

MINIREVIEW

Roads to the development of improved pertussis vaccines paved by immunology

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One sentence summary: To develop improved pertussis vaccines capable of inducing long-lived protective immunity, lessons have to be learned from immunology of *Bordetella pertussis* infection and current vaccination.

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ABSTRACT

Current acellular pertussis vaccines have various shortcomings, which may contribute to their suboptimal efficacy and waning immunity in vaccinated populations. This calls for the development of new pertussis vaccines capable of inducing long-lived protective immunity. Immunization with whole cell pertussis vaccines and natural infection with *Bordetella pertussis* induce distinct and more protective immune responses when compared with immunization with acellular pertussis vaccines. Therefore, the immune responses induced with whole cell vaccine or after infection can be used as a benchmark for the development of third-generation vaccines against pertussis. Here, we review the literature on the immunology of *B. pertussis* infection and vaccination and discuss the lessons learned that will help in the design of improved pertussis vaccines.

Keywords: *Bordetella pertussis*; vaccine; T cell; Toll-like receptor agonist; immunology

Introduction

Bordetella pertussis is a respiratory tract pathogen that causes the severe disease whooping cough (pertussis) in infants and young children. It can also infect adolescents and adults. The disease was largely controlled in developed countries following the introduction of whole cell pertussis (wP) vaccines in the 1940s/1950s. However, pertussis still kills over 200 000 infants yearly worldwide, mostly in developing countries, but the incidence of disease is also increasing in many developed countries (Black et al. 2010). This has been attributed to the introduction of acellular pertussis (aP) vaccines in the 1990s, which were developed in response to concerns around the safety of wP vaccines. Unlike wP vaccines, which are composed of killed

bacteria with a wide range of antigens and pathogen-associated molecular patterns (PAMPs) that bind to pattern recognition receptors (PRRs) and activate innate immune cells, aP vaccines are composed of three to five *B. pertussis* antigens absorbed to alum as the adjuvant. A monocomponent vaccine based on hydrogen peroxide-detoxified pertussis toxin (PT) was also developed (Trollfors et al. 1995), but had lower efficacy than the three and five component aP vaccines. Although initial reports from phase 3 clinical trials carried out in the 1990s suggested that aP vaccines were at least as effective as wP vaccines (Greco et al. 1996; Gustafsson et al. 1996), recent studies have demonstrated a rapid and alarming drop in protection over time, suggesting a failure to sustain immunity (Klein et al. 2012).

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Table 1. The putative roles of different immune cells in protective immunity to *B. pertussis*.

Cell Type	Infection ^a	wP	aP	Species	Function	Reference
Th1 cell	+++	++	+/-	Man	IFN γ production, macrophage activation, opsonizing Ab, prevents dissemination of Bp	(Ryan et al. 1997, 1998b; Ausiello et al. 1999; Mascart et al. 2003; Mascart et al. 2007; Schure et al. 2012) (Warfel and Merkel 2013) (Mills et al. 1993; Ross et al. 2013; Raeven et al. 2014; Brummelman et al. 2015)
	+++	+	+	Baboon		
	+++	++	+/-	Mouse		
Th2 cell	+/-	+/-	+++	Man	No identified role in mice; unknown role in baboons and humans	(Ryan et al. 1997, 1998b; Ausiello et al. 1999; Mascart et al. 2003; Mascart et al. 2007; Schure et al. 2012), van Twillert unpublished (Warfel and Merkel 2013) (Mills et al. 1993; Ross et al. 2013; Raeven et al. 2014; Brummelman et al. 2015)
	-	-	+++	Baboon		
	-	-	+++	Mouse		
Th17 cell	+++	++	+/-	Man	IL-17 production, neutrophil recruitment and activation	(Schure et al. 2012), van Twillert, unpublished (Warfel, Zimmerman and Merkel 2014) (Ross et al. 2013; Raeven et al. 2014; Brummelman et al. 2015)
	+++	++	-	Baboon		
	+++	++	+/-	Mouse		
T _{RM} cell	+++	++	-	Mouse	Sustains local cellular immunity in the respiratory tract?	Wilk and Mills, unpublished
T _{FH} cell	+++	++	+	Mouse	Activates Ab production and memory B cells?	Wilk, Allen and Mills, unpublished
T _{CM} cell	?	+	+	Man and Mouse	Maintains long-term immunity	(Brummelman et al. 2015; de Rond et al. 2015).
T _{EM} cell	?	+	++	Man and Mouse	Immediate effector function	(Brummelman et al. 2015, de Rond et al. 2015).
T _{TD} cell	?	+	++	Man	Waning immunity?	(de Rond et al. 2015).
$\gamma\delta$ T cell	++	?	?	Mouse	Immune regulation early in infection	(Zachariadis et al. 2006)
B cell	+	++	+++	Mouse	Ab production	(Mahon et al. 1997; Leef et al. 2000; Stenger et al. 2010) (Bernard et al. 2015)
Alveolar Mac	+++	+	-	Mouse	Early response to infection, phagocytosis and killing of Bp	(Dunne et al. 2009)
DC	+++	?	?	Mouse	Activation of naive T cells in lymph nodes	(Dunne et al. 2009)
NK cell	+++	?	?	Mouse	Early IFN γ , prevents dissemination of Bp	(Byrne et al. 2004)
Neutrophil	+++	++	-	Mouse	Ab-dependent phagocytosis and killing of opsonized Bp	(Andreasen and Carbonetti 2009; Ross et al. 2013; Eby, Gray and Hewlett 2014).

^aResponses denoted as +++, ++, +, +/-, - and ? equate to strong, medium, weak, weak/ inconsistent, undetectable responses or not tested respectively; Bp, *B. pertussis*, Ab, antibody.

