by the operon/gene cluster method (Strong et al., 2003; Bowers et al., 2004) suggests that the PE and PPE families are functionally linked (Gey van Pittius et al., 2006; Tekaia et al., 1999; Strong et al., 2006; Tundup et al., 2006). That is, the two genes tend to be in close chromosomal proximity on the *Mtb* genome (Strong et al., 2003; Bowers et al., 2004). Based on their short intergenic distance (56 bp) and same transcription direction, *Rv2107* and *Rv2108* were assumed to belong to the same operon (Fig. 1) and so be co-transcribed. In *Mtb* genome, these same-operon PE/PPE pairs comprise less than 10% of the total number of PE and PPE genes (14 pairs of PE and PPE genes are found adjacent — same orientation, minimal intergenic distance — in the genome) (Riley et al., 2008). Genes separated by short intergenic sequences tend to have related function and interact physically (Jacob & Monod, 1961). The structure of a complex of one PE/PPE protein pair was recently characterized (Strong et al., 2006; Tundup et al., 2006). These results indicate that there may be many other instances of interactions between PE and PPE proteins. Like the PE and PPE proteins from the gene *Rv2431c* (PE25) and *Rv2430c* (PPE41) that interact together in vitro as probably *in vivo* (Strong et al., 2006; Tundup et al., 2006), it is stongly probable that PPE36 and PE22 have the same behavior. In fact, computational methods predict that the PE22/PPE36 interaction probability is almost the strongest of all the PE/PPE possible combinations tested (Riley et al., 2008). Furthermore, according to this analysis, this putative complexe is predicted to interact specifically, that is, PPE36 do not appear to interact with PEs other than its operon partner PE22, and vice versa (Riley et al., 2008) but this supposition would need to be experimentaly confirmed. However due to the fact that *Rv2108* is absent in *M. tuberculosis strain C* and *Rv2107* is absent in *M. tuberculosis strain F11*, it is possible that another interacting partner is able to interact with the orphaned gene, possibly restoring the PE/PPE complex's function, or introducing new complexes that help these strains survive in their environmental niches (Riley et al., 2008). A putative interaction PE22/PPE36 is probably under the form of a 1:1 heterodimeric complex (Strong et al., 2003). In their study, they found, as us (Le Moigne et al., 2005), that PPE36 is insoluble when expressed alone. The association with the relative PE protein would lead to a soluble complex: their experiments showed that proteins PE Rv2431c and PPE Rv2430 that are insosuble when expressed on

their own are soluble when they are expressed together (Strong et al., 2006; Tundup et al., 2006).

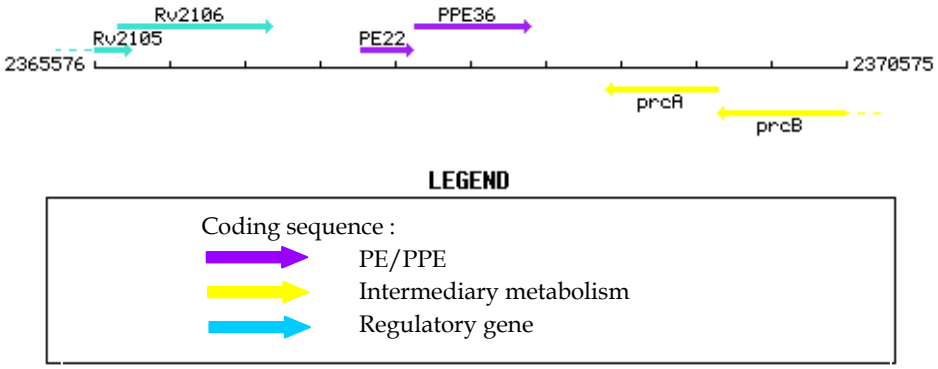

Fig. 1. Genomic environment of *Rv2108* gene. (adapted from TubercuList, http://genolist.pasteur.fr/TubercuList/)

2.3 Regulation expression of *Rv2108*

A fundamental step in understanding the role of *pe* and *ppe* genes is elucidating how their expression is regulated. Although various studies have demonstrated that *pe* and *ppe* genes are expressed under a range of *in vitro* and *in vivo* conditions, they have not revealed any obvious indication of global *pe* and *ppe* gene regulation (Voskuil et al., 2004 (b)). This group of Voskuil and Smith has tested a large variety of diverse conditions to analyse gene expression in *Mtb* (Manganelli et al., 2001; Sherman et al., 2001; Manganelli et al., 2002; Rodriguez et al., 2002; Schnappinger et al., 2003; Voskuil et al., 2003; Voskuil et al., 2004 (a); Voskuil et al., 2004 (b)). Among them, only two conditions induce a variation in *Rv2108* expression (at least two fold): in presence of 0.05% sodium dodecyl sulfate (SDS) 90 min, *Rv2108* expression is repressed (Manganelli et al., 2001) as after 14 days of stationnary phase culture (Voskuil et al., 2004 (a)). Other conditions (Macrophage IFNγ activated, 24 h; diethylenetriamine/nitric oxide adduct (DETA/NO or DNO) 0.5 mM, 40 min; hydrogen peroxide (H_2O_2) 10 mM, 40 min; hypoxia (oxygen from 20% to 0.20%), 2 h (Sherman et al., 2001; Rustad et al., 2008); palmitic acid 50 µm, 4h; non-replicating persistence (NRP) dormancy model 20 days; Iron high vs. low; diamide 5 mM, 1 h; potassium cyanide (KCN) 0.5 mM, 1 h; carbonyl-cyanide 3-chlorophenylhydrazone (CCCP) 0.5 mM, 1 h; ethambutol 10 µm, 24 h; nutrient starvation 24 h (Betts et al., 2002); heat shock (45°C), 30 min (Stewart et al., 2002); acid shock (pH 5.5 vs. 6.9) (Fisher et al., 2002)) do not appear to generate variation (more than 2 fold) in *Rv2108* expression. The associated *pe* gene, *Rv2107* (*pe22*), is found to be induced in macrophage culture of *Mtb* (Schnappinger et al., 2003) and in presence of 0.5 mM DETA/NO (Voskuil et al., 2003).

However, inversely, Park et al. (2003) found that *Rv2108* gene is induced by hypoxia (even if this needs confirmation since standard error deviation is elevated). However, contrarly to the majority of genes powerfully regulated by hypoxia, its induction does not require the putative transcription factor Rv3133/DosR.

Like the majority of other PPE gene (54 of 69), Lsr2, a small basic protein highly conserved in mycobacteria that binds DNA and is implicated in gene regulation, is able to bind Rv2108 sequence (Gordon et al., 2010). The binding of Lsr2 to the majority of *pe/ppe* genes suggests that this factor may negatively affect the expression of these antigenic proteins to modulate interactions with the host.

More generally, *Rv2108* has a low expression in the diverse *M. tuberculosis* strains that have been tested (Gao et al., 2005) and it does not seem that there is a difference of *Rv2108* gene expression between *M. bovis* and *M. tuberculosis* in microarray analysis (Rehren et al., 2007).

Furthermore, a study showed, by high density mutagenesis experiments, that *Rv2108* is not an essential gene for mycobacterial growth (Sassetti et al., 2003). In these experiments, only three of *pe* and *ppe* genes met the criteria for defining growth-attenuating mutations (*Rv1807*, *Rv3872*, and *Rv3873*). Although mutations in several other *pe* and *ppe* genes appeared to have subtle defects, the fact that such a small fraction are detected in this system suggests either that most of these genes are able to functionally complement each other, or that they are required under conditions that have not been testes. In the same study, the *Mycobacterium leprae* gene *ML0411* is presented as an orthologue of *Rv2108*. In the Sanger Institute *Mycobacterium leprae* genome project, *ML0411* is in fact described as being similar to *Rv2108*. ML0411 is coding for a protein 408-amino acid long named as a serine-rich antigen (Sra) that have been largelly described (Vega-Lopez et al., 1993; Rinke de Wit et al., 1993; Macfarlane et al., 2001; Parkash et al.,2006) *Rv2108* belongs the the 27% of genes that are not required for *in vitro* growth having *M. leprae* orthologues while the majority (78%) of the genes that they predict to be required for the optimal growth of *M. tuberculosis* have an orthologue in *M. leprae* genome. Thus, *M. leprae* appears to have selectively conserved the majority of genes that are necessary for optimal growth (Cole et al., 2001).

3. Characterization of P27-PPE36 protein

The *Rv2108* nucleotide sequence encoded for a 243 amino acid length protein. The P27-PPE36 antigen belongs to the PPE protein family, large family of protein present in *Mtb*, which represent ≈3% of the genome of this bacterium (Cole et al., 1998). With the related PE protein family, they account for 10% of the genome. These families appear to have originated in the fast growing mycobacterial species before undergoing extensive expansion

and diversification in certain slow growing species, particularly *M. ulcerans*, *M. marinum* and members of the *M. tuberculosis* complex (Gey van Pittius et al., 2006). This asparagine or glycine-rich protein family containing 69 members has been termed PPE after the characteristic Pro-Pro-Glu motifs near the N-termini, in position 8-10. The relatively conserved N-terminal domain is about 180 amino acids lenght while C-terminal segments vary in sequence and length. According to this C-terminal region, the PPE proteins are classified into four subfamilies: the first subfamily (24 members), named PPE-SVP, has the well conserved motif Gly–X–X–Ser–Val–Pro—X–X–Trp located approximately at position 350; the second (23 members) constitutes the major polymorphic tandem repeats (MPTR) subfamily and is characterized by the presence of multiple tandem repeats of the motif Asn–X–Gly–X–Gly–Asn–X–Gly; the third subfamily (10 members), named PPE-PPW, is characterized by a highly conserved region comprising Gly–Phe–X–Gly–Thr and Pro–X–X–Pro–X–X–Trp motifs; and the last PPE subfamily (12 members) includes proteins with a low

percentage of homology at the C-terminus that are unrelated other than having the PPE motif (Gordon et al., 2001; Adindla & Guruprasad, 2003; Gey van Pittius et al., 2006). P27-PPE36 belongs to this last subfamily. A recent phylogenetic analysis of the 69 *ppe* genes present in the *M. tuberculosis* reference strain H37Rv has uncovered their evolutionary relationships and reveals that they can be divided into 5 sublineages which globally match the subfamilies described above (Gey van Pittius et al., 2006). *Rv2108* is classified in the sublineage III, having the most similarity with *Rv3892c*.