There have been a number of explanations put forward to account for recent epidemics of pertussis in highly vaccinated populations. First, there is evidence of antigen variation in some of the key protective antigens and components of aP vaccines, namely pertussis toxin (PT), pertactin (Prn) and Fimbriae (Fim2 and Fim3) (van Loo et al. 2002; Tsang et al. 2004; Bart et al. 2014). Moreover, Prn-deficient strains have emerged following introduction of aP vaccination in Europe (Bouchez et al. 2009; Barkoff et al. 2012; Hegerle et al. 2012; Zeddeman et al. 2014), Australia (Lam et al. 2014), Japan (Otsuka et al. 2012) and the USA (Queenan, Cassidy and Evangelista 2013; Pawloski et al. 2014; Martin et al. 2015). Therefore, it is possible that the immune responses induced by three to five antigens from the single isolate used to manufacture the aP vaccine do not protect against at least some

of the current circulating strains. Secondly, the waning protective immunity observed following immunization with aP vaccines suggests that these vaccines fail to generate effective immunological memory (Klein et al. 2012, 2013; Sheridan et al. 2012; Liko, Robison and Cieslak 2013; Witt et al. 2013).

Finally, there is convincing evidence that the aP vaccines do not induce the optimum profile of immune responses required for protection against infection with *B. pertussis* and that these differ significantly from those induced by natural infection or immunization with wP vaccines. The consensus view from studies in the mouse model is that wP vaccines and previous infection confer better protective immunity than aP vaccines because they induce Th1 cells and associated opsonizing antibodies, with a minor contribution by Th17 cells (Table 1). In contrast,

the less effective aP vaccines induce a mixed Th2 and Th17 response (Ross et al. 2013), but the Th2 component appears to be redundant to protection and, together with associated IgE (Ryan et al. 2000), may even be the culprits in rare type hypersensitivity reactions seen in children after a fourth or fifth dose of the aP vaccines (Rennels et al. 2008). This pattern of immune responses induced by the pertussis vaccines is generally similar in humans and mice. Recent evidence from the new animal model of experimental pertussis in baboons has uncovered a major deficit in protective immunity induced by aP vaccination. While the immune responses induced by immunization with wP vaccines were able to prevent disease and enhanced clearance of the infection (though not as effectively as previous infection), immunization with the aP vaccine prevented disease, but did not prevent infection or transmission of *B. pertussis* to naive baboons (Warfel, Zimmerman and Merkel 2014). Whilst it has not been conclusively proven in the baboon, it appears that the failure of aP vaccines to prevent infection reflects its failure to induce appropriate cellular immune responses, especially Th1 cells. In this minireview, immunological evidence from infection and vaccination studies will be discussed in order to guide the development of improved pertussis vaccines.

CURRENT STATE OF THE ART ON THE MECHANISMS OF NATURAL IMMUNITY TO *B. PERTUSSIS*

The innate immune response plays a crucial role in controlling the initial stages of *B. pertussis* infection and helps to shape the subsequent adaptive immune responses. *B. pertussis* attaches to

ciliated epithelial cells on the upper respiratory tract (Coutte et al. 2003; Carbonetti et al. 2007). These cells may also provide a first barrier, by preventing pathogen penetration and through production of mucine by secretory goblet cells which can protect the lungs from pathogens that are inhaled into the respiratory track. Two resident innate immune cell types, airway mucosal dendritic cells (AMDCs) and alveolar macrophages (AMs), which are in close contact with the lung epithelium, mediate the first cellular response to *B. pertussis*. AMDCs are strategically positioned between epithelial cells to take up antigen directly from the airway lumen and prime T cells after migration to the lymph node (Stumbles et al. 1998; Jahnsen et al. 2006), whereas AMs reside within the mucous layer and appear to be important for phagocytosis and killing of *B. pertussis* (Lambrecht 2006). These lung-resident innate immune cells provide a first line of immediate immune defense against *B. pertussis* infection. Additionally, they initiate and orchestrate complex and tightly regulated processes that involve activation and recruitment of other immune cells and generation of long-lasting adaptive immunity (Fig. 1 and Table 1).

B. pertussis was classically considered to be an extracellular pathogen that infects the upper respiratory tract, but it can also penetrate the lungs and has been found inside ciliated respiratory epithelial cells and in lung macrophages (Paddock et al. 2008; Lamberti et al. 2010, 2013). The recognition of *B. pertussis* PAMPs and virulence factors including lipooligosaccharide (LOS), adenylate cyclase toxin (ACT), filamentous hemagglutinin (FHA) and TLR2 lipoproteins by PRRs expressed by macrophages and DCs (and other cells of the innate immune system) leads to their maturation and production of cytokines and chemokines that

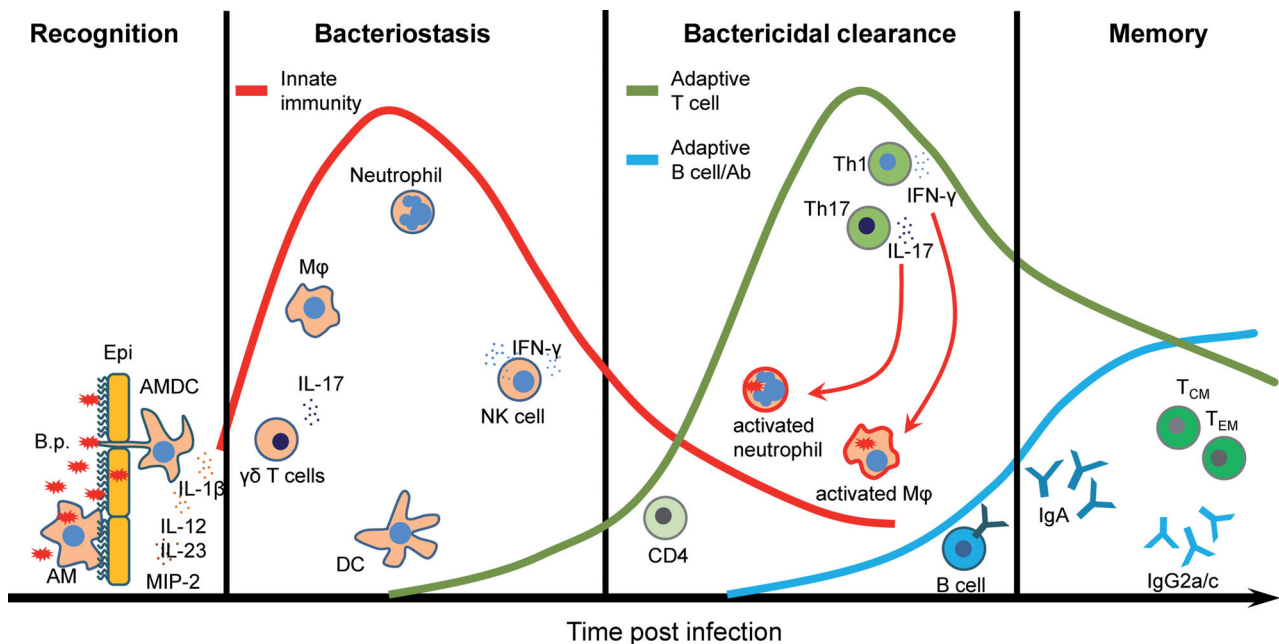


Figure 1. Relative contribution of the cell subtypes to the induction of immune response to *B. pertussis*. The immune response to *B. pertussis* is a complex process that involves activation and recruitment of immune cells to the respiratory tract and generation of long-lasting adaptive immunity. Attachment of *B. pertussis* to ciliated epithelial cells and recognition by AMDCs and AMs provide a first line of immediate defense against *B. pertussis* infection. Secretion of cytokines and chemokines promotes recruitment of innate immune cells. Innate IL-17 together with CXCL2 (or MIP-2) secreted by activated macrophages and epithelial cells promote neutrophils recruitment. NK cells play a protective role through the secretion of IFN γ , which enhances the antimicrobial activity of macrophages as well as induces Th1 cells. Activated neutrophils and macrophages participate in an antibody-mediated phagocytosis and intracellular killing of *B. pertussis*. DCs migrate to the lymph nodes to present the antigen to the naive T cells. Primed T cells proliferate and differentiate into Th1 and Th17 cells that migrate to the lungs to further activate neutrophils and macrophages by production of IFN γ and IL-17, respectively. Activated B cells differentiate into plasma cells that produce *B. pertussis*-specific IgA or IgG2a/c antibodies (mouse). Finally, a small fraction of T and B cells become memory cells providing an effective protection after reinfection. B.p., *Bordetella pertussis*; Epi, epithelium; AMs, alveolar macrophages; AMDCs, airway mucosal dendritic cells; DCs, dendritic cells; M ϕ , macrophages; Neu, neutrophils; Th, T helper cells; T_{CM}, central memory T cells; T_{EM}, effector memory T cells.

mediate and regulate immune responses to the bacteria. It was also shown that PT can act as PAMP, triggering the TLR4 signaling pathway (Wang et al. 2006; Nishida et al. 2010) and enhancing IL-12 production, thereby acting as an adjuvant to promote Th1 responses (Ryan et al. 1998a; Ausiello et al. 2002; Tonon et al. 2002). Binding of LOS to TLR4 results in DC maturation and production of IL-12 and IFN γ , cytokines required for development of Th1 responses (Higgins et al. 2003). TLR4 activation of DCs with LOS can also induce IL-10 production, consequently generating IL-10-secreting type-1 regulatory T cells (Tr1) that may dampen Th1 responses and limit inflammatory pathology in *B. pertussis*-infected lungs (Higgins et al. 2003). DCs can also be activated by intrinsic recognition of other *B. pertussis* virulence factors and toxins. It was shown that ACT activates the NLRP3 inflammasome and caspase-1, leading to production of mature bioactive IL-1 β . Active IL-1 β together with IL-23 promotes expansion of murine Th17 cells, which help to recruit neutrophils that promote killing of *B. pertussis* (Dunne et al. 2010). Furthermore, studies with human DCs have demonstrated that ACT enhances Th17 responses but suppresses Th1 responses by inhibiting IL-12p70 production (Spensieri et al. 2006). ACT also modulates TLR-induced signaling to upregulate IL-10 production and promote the development of Treg cells (Hickey, Brereton and Mills 2008).

It was shown that TLR4-deficient C3H/HeJ mice had a more severe course of *B. pertussis* infection when compared with TLR4-sufficient C3H/HeN mice (Mann et al. 2005; Higgins et al. 2006; Banus et al. 2008). TLR4 expressed by DCs and macrophages plays a central role in protective immunity to this pathogen. Furthermore, an early burst of proinflammatory cytokines and chemokines produced by AMs soon after infection depends on TLR4 signaling pathway. Mice that lack MyD88 adaptor-like protein (Mal; also known as TIRAP) were unable to control infection (Bernard et al. 2015). The bacteria disseminated from the lungs causing lethality in nearly 50% of MAL $^{-/-}$ mice. Additionally, AMs were completely depleted from the lungs of Mal $^{-/-}$ mice early after infection, suggesting that these cells are critical for mediating protection to *B. pertussis* (Bernard et al. 2015).

The initial recruitment of DCs and macrophages into the lungs of *B. pertussis*-infected mice is followed by recruitment or expansion of $\gamma\delta$ T cells and infiltration of neutrophils and NK cells. It is thought that $\gamma\delta$ T cells orchestrate the early cell trafficking into the lungs by producing IL-17 (Zachariadis et al. 2006). This cytokine together with CXCL2 (MIP-2) secreted by activated macrophages and epithelial cells is required for neutrophil recruitment as well as induction of Th1 and Th17 responses to *B. pertussis*.

A primary role of neutrophils during the microbial infection is antibody-mediated phagocytosis and intracellular killing of pathogenic bacteria and formation of neutrophil extracellular traps (Andreasen and Carbonetti 2009; Eby, Gray and Hewlett 2014). It was shown that ACT can inhibit neutrophil function but does not impact on protective immunity to *B. pertussis*. It was suggested that neutrophils are important, but not essential, in the controlling of bacteriostasis of *B. pertussis*, but are critical for clearance of *B. bronchiseptica*; infection of neutrophil-deficient mice with *B. bronchiseptica* had a lethal outcome (Harvill, Cotter and Miller 1999). It was proposed that *B. bronchiseptica* can kill resident and recruited phagocytes in the lungs or AMs are sufficient to control *B. pertussis* infection (Harvill, Cotter and Miller 1999).

NK cells play a protective role in immunity to *B. pertussis* through secretion of IFN γ , which enhances the antimicrobial activity of macrophages. Production of IFN γ by NK cells is depen-

dent on IL-12 from *B. pertussis*-activated DCs. Depletion of NK cells from *B. pertussis*-infected mice resulted in the dissemination of the bacteria from the respiratory tract to the liver and this was associated with reduced IFN γ production and Th1 responses (Byrne et al. 2004). Thus, NK cells play a critical role in early innate immune control of infection and in shaping the adaptive immune response to *B. pertussis* (Fig. 1).