The role of the PPE proteins stills unknown. Firstly, they have been thought to be implicated in increasing antigenic variation and immune evasion due to the highly polymorphic nature of their C-terminal domains (Cole et al., 1998 ; Cole, 1999; Karboul et al., 2008). Concerning this, an interessant study realized by Plotkin et al. (2004) shows that PE/PPE proteins are under strong selection for amino acids substitution. They calculate volatility of codons which is the proportion of their point-mutaions neighbours that encode different amino acids. The volatility of a codon is used to quantify the chance that the most recent nucleotide mutation to that codon caused an amino-acid subtitution. According their calcul, *Rv2108* has a volatility value of 0.1029, which place it at the 594th rank of genes with the higher volatility among the 4099 genes values calculated. Furthermore, in agreement with the theory of an antigenic variation role, it has been observed that many PPE proteins present high levels of polymorphism like for exemple PPE38 (*Rv2352c*), PPE39 (*Rv2353c*) and PPE40 (*Rv2356c*) (McEvoy et al., 2009), PPE34 (*Rv1917c*) (Sampson et al., 2001(a)), PPE42 (Rv2608) (Chakhaiyar et al., 2004), PPE8 (*Rv0355c*) (Srivastava et al., 2006) or PPE18 (*Rv1196*) (Hebert et al., 2007) and sequence variation has been observed between the orthologues of the PE and PPE protein families in *in silico* analyses of the sequenced genomes of *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551 and *M. bovis* (Gordon et al., 2001; Fleischmann et al., 2002; Garnier et al., 2003). However, this variability can not be extended to all *pe/ppe* family members since some are in fact conserved across strains and species (Cubillos-Ruiz et al., 2008). It has then been suggested that the PPE proteins may play a role in the virulence of *Mtb* (Rindi et al., 1999; Li et al., 2005), in the maintenance of bacterial growth in macrophages (Camacho et al.,1999; Dubnau et al., 2002; Hou et al., 2002; Li et al., 2005; Sassetti et al., 2003) and in the regulation of bacterial iron starvation and oxidative stress responses (Rodriguez at al., 1999; Rodriguez at al., 2002). In addition, PPE might be a target for the protective immune response in experimental mouse models (Skeiky et al., 2000). It has also be emitted the hypothesis that PPE proteins, due to their abundance of asparagine, could have a possible storage function for this amino acid which is one of the preferred nitrogen sources of the tubercle bacilli (Tekaia et al., 1999). Some PPE proteins, like PPE31 (*Rv1807*) could be involved in the protection from antibiotic stress targeting the envelope and help to confer the basal level of Mtb resistance to antibacterial drugs (Provvedi et al., 2009). Many PPE proteins are also known to induce a strong T cell and B cell responses and associate with the cell wall. Following surface exposure, these PPE proteins could act as agonists to various surface receptors of APCs resulting in modulation of the host immune responses (Choudhary et al., 2003; Tundup et al., 2008; Mishra et al., 2008; Chaitra et al., 2008 (a); Chaitra et al., 2008 (b)). Recently, two PPE proteins, PPE18 (*Rv1196*) and PPE34 (*Rv1917c*), were found to specifically interact with the innate immune receptor TLR2 (Nair et al., 2009; Bansal et al., 2010).

Very little is known about the protein encoded by the *Rv2108* gene. Theoretical properties of P27-PPE36 protein are a low pH_i (4.59) and representative amino acid composition is 12% for alanine and 9% for glutamic acid. Predictive secondary structure shows that this protein would be mainly constitued of alpha-helix (58,5%) and the absence of β-feuillet. The resting amino acids (31%) would be in random coil.

3.1 Expression and purification of the PPE36 protein

The *Rv2108* gene was amplified, inserted into bacterial vectors, sequenced, and expressed as a recombinant protein. Either the GST (pGEX-4T-3) in *E. coli* DH5α or the pET (pET15b) in *E. coli* BL21 (DE3) plasmid were used. Induction of the PPPE36 protein by these various expression systems lead to the expression of a protein with an apparent molecular mass of 43 kDa in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 2A and B).