The lungs of naïve mice contain very low numbers of T cells. However, during the course of infection with *B. pertussis* the adaptive immune response slowly develops and T cells are recruited to or expanded in the respiratory tract and mediate subsequent bacterial clearance of *B. pertussis* from the respiratory tract. Human infants without fully developed adaptive immunity are particularly susceptible to the severe symptoms of whooping cough, which can be lethal. Studies in mice have demonstrated that CD4 $^{+}$ T cells play a key role in the clearance of *B. pertussis* infection. Primary infection of athymic *nu/nu* mice, which lack all T cells, results in a persistent or even lethal infection with *B. pertussis* (Mills et al. 1993). Adoptive transfer of immune splenic T cells into athymic mice reversed chronic infection and resulted in bacterial clearance within 14 to 21 days post-challenge. Similarly, transfer of *B. pertussis*-specific CD4 $^{+}$ T cells into immunosuppressed sublethally irradiated naïve BALB/c mice before infection resulted in clearance of the pathogen. In contrast, sublethally irradiated mice were not able to clear primary infection after transfer of *B. pertussis*-specific CD8 $^{+}$ cells (Mills et al. 1993). In addition, antigen-specific CD4 $^{+}$ T cells from infected mice produce IFN γ and/or IL-17, but not Th2-type cytokines, suggesting that Th1 and Th17 cells are involved in protective immunity to *B. pertussis* infection. Indeed, IFN γ $^{-/-}$ mice exhibit impaired ability to clear the pathogen from the lungs (Barbic et al. 1997), whereas IFN γ R $^{-/-}$ mice develop a lethal disseminated infection. Interestingly, IFN γ R $^{-/-}$ mice that survive the initial infection develop neither Th1 nor Th2 response, suggesting that Th17 cells might be involved in developing protective immunity to *B. pertussis* (Mahon et al. 1997). Indeed, it has been demonstrated that IL-17 $^{-/-}$ mice infected with *B. pertussis* have a significantly greater bacterial load in comparison to wild-type (WT) mice (Ross et al. 2013). This impaired ability of IL-17 $^{-/-}$ mice to clear the bacteria correlated with a significant decreased frequency of neutrophils in the lungs during the course on infection (Ross et al. 2013). Taken together, the published studies demonstrate that Th1 and Th17 cells both contribute to the protective immunity induced by primary *B. pertussis* infection in mice (Fig. 1).

Humoral immunity may play an auxiliary role in the clearance of infection from the lungs and contribute to a development of the long-lasting, cell-mediated immunity to *B. pertussis*. The number of B cells significantly increases in the lungs during the course of infection reaching the highest number when the infection is almost cleared. Similarly, *B. pertussis*-specific IgG antibodies are only detectable in the serum at significant levels when the pathogen is almost cleared from the lungs (Mills et al. 1993; Kirimanjesswara, Mann and Harvill 2003). However, IgA was detected in the lungs 2 weeks after challenge (Mills et al. 1993). This suggests that IgG antibodies do not play a major role in the clearance of a primary natural infection, but may be more important in adaptive immunity induced by previous infection or vaccination. Leef et al. showed that B cells may contribute to the protective immunity by different mechanisms than specific antibody production. Although, *B. pertussis*-infected Ig $^{-/-}$ mice developed a chronic infection and could not clear the infection, these mice also lack mature B cells and fail to generate an effective T-cell response to *B. pertussis* (Mahon et al. 1997). It is also

possible that B cells may contribute to the induction of memory CD4⁺ T cells by acting as the antigen-presenting cells (Leef et al. 2000; Linton, Harbertson and Bradley 2000).

Convalescent mice are able to clear the pathogen from the lungs as soon as 3–7 days after reinfection (Mills et al. 1993). Baboons recovered from a *B. pertussis* infection are protected from disease and do not transmit the pathogen upon subsequent challenge and this was associated with a long-lived mixed Th1/Th17-cell response (Warfel and Merkel 2013; Warfel, Zimmerman and Merkel 2014). Hence, natural infection induces strong and persistent immune protection against reinfection with *B. pertussis*, although it does not result in life-long immunity.

MECHANISMS OF VACCINE-INDUCED IMMUNITY AND LIMITATIONS OF CURRENT AP VACCINES

Due to their high reactogenicity, wP vaccines were replaced in the 1990s in most developed countries by aP vaccines containing a high dose of three to five immunogenic *B. pertussis* antigens, namely PT, Prn, FHA, Fim2 and Fim3. Both vaccine types provide protection against severe disease, however, with important underlying differences. aP vaccine-induced protective immune responses are less durable, and in contrast to wP vaccine-induced immunity only protect against clinical symptoms of pertussis but not against colonization and transmission of *B. pertussis* (Warfel, Zimmerman and Merkel 2014).

Besides its narrow specificity, various other features of aP vaccine-induced immunity may account for these differences. First, aP-induced T-cell responses have a distinct functional programming, compared to those induced by wP vaccines, as evidenced by both humoral and cell-mediated responses. While the two vaccine types readily induce significant levels of *B. pertussis* antigen-specific IgG antibodies, as reported in humans (Edwards et al. 1995; Hendriks et al. 2011) and in mice (Redhead et al. 1993; Stenger et al. 2010; Raeven et al. 2015), there is a difference in class switching patterns. In mice, aP vaccines induce predominantly antibodies of the IgG1 subclass (Stenger et al. 2010; Brummelman et al. 2015), reflecting the induction of a Th2-type response, whereas wP vaccines induce predominantly IgG2a/b/c, as well as IgG1, and IgG3 (Raeven et al. 2015), which is consistent with the strong induction of Th1 cells. A comparison of the antibody isotype in cohorts of children that had been primed with either wP or aP vaccines, and followed between 3 and 9 years of age, showed that IgG1 was the predominant IgG subclass induced by both vaccines (Hendriks et al. 2011). In humans, however, this complement activating and opsonizing IgG subclass is thought to be driven by Th1 cells (Hjelholt et al. 2013). Only low levels of the IgG2 subclass were observed in both wP- and aP-primed children (Hendriks et al. 2011). The Th2-associated *B. pertussis*-specific IgG4 subclass was detected only after aP vaccination, with higher concentration after a greater number of doses (Hendriks et al. 2011). Moreover, these IgG4 antibodies strongly correlate with the total IgE production in these aP-vaccinated children (Hendriks et al. 2011), indicating that Th2-skewed immune responses are induced by aP vaccination in humans as well as in mice.

Further evidence for different programming of immune responses induced with aP and wP vaccines comes from studies focused on CD4⁺ T-cell responses and assessing the importance of their secreted cytokine profiles in the context of protective immunity in different species (Table 1). In mice, aP

vaccination is associated with induction of CD4⁺ T cells that produce IL-4, IL-5 and IL-17, but relatively lower concentrations of IFN γ (Ross et al. 2013; Brummelman et al. 2015), representing a mixed Th2/Th17 response, while wP vaccines induce a mixed IFN γ /IL17A (Th1/Th17) response (Ross et al. 2013). Studies using cytokine-defective mice have shown that protection against *B. pertussis* induced by immunization with an aP vaccine is as effective in IL-4^{-/-} or IFN γ ^{-/-} mice as in WT mice, whereas protection is significantly diminished in IL-17A^{-/-} mice (Ross et al. 2013). This indicates that Th17 cells play an essential role in the protective immune response induced by the aP vaccine, while the Th2 cells do not contribute to protection. In contrast, Th1 are required for protection induced with wP vaccines (Ross et al. 2013).

In the baboon model, immunization with an aP vaccine, which conferred protection against disease but not infection or transmission, induces a mixed Th1/Th2 type of CD4⁺ T-cell response. In contrast, wP vaccination, providing protection against colonization and host-to-host transmission, induced a mixed Th1/Th17 memory response (Warfel, Zimmerman and Merkel 2014), suggesting that both Th1 and Th17 cells may be important in prevention of *B. pertussis* colonization in this model.

In humans, aP vaccination has been shown to induce a Th2-dominated, yet mixed Th2/Th1/Th17 type of CD4⁺ T-cell response in children (Ryan et al. 1998b; Ausiello et al. 1999; Mascart et al. 2007; Schure et al. 2012). In contrast, wP vaccines induce a mostly mixed Th1/Th17-type CD4⁺ T-cell response, similar to natural infection (Ryan et al. 1998b, 2000; Mascart et al. 2003, 2007; Rowe et al. 2005; Vermeulen et al. 2010; Ross et al. 2013). Notably, some of the Th1 responses in certain aP-vaccinated children may be attributed to exposure to or subclinical infections with *B. pertussis* (Ryan et al. 1998b; Ausiello et al. 1999; Mascart et al. 2003; Schure et al. 2012). In general, the studies in humans are consistent with the data from animal models, i.e. indicating Th2 dominance associated with aP vaccination and a mixed Th1/Th17 profile after wP vaccination, implying that aP vaccines may have a poorly protective functional T-cell profile in humans.

A second feature of the immune response that might contribute to the difference in long-term effectiveness of aP and wP vaccines has recently emerged from the series of head-to-head comparisons of the cellular immunity in aP- and wP-primed cohorts of children by Buisman and coworkers. It was found that PBMC from aP-primed 4-year-old children, three years after their primary pertussis vaccine series, produced higher specific levels of CD4⁺ T-cell cytokines than those from wP-primed children; however, this response was not boosted after a fifth dose of aP vaccine, in contrast to the response in wP-primed children (Schure et al. 2012). Yet, at the age of 6, two years after this booster dose, PBMC from aP-primed children produced lower levels *B. pertussis*-specific IL-17 when compared with PBMC from wP-primed children (Schure et al. 2013). Furthermore, assessment of CCR7 and CD45RA expression, which allowed CD4⁺ T cells to be categorized as naive T cells (T_N), effector memory T cells (T_{EM}), central memory T cells (T_{CM}) or terminally differentiated T cells (T_{TD}), showed that the proportion of *B. pertussis* antigen-specific T_{TD} cells was higher, in the aP-primed cohort (de Rond et al. 2015). Together, these data suggest that aP-primed CD4⁺ T-cell responses might be more end-stage differentiated than those induced by wP vaccines. The high antigen dose in aP vaccines compared to wP vaccines may contribute to this phenomenon. High antigen and adjuvant dose may induce vigorous short-term immunity, but can also exhaust the immune system and impede long-term immunity (Darrach et al. 2007; Joshi et al. 2007; Gattinoni, Klebanoff and Restifo 2012; Tubo and Jenkins

2014). For example, in the Leishmania model, high dose vaccination with a Leishmania protein-expressing adenoviral vector in mice elicited higher numbers of total IFN γ -producing CD4⁺ T cells compared to low dose vaccination; however, high dose vaccination resulted in poor protective immunity (Darrah et al. 2007).

Thus, aP vaccine-induced immunity has various shortcomings, including immune responses of narrow specificity and with the wrong type of Th-cell subtypes and end-stage differentiated memory T cells which may collectively contribute to its suboptimal efficacy.

APPROACHES TO IMPROVE EFFICACY OF CURRENT AP VACCINATION BY TLR LIGATION

The lesson from studies on T cells in mouse models and humans to date have suggested that next-generation aP vaccines should induce CD4⁺ T cells with a T_{CM} cell phenotype a mixed Th1/Th17-cell profile, like those induced by infection or immunization with wP vaccine. An important factor in natural infection with *B. pertussis* and immunization with wP vaccines that steers protective adaptive immune responses is the presence of PAMPs, including LOS that binds to TLR4 and lipoproteins that bind to TLR2 (Dunne et al. 2015). These PAMPs activate innate cells via PRRs and thereby promote DC maturation and production of proinflammatory cytokines that direct the induction of Th1 and Th17 cells (Fedele et al. 2008). Current aP vaccines do not contain classic PAMPs. Although active PT has adjuvant properties and might be considered to be a PAMP, the PT preparations in aP vaccines are aldehyde treated which destroys immunostimulatory activity (Ryan et al. 1998a). Overall, PT-containing aP vaccine induces a Th2-dominated response, likely due to the Th2-promoting properties of the adjuvant alum. One approach to enhance the efficacy of aP vaccines could be to address this shortfall by replacing or complementing alum with PAMPs, which are known to induce Th1 responses. Indeed, there is evidence from mouse studies that several TLR agonists may be effective Th1 promoting adjuvants for experimental aP vaccines.