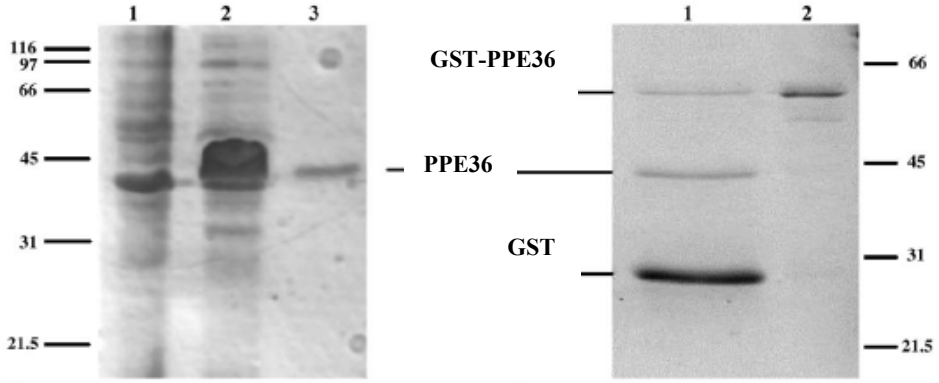

Fig. 2. Coomassie blue staining of bacterial lysates and purified recombinant PPE36 protein expressed with His-Tag (A) or with GST (B).
(A): Lanes 1 and 2: bacterial extracts of E. coli BL21 (DE3) without or with IPTG induction, respectively. Lane 3: purified PPE36 protein. (B): Lanes 1 and 2: PPE36, GST-PPE36 fusion protein, partially cleaved or not cleaved by thrombin, respectively.

This value was higher than the theoretical mass predicted by its DNA sequence translation of 27 kDa. Mass spectrometric analysis of the expressed protein in the pET system revealed a molecule at 29 kDa, which corresponds to the P27-PPE36 putative protein estimated mass plus 2 kDa for the polyHistidine fusion Tag (Le Moigne et al., 2005). This result was confirmed by partial sequencing of the N-terminal region of the recombinant protein. The reason for this difference may be due to the nature of the P27-PPE36 protein, which belongs to a family of intrinsically unstructured proteins (IUP) with an atypical composition of amino acid sequences (Tompa, 2002). It presents notably a high proportion of Proline dimers (3 for 243 amino acids). These proteins bind less to SDS than most other proteins and their

apparent molecular mass is often 1.2–1.8 times higher than the real value calculated from sequence data or measured by mass spectrometry (Dunker et al., 2001). Such a phenomen of electrophoresis abnormal migration has been observed for another protein belonging to the PE protein family of *Mtb*: the product of the gene *Rv1441c* has an apparent molecular weight of about 60 kDa instead of a theorical MW of 40,7 kDa (Banu et al., 2002).

Generally, PE and PPE proteins did not express well or expressed in insoluble or unfolded forms (Strong et al., 2006). Our attemps to express P27-PPE36 under the form of a recombinant proteins confirm this rules and lead to the obtention of an insoluble protein (Le Moigne et al., 2005), as confirmed later by an other study (Strong et al., 2006). The lack of apparent transmembrane elements is a possible explanation for their failure to express on their own is that they need protein partners to fold (Strong et al., 2006) like explained above in the *Genomic organization* paragraph.

3.2 Physico-chemical caracteristic of the PPE36 protein

Based on the DNA and protein sequences, the expected pI value of the P27 protein should be 4.8. To determine the PI value of the expressed p27 protein, a two-dimensional gel was applied to the cell lysates from the BCG strain. After gel transfer to a nitrocellulose membrane and blotting with the P27-PPE36-specific antibodies, only one spot with a pI between 4.5 and 5 at the same molecular mass level observed by SDS-PAGE was recognized on the membrane (Le Moigne et al., 2005).

4. Anti-P27-PPE36 antibodies production and localization of P27-PPE36

Very little is known about the cellular localization of the PPE protein family, a 143 kDa PPE protein encoded by the *Rv1917c* gene (PPE34) was found to be a cell-wall associated protein and probably surface exposed (Sampson et al., 2001) as well as the PPE68 protein (*Rv3873* gene) located in the cell envelope (Pym et al., 2002; Okkels et al., 2003; Demangel et al., 2004).

We have generated specific mouse monoclonal and rabbit polyclonal antibodies to P27-PPE36 and used them for the immunochemical characterization and cellular localization of this protein. Specific immunoblot analysis confirmed the presence of the P27-PPE36 antigen in *Mycobacterium bovis* BCG strain and in human clinical isolates of *M. tuberculosis* from infected patients (Fig. 3), but not in other mycobacteria tested which does not belong to the *Mtb* complex (Le Moigne et al., 2005).

Then, after demonstrating that the P27-PPE36 protein was present in the *M. bovis* BCG strain and in clinical isolates of *M. tuberculosis*, we attempted to localize this PPE protein in the BCG strain. To achieve this, bacteria were washed, fixed and ultrathin sections were prepared to be analysed by electron microscopy using immunohistochemistry test with specific anti-P27-PPE36 antibodies. Results generated with monoclonal (Fig. 4 A) and polyclonal antibodies (Fig. 4 B) revealed a peripheral localization of this protein on the cell membrane. Similar results were obtained using western-blot analysis (Fig. 4 D) of the *Mtb* cell fractions with the monoclonal anti-P27-PPE36 antibody indicating that the P27-PPE36 protein is localized in the membrane of the cell (Le Moigne et al., 2005).

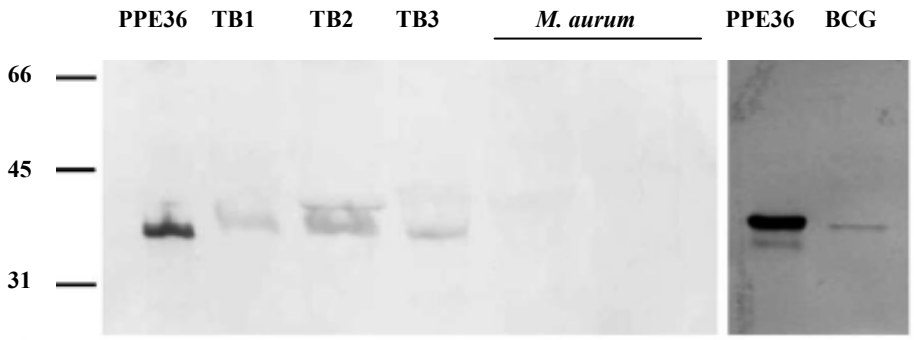

Fig. 3. Western blot analysis of bacterial lysate from different mycobacterial species. P27-PPE36 is the recombinant PPE36 protein. TB1, TB2 and TB3 are *Mycobacterium tuberculosis* clinical strains isolated from infected patients. *Mycobacterium aurum* is a fast-growing mycobacteria, and BCG is the Calmette-Guérin bacillus. The first antibody is a mouse monoclonal IgG antibody directed against PPE36.

This protein was the third member of its family to be localized at the periphery of the cell (Sampson et al., 2001; Pym et al., 2002; Okkels et al., 2003; Demangel et al., 2004) and since, the same localization was assigned to other PPE proteins like for exemple Map3420c and Map1506 in *Mycobacterium avium* subsp. p*aratuberculosis* (Newton et al., 2009). In *Mycobacterium immunogenum*, a PPE protein (accessio no. YP_001288073) have been found to be a cell-membrane-associated antigen (Gupta et al., 2009). In a recent detailed analysis of the *Mycobacterium marinum* capsule using cryoelectron microscopy in conjunction with liquid chromatography mass spectrometry (LC-MS) demonstrated that 5 (MM1129, MM1402, MM0186, MM5047 and MM1497) of the 25 major cell surface proteins were members of the PPE familie (Sani et al., 2010). Similarly high-throughput proteomics MALDI-MS and LC-MS approaches have been utilized by Målen et al. (2010) to identify 8 PPEs in the *M. tuberculosis* envelope fractions (PPE18, PPE20, PPE26, PPE32, PPE33, PPE51, PPE60 and PPE68).

Therefore, these results suggest that cell wall/surface localization is a characteristic of several PE/PPE proteins although another PPE protein, PPE41, have been shown to be secreted by pathogenic mycobacteria (Abdallah et al., 2006). So, if for the majority of PE and PPE proteins are localize to the cell wall, some of them could be secreted into the extracellular environment.

Like explained above, P27-PPE36 should be, as a disordered protein which need a partner to fold, associated with the PE protein PE22 (Gey van Pittius et al., 2006; Strong et al., 2006). Moreover, this putative complex PPE36–PE22 could be associated with a system dedicated to the secretion of members of the potent T-cell antigen 6-kDa Early Secreted Antigenic Target (ESAT-6) family (Gey van Pittius et al., 2006). According this last computational study constructing an evolutionary history of the *pe* and *ppe* genes families, *Rv2107* and *Rv2108* genes are hypothesized to have been duplicated from the ESAT-6 (esx) gene cluster regions, as they are very homologous to their paralogues within the ESAT-6 (*esx*) gene clusters and have the same paired genomic orientation. These esx clusters encode the so-

called Type VII or ESX secretion systems, of which there are 5 in *Mtb* (Gey van Pittius et al., 2001). Thus, *Rv2107* and *Rv2108* would derive from ESAT-6 gene cluster Region 2, i.e. from *Rv3893c* (PE36) and *Rv3892c* (PPE69).

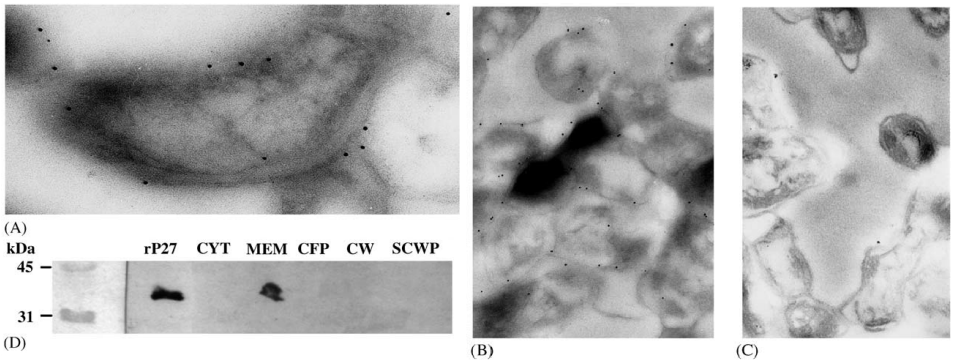

Fig. 4. Localization of P27-PPE36 antigen: Immunogold electron microscopic image (A–C) showing the peripheral localization of the P27-PPE36 protein in cryosectioned *M. bovis* BCG and western-blot on various *M. tuberculosis* cell fractions (D). Incubation was realized either with a mouse monoclonal antibody (A), or rabbit polyclonal anti-P27-PPE36 antibodies (B). Negative control was done using normal rabbit serum (C). (A: ×49 000, B and C: ×23 000). (D): Immunoblot analysis of different cell fractions of *M. tuberculosis* obtained from the Tuberculosis Research Materials and Vaccine Testing Laboratory, Colorado State University using monoclonal anti-P27-PPE36 antibody. Recombinant P27-PPE36 (rP27), cytosol fraction (CYT), cell membrane fraction (MEM), culture filtrate proteins (CFP), cell wall fraction (CW), and SDS-soluble cell wall proteins (SCWP).