A natural TLR2 ligand of *B. pertussis*, BP1569, was recently identified (Dunne et al. 2015). Immunization of mice with an experimental aP vaccine containing a corresponding synthetic lipopeptide LP1569 induced a higher level of protection against aerosol *B. pertussis* challenge when compared with the same vaccine formulated with alum. Furthermore, when compared with an alum-adjuvanted vaccine, the same vaccine formulated with the TLR2 lipoprotein was found to induce significantly higher IgG2a antibodies and promote higher IL-17, IFN γ and lower IL-5 production by spleen cells after antigen stimulation *ex vivo*.

The TLR4 agonist LOS is an important PAMP in wP vaccines enhancing Th1 responsiveness, but was also the main mediator of their high reactogenicity (Donnelly et al. 2001). Although mice are less sensitive to endotoxin than humans (Munford 2010), LOS from *B. pertussis* is less effective in activating human macrophages and DCs than LPS from other bacteria (Fedele et al. 2008). Therefore, well-defined detoxified LPS derivatives from other Gram-negative bacteria with Th1-promoting capacity have been tested as adjuvant for an experimental aP vaccine. Immunization of mice with an aP vaccine adjuvanted with monophosphoryl lipid A (MPL), the hydrophobic biologically active Lipid A part of *Salmonella minnesota* LPS, was more effective in protecting against *B. pertussis* challenge than the aP vaccine adjuvanted with alum (Geurtsen et al. 2008). Furthermore, *B. pertussis*-specific IL-5 production by spleen cells was lower

following immunization with MPL-adjuvanted vaccine, indicating that this adjuvant can skew the immune response away from a Th2 response. Substantiating these data, the authors recently found, at the single T-cell level, that addition of the water-soluble genetically engineered derivative from *Neisseria meningitidis* LPS, LpxL1 (Zariri and van der Ley 2015), to the aP vaccine increased the percentage of specific IFN γ and IL-17-producing CD4⁺ T cells and diminished the percentage of IL-5-producing CD4⁺ T cells (Brummelman et al. 2015). Combining direct MHC class II tetramer staining of Prn-specific CD4⁺ T cells with the memory markers CD44 and CD62L in this model indicated that the ratio of T_{CM} and T_{EM} was not altered by addition of the adjuvant; however, there was a net benefit in the number of T_{CM}, since higher numbers of *B. pertussis*-specific memory CD4⁺ T cells were detected after vaccination with the LpxL1-containing aP vaccine. Hence, this analysis confirmed that there was no trade-off for the altered cytokine profile after TLR4 engaging aP vaccination by a reduction of induced T_{CM} cells. Since there are species differences in specificity of ligands for TLR4 (Marr et al. 2010; Bryant and Monie 2012), caution should be exercised in translating studies on LPS-based adjuvants from mice to humans.

TLR9 agonists, including CpG oligonucleotides from bacterial DNA, can also induce Th1 and Th17 responses and have been used as adjuvants for experimental aP vaccines tested in mice. The complementation or replacement of alum in an aP vaccine by CpG increased the anti-PT IgG2a titers, and the IgG2a/IgG1 ratio, providing indirect evidence for induction of Th1 responses (Sugai et al. 2005). In addition, *B. pertussis*-specific IFN γ production by spleen cells was found to increase when PT, FHA and Prn were administered intranasally with both CpG and alum, but not with CpG alone (Asokanathan, Corbel and Xing 2013). Finally, in another report substituting alum with CpG in an experimental parenterally delivered aP vaccine enhanced its efficacy against aerosol challenge with *B. pertussis* and was associated with stronger *B. pertussis*-specific IFN γ and IL-17, but lower IL-4 and IL-13 production by spleen cells, and higher IgG2a concentrations in sera (Ross et al. 2013). The failure of the study by Asokanathan et al. to detect IFN γ following immunization with pertussis antigens and CpG (without alum) may be due to the route of immunization (i.n.) and the lower dose of CpG (30 μ g) employed. Together, these studies demonstrate that TLR agonists such as TLR2, TLR4 and TLR9 ligands can steer the aP vaccine-induced CD4⁺ T-cell response towards a more favorable protective Th1/Th17 response, and that pertussis vaccine formulations containing experimentally selected adjuvants could be considered as more effective alternatives for current aP vaccines.

In addition to the type of adjuvant, the antigen composition and dose of both adjuvant and antigen may influence the induction of T-cell response with aP vaccines. Therefore, careful evaluation of choice of adjuvant and antigen and their dose will be required to ensure that a vaccine induces the appropriate functional T-cell subtype and profile of memory CD4⁺ T cells required for complete and sustained protective immunity against infection (Fig. 2).

Several pertussis vaccine candidates in development address the absence of functional PRR triggering by current aP vaccines by expressing endogenous PAMPs. BPZE1, a live-attenuated *B. pertussis* vaccine candidate has been shown to induce FHA- and PT-specific Th1 responses in immunized neonatal mice and confer protection against challenge with virulent *B. pertussis* (Feunou et al. 2014). Furthermore, BPZE1 has been shown to induce maturation of human DCs and subsequent induction

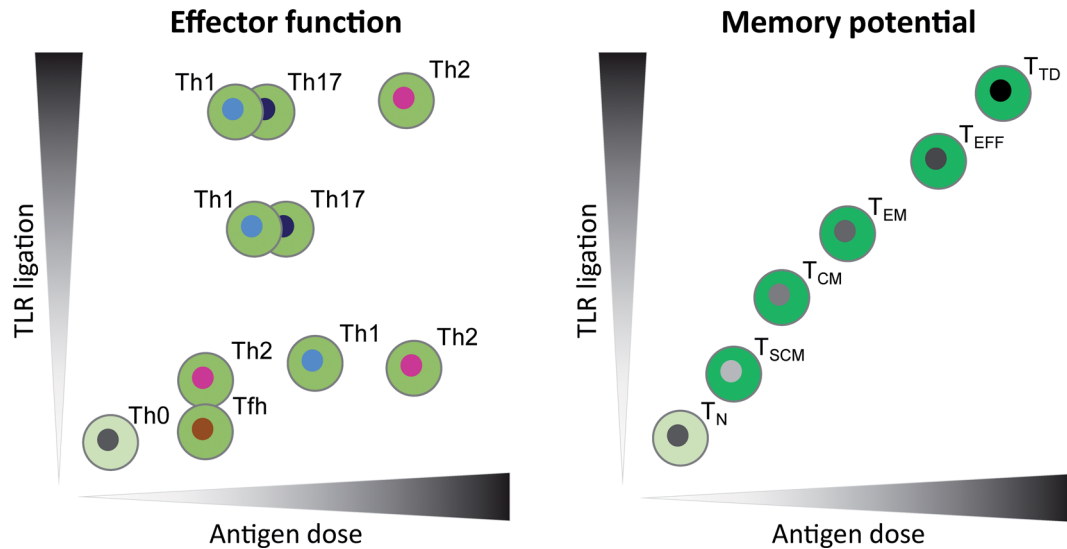


Figure 2. Models for the influence of antigen dose and the strength of TLR ligation on CD4⁺ T-cell differentiation. Vaccine-induced CD4⁺ T-cell responses can be evaluated based on effector function i.e. Th type (left panel) and memory potential i.e. memory differentiation stage (right panel). Antigen dose can affect TCR signal strength, thereby influencing Th type and memory T-cell differentiation (Gattinoni, Klebanoff and Restifo 2012; Tubo and Jenkins 2014). Inflammatory signals, such as induced by TLR ligation, during T-cell priming can also influence the Th type and memory potential of antigen-specific T cells via innate mechanisms (Joshi et al. 2007; Tubo and Jenkins 2014). The x axes depict the range of antigen dose and the y axes show the amount of TLR ligation present during vaccination or infection. *In vivo*, antigen and TLR ligand dose may form a gradient of local concentrations, possibly inducing an array of differentiation states. In the presence of low dose TLR ligands, low antigen dose will steer towards Th2 or Tfh cells, while an intermediate dose promotes Th1 cells and a high dose Th2 cells. Increasing TLR ligation induces Th17 and Th1 cells, yet high exposure to both TLR ligation as antigen dose will promote Th2 cells (Tubo and Jenkins 2014). In parallel, both low and high antigen dose and TLR ligation strength may also induce regulatory functions in cell populations (not shown). The memory differentiation stage is correlated with both the antigen dose and TLR ligation, meaning that the memory potential of the cells decreases when the cells are exposed to increasing TLR ligation and antigen dose (Joshi et al. 2007; Gattinoni, Klebanoff and Restifo 2012). The color gradient of memory phenotypes from light to dark indicates loss of long-term memory potential. TLR: Toll-like-receptor; T_N: naïve T cell; T_{SCM}: stem-cell memory T cell; T_{CM}: central memory T cell; T_{EM}: effector memory T cell; T_{EFF}: effector T cell; T_{TD}: terminally differentiated T cell.

of IFN γ - and IL-17- producing T cells, indicating that BPZE1 should be capable of inducing the favorable Th1/Th17 response in humans (Schiavoni et al. 2014). This vaccine is currently being tested in the baboon model and is in clinical development (Thorstensson et al. 2014). Another vaccine concept based on *B. pertussis* outer membrane vesicles also has been shown to induce a mixed Th1/Th17-type response in mice and to induce protection against *B. pertussis* challenge (Gaillard et al. 2014) (Raeven and Brummelman et al. in preparation). These vaccine candidates also address other limitations of aP vaccines, by expressing ‘natural’ (low) doses antigen (preventing an exhausted immune response) and a broader panel of antigens, important to ensure maintenance of long-lasting protective immunity against circulating *B. pertussis* strains with altered antigenic make up.

IMMUNOLOGICAL EVALUATION OF NEW PERTUSSIS VACCINES IN (PRE-) CLINICAL DEVELOPMENT

The development of new pertussis vaccines requires immunological tools to determine the functional differentiation, memory potential, breadth and protective capacity of the induced immune responses in reliable preclinical animal models, important to predict efficacy in humans. Mouse and more recently baboon models have been used to evaluate candidate pertussis vaccines. One of the problems with these models is that the level of protection is usually assessed shortly after vaccination (van der Ark et al. 2012). Furthermore, mice are not the natural host of *B. pertussis* and relatively high vaccine doses are often used in these challenge models to induce measurable im-

mune responses; thus, the results may be difficult to translate to the humans. Future studies in these models should address the issue of waning immunity by assessing the long-term protection of experimental aP vaccines, and the protection induced by lower doses. While it is possible to do medium-term (6–12 month) studies in the mouse model, studies of greater than 1 year will require experiments in the recently developed baboon model. Warfel and Merkel (2013) have used species-specific tools to assess waning of the vaccine induced CD4⁺ T-cell response up to 15–24 month after *B. pertussis* infection in baboons. However, cost and animal availability will be significant limitations to extensive use of this model. In both mice and baboons, identifying early biomarkers that predict long-term immunity would be valuable.

One such biomarker of long-term immunity in pre-clinical and clinical vaccination settings is the memory phenotype of the induced specific CD4⁺ T cells, since T_{CM} are important for long-term immunity (Sallusto et al. 1999; Kaech, Wherry and Ahmed 2002; Seder and Ahmed 2003; Sallusto, Geginat and Lanzavecchia 2004) (Fig. 2). Furthermore, there is emerging evidence from other infectious diseases that follicular helper T (T_{FH}) cells (Yoo, Fish and Braciale 2012; Zimara et al. 2014) and tissue resident memory (T_{RM}) (Teijaro et al. 2011; Iijima and Iwasaki 2014) may be key to long-term immunity and these have yet to be addressed in the field of pertussis vaccines. Memory phenotypes can be determined in the early stage after vaccination. Recently, we showed that MHC class II-tetramers specific for the immunodominant Prn epitope can be used to evaluate the CD4⁺ T-cell memory phenotype on a single cell base in both mouse and humans (Brummelman et al. 2015; Han et al. 2015). The knowledge on human pertussis MHC class II T-cell epitopes is increasing by

studies using prediction methods, and advanced immunoproteomics analysis (Han *et al.* 2013; Stenger *et al.* 2014), however, is still very limited (De Magistris *et al.* 1989; Peppoloni *et al.* 1991; Vaughan *et al.* 2014). However, routine assessment of memory T cells induced by pertussis vaccine will require optimization and standardization of techniques. The availability of MHC class II tetramer with peptides from *B. pertussis* antigens together with single cell analysis using intracellular cytokine staining is one approach that may facilitate accurate quantification of *B. pertussis*-specific memory T cells induced with pertussis vaccines.