5. Serological studies

Diverse reports point out the potential immunodominant nature of PPE proteins. Presence of antibodies against other PPE proteins have been found in mycobacterium infected human or animals: in human against the PPE17 (Rv1168c) (Khan et al., 2008), PPE41 (*Rv2340c*) (Choudhary et al., 2003), PPE42 (*Rv2608*) (Chakhaiyar et al., 2004), PPE55 (*Rv3347c*) (Singh et al., 2005), PPE57 (*Rv3425*) (Zhang et al., 2007), in human and mice against PPE68 (*Rv3873*) (Daugelat et al., 2003), in human (Rindi et al., 2007) and mice (Romano et al., 2008; Bonanni et al., 2005) against the PPE44 (*Rv2770c*), in cattle against PPE68 (*Rv3873*) (Cockle et al., 2002), and against a PPE protein of *Mycobacterium avium subsp paratuberculosis* (Newton et al., 2008). Other studies highlight the capacity of PPE proteins to induce high B cell response in TB patients like PPE41 (*Rv2340c*) (Tundup et al., 2008). Inversely, a study shows that patients with tuberculosis do not develop a strong humoral response against the PPE44 protein (Zanetti et al., 2005). In cattle, no difference is seen in the humoral response to the PPE44 (Rv2770c) between infected and TB-free animals (Molicotti et al., 2008).

The P27-PPE36 expressed protein is immunologically active, and reacts, in western-blot and ELISA, with antibodies from sera of patients infected with *Mtb* (Le Moigne et al., 2005). (Fig. 5).

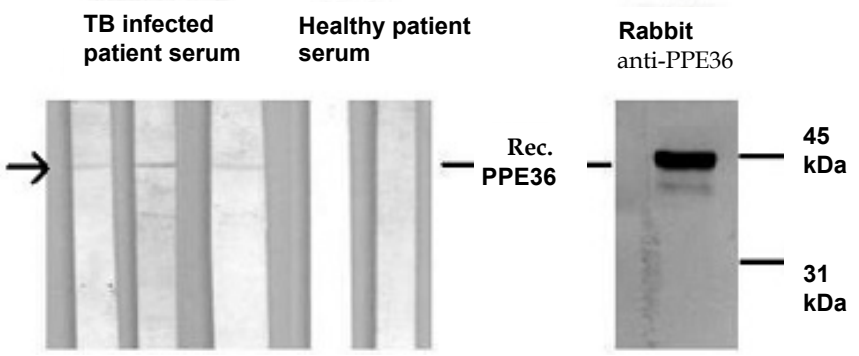

Fig. 5. Western blot analysis of the presence of anti-recombinant PPE36 antibodies in the sera of TB patients in comparison with serum from healthy donors and recombinant PPE36-hyperimmunized rabbit.

So, we then studied PPE36 specific antibody isotype distribution in sera of pulmonary tuberculosis patients and compared them to those in sera from healthy control by enzyme-linked immunosorbent assay (ELISA). Our result showed a significant increase of IgA antibody response in patient's sera, but a less important IgM response accompanied by total absence of IgG2, 3, and 4 responses and a weak IgG1 response in few patients' sera (unpublished results).

The absence of IgG response in the sera of patients allowed verifying for the presence of immune complexes that may inhibit the interaction of antibodies with our antigen on the plate. Using an immunoprecipitation test with goat anti human immunoglobulin antibodies, no immune complex containing P27-PPE36 was present in the patient's sera.

The significance of IgA and IgM is not clear. The IgA response is the more interesting and intriguing results for this protein, because this is the first study showing the presence of IgA alone and the absence of an IgG response against a peptidic antigen. The IgA response is often considered to be local (mucosa and body surface) and non systemic (sera). It has also been reported as a more specific for the non peptidic antigens comparing to the IgG response, which was more reactive (Julián et al., 2005). The IgM response is in general related to the natural auto antibodies found in the sera of healthy and infected peoples and animals, and we couldn't ascribe it a diagnostic value, though we note its augmentation during infection. These antibodies are in general polyspecific with weak affinity for their antigens.

The occurrence of antibodies against the PPE proteins is highly controversial; different studies highlighted the capacity of PPE proteins to induce high B cell response in TB human patients or infected animals (Tundup et al., 2008; Singh et al. 2005). Inversely, a study showed that patients with tuberculosis do not develop a strong humoral response against a PPE protein (Zanetti et al., 2005).