Furthermore, systems biology approaches could aid in identifying novel early biomarkers that predict the outcome of the adaptive immune response. Using this approach, a recent study revealed molecular and cellular immunological processes in response to live *B. pertussis* infection and may provide guidance in evaluating new candidate vaccines (Raeven *et al.* 2014). State-of-the-art tools, such as MHC class II tetramers and systems biology, are needed for mouse, baboon and humans to evaluate the quality of the immune response induced by current and potential new pertussis vaccines.

CONCLUSIONS AND FUTURE PERSPECTIVE

The potential shortcomings of current aP vaccines are still a subject of debate. While the switch from wP to less effective aP vaccines is one explanation for the resurgence of pertussis, it has also been argued that recent epidemics may be part of natural cycles of disease that have always been with us, even in the wP vaccine era (Riolo, King and Rohani 2013). Nevertheless, the fact remains that the incidence of pertussis is higher than of any other vaccine-preventable infectious disease. There is now growing acceptance that we do have a problem and must come up with short- and long-term solutions. While a rationally designed third-generation pertussis vaccine would appear to be the ideal solution to improve the aP vaccine in use today, the development and especially the clinical evaluation, regulation, licensing and implementation of new aP vaccines into pediatric immunization programs will face many logistic as well as scientific hurdles.

In the interim, there are a number of short-term measures that are being introduced or considered. These include (1) maternal immunization to protect disease in the newborn by passive transfer of antibody, which has proved very effective in prevent infant death in certain countries (e.g. UK); (2) additional booster vaccination with current aP vaccines in adolescents, to tackle the problem of waning immunity; (3) immunization of adults with a vaccine that prevented infection as well as diseases to reduce the carriage and spread of the bacteria; and (4) cocooning strategies to contain local outbreaks of disease.

Before considering a new pertussis vaccine for infants, the first step may be to develop an alternative aP formulation that includes a more powerful new generation adjuvant, such as a TLR agonists, instead of or in addition to alum, and to evaluate it as part of a pertussis only vaccine for boosting adolescents and maybe adults. While this will still require clear evidence of improved immunogenicity over the existing vaccine and stringent safety data before being licensed, it would be logistically less difficult than introducing a new pertussis vaccine for pediatric use. However, one scientific issue that may mitigate against success of this approach is that there is evidence that the immune responses is 'set' after the initial immunization and attempts to induce *B. pertussis*-specific Th1 responses in individuals

that have received a full course of aP vaccination may be difficult even through the use of Th1-inducing adjuvants. So switching from Th2 to Th1 by changing the adjuvants may not be so straightforward in a primed population, but should be less of an issue in naive individuals. Although it has been argued that the immune responses is Th2 skewed in neonates (Zepp *et al.* 1996), it has been demonstrated that infection with *B. pertussis* or immunization with wP vaccines can induce potent Th1-skewed CD4 T-cells responses in human infants (Ryan *et al.* 1998b, 2000; Rowe *et al.* 2005; Mascart *et al.* 2007), suggesting that it should be possible to induce Th1 responses in infants with an aP vaccine formulated with the appropriate Th1-promoting adjuvant.

In the longer term, a new pertussis vaccine will be required for primary and booster vaccination of infants and children. There is now convincing evidence from clinical studies that the current aP vaccine does not induce sustained protective immunity (Klein *et al.* 2012), and that *B. pertussis* is evolving to escape immune responses that the vaccines generate (Mooi, Van Der Maas and De Melker 2014). Furthermore, studies in mice complemented by those in humans have indicated that the aP vaccine fails to induce protective Th1 responses (Ryan *et al.* 1998b; Ross *et al.* 2013). The development of a new aP vaccine should address these limitations and should be capable of inducing Th1 responses and sustained immunity mediated by effective T- and B-cell memory. Preliminary studies have suggested that current aP vaccines are less effective than wP vaccines in inducing T_{FH} and T_{RM} cells (Wilk and Mills, unpublished). T_{FH} play a crucial role in T-cell help for antibody production and in memory B-cell formation, whereas T_{RM} appear to be critical for sustained cellular immunity at mucosal surfaces. Therefore, new generation vaccines also need to consider the induction of these T-cell types. The inclusion of additional antigen(s), such as ACT (Sebo, Osicka and Masin 2014) or the autotransporter BrkA (Marr *et al.* 2008), could help to broaden the immune responses and reduce the problem associated with immune escape but will add further complexity, especially for manufacturer and regulatory agency approval.

The clinical evaluation and regulatory approval of a new pertussis vaccine will not be straightforward. At this stage, the criteria for testing and licensing of a new vaccine are not clear. Large-scale phase 3 clinical trials, such as those carried out in Sweden and Italy in the 1990s, to estimate the efficacy of current aP vaccines (Greco *et al.* 1996; Gustafsson *et al.* 1996) will not be possible, because there is no equivalent cohort of non-vaccinated individuals, such as those available in Sweden and Italy in the 1990s when these countries had stopped using wP vaccines. Evidence of immunogenicity and safety in smaller clinical trials combined with estimates of efficacy from an animal model is one approach that may be considered. Alternatively, human challenge studies are being considered and have the obvious advantage over mice and baboons of being in the target species, albeit in adults rather than in children. However, most, if not all, adult volunteers, even if they lack circulating anti-*B. pertussis* antibodies, are likely to have memory T and B cells specific for *B. pertussis* antigens induced either by previous immunization or exposure to live *B. pertussis* through clinical or subclinical infections. Therefore, while human challenge may be useful for comparing immune responses and protection against infection induced by new compared with existing aP vaccines in primed individuals, they are unlikely to tell us what type of immune responses these vaccines will induce in unprimed individuals or whether they will protect naive individuals and more importantly naive children. Regardless of the complexity of the task, the obstacles to development, assessment and

regulation of new pertussis vaccines must be overcome using lessons from immunology in order to tackle the growing threat from this vaccine-preventable infectious disease.

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