In comparison with other PPE proteins, P27-PPE36 proved to be less useful as a basis for the development of a TB diagnostic test. However, the presence of an IgA response in the

absence of an IgG one, could be exploited as an indicator for *Mtb* diagnosis. A large number of sera should be tested to gather further information on the immune responses to this antigen.

6. Immune response against P27-PPE36 by different immunisation ways

We have studied the immune response of mice against the *Mtb* P27-PPE36 protein.

The peripheral localization of the P27-PPE36 protein led to the belief that they might play an important immunological role either in diagnosis or in protection. So, we examined the immune response against the P27-PPE36 protein using different Pathogen associated molecular patterns (PAMPs) as adjuvants and vectors for immunization. PAMPs are expressed only by micro-organisms and are recognized by the eukaryotic cells through the pattern recognition receptors (PRRs) of the innate immune system such as the Toll-like receptors (TLRs) (Medzhitov & Janeway, 2000). The interaction of PAMPs with their corresponding TLRs helps to identify the nature of the PAMP and to guide the adequate adaptive immune response (Medzhitov & Janeway, 2000). Muramyl dipeptides, a major element of the Freund's complete adjuvant, bacterial DNA, and bacterial flagellin are three PAMPs recognized by TLR2, TLR9, and TLR5, respectively.

Different immunization protocols were used to study immunological potential of the P27-PPE36 protein. BALB/c mice were immunized either with the P27-PPE36 recombinant protein in Freund's adjuvant or in phosphate saline buffer (PBS) (classical immunization), with a pcDNA3 plasmid containing the gene encoding the P27-PPE36 protein (DNA immunization), or with the *Escherichia coli* bacteria expressing the P27-PPE36 protein genetically fused into the flagellin (flagellin immunization) (Le Moigne et al., 2008).

We found that P27-PPE36 expressed into the flagellin led to the strongest cellular responses, where we obtained the highest production of IFN-γ (Fig. 6 B) and cell proliferation (Fig. 6 A), an indication of specific Th1-like orientation of the immune response. DNA immunization was less potent in the induction of such responses. We confirmed the role of flagellin in this response by using different immunization combinations (Le Moigne et al., 2008). However, the specific antibody response was weak with either method (Fig. 6 C). On the other hand, classical immunization with the recombinant protein, soluble or incorporated in Freund's adjuvant still yielded the best antibody response (Fig. 6 C). The best cellular and humoral responses were obtained in the group of mice primed with the recombinant protein and boosted by the antigen presented on the modified flagellin (Le Moigne et al., 2008). In general, the P27-PPE36 PPE antigen induced a strong proliferative response accompanied by high production of IFN-γ and low amount of IL-4 (Le Moigne et al., 2008), independently of the PAMP used. The results indicated that this antigen may be involved in the establishment of the host cellular immune responses against the *Mtb*.

Protective anti-mycobacterial immunity is primarily mediated by cellular immune responses (Flynn et al., 1992; Caruso et al., 1999). *Mtb* is rich in antigens that induce IFN-γ secretion, and the presence of such antigens has been reported in purified cell walls, the cytosolic fraction, and short-term culture filtrates (ST-CF) (Mustafa, 2001). The importance of antibodies in tuberculosis is much debated, but it has been suggested that certain antibody specificities against bacterial surface epitopes and with the correct isotype may confer protection against intracellular infections (Glatman-Freedman, 2003; Glatman-Freedman and Casadevall, 1998; Casadevall, 1995).

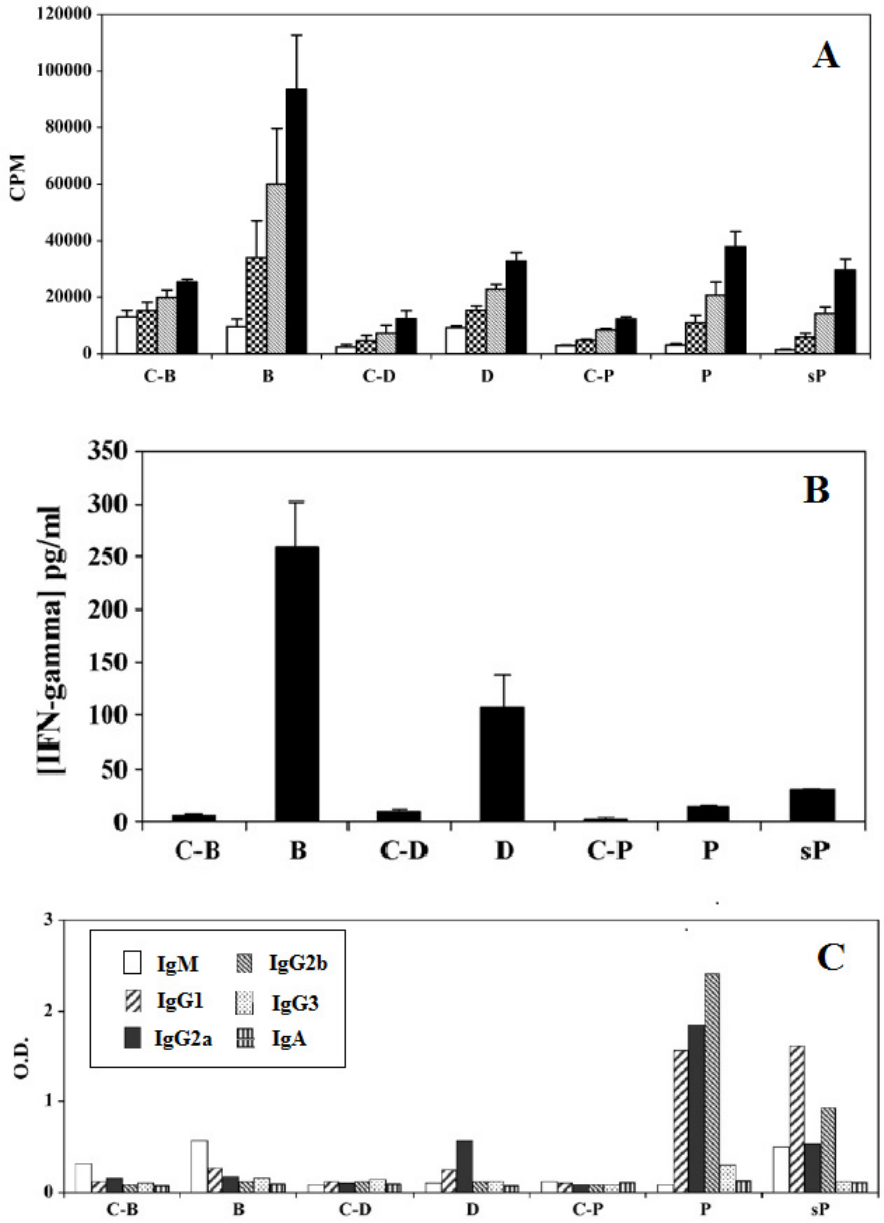

Fig. 6. Immune response generated against P27-PPE36 from mice immunized either with flagellin-modified bacteria (B), DNA plasmid containing the *Rv2108* gene (D) or with the P27-PPE36 recombinant protein associated (P) or not (sP) with Freund's adjuvant. Control groups have been immunized with non-modified bacteria (C-B), the empty pcDNA3 plasmid (C-D) or with PBS in Freund's adjuvant (C-P).

(**A**): Proliferation of splenic cells of immunized mice after incubation *in vitro* with different concentrations (□) 0.0 µg/ml, (▩) 1.1µg/ml, (▒) 3.3 µg/ml and (■) 10µg/ml) of purified p27 recombinant protein. The proliferation was monitored by [^{3}H] thymidine uptake at 66 h after stimulation. (**B**): Cytokine secretion by splenic cells of immunized mice. Splenic cells were stimulated *in vitro* by the recombinant P27-PPE36 protein and IFN-γ was quantified in the supernatant after one week of culture. Results are presented as mean cytokine concentrations (±standard errors) compared to a standard curve of purified cytokines. (**C**): Specific anti-P27-PPE36 antibodies responses. Mice sera diluted at 1/500 were tested in ELISA for the presence of anti-P27-PPE36 antibodies of the different isotypes IgG1, IgG2a, IgG2b, IgG3, IgM and IgA one week after the third immunization. The results are presented as the optical density of the different isotypes.

Other PPE proteins have been reported to be strongly immunogenic (Choudhary et al., 2003; Demangel et al., 2004, Okkels et al., 2003; Dillon et al., 1999; Skeiky et al., 2000). Antibodies against PPE41 (*Rv2430c*) are present in TB patients and not in healthy individuals (Choudhary et al, 2003); PPE68 (*Rv3873*) induces IFN-γ production from splenocytes of *M. tuberculosis*-infected mice and from peripheral blood mononuclear cells of TB patients and PPD+ healthy individuals (Demangel et al., 2004, Okkels et al., 2003) and from cattle blood cells (Cockle et al., 2002; Mustafa et al., 2002). Immune responses elicited by PPE18 (*Rv1196*) and PPE14 (*Rv0915c*) have been shown to provide some protection in mice infected with *M. tuberculosis* (Dillon et al., 1999; Skeiky et al., 2000). Together, these studies suggest that several PPE proteins are expressed in vivo. In other mycobacteries, other PPE proteins have been shown to induce immune responses. For exemple in *M. avium subs. paratuberculosis*, two PPE proteins named Map39 and Map41 significantly elicited IFN-γ production in peripheral blood mononuclear cells from infected cattle (Nagata et al., 2005). When immunized in mice, PPE57 (*Rv3425*) and PPE46 (*Rv3018c*) induce also strong humoral and cellular responses (Wang et al., 2008; Chaitra et al., 2007)

7. Conclusion

The P27-PPE36 protein is the third member of its family to be localized at the periphery of the cell (Sampson et al., 2001; Pym et al., 2002; Okkels et al., 2003). Now others PPE have been found to have a similar localization. This may shed some light on its role in the diagnosis and pathogenesis of Mtb.

In conclusion, the P27-PPE36 protein was found to be a specific antigen for the *Mtb* complex and was recognized by sera of tuberculosis patients and localized in the membrane of the bacterial cell.

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9. References

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