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## The Key Players of Coxsackievirus-Induced Myocarditis

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### 1. Introduction

Myocarditis is a devastating cardiac disease causing death in children and young adults worldwide (Esfandiarei et al. 2008). The disease is clinically characterized by inflammation of the myocardium and degeneration of myocytes. Clinical symptoms of viral myocarditis range from flu-like and/or gastrointestinal illness to ventricular dysfunction ending commonly in heart failure. Acute disease is accompanied by multi-organ abnormalities and presents mostly in neonates and young children. Chronic disease occurs in one third of patients and is likely a consequence of autoimmune-mediated myocardial injury and viral persistence. As cardiac myocytes are destroyed by virus and/or self-induced cytopathic immune effects, excessive repair or fibrosis of myocardial tissue impairs disease progression rather than protects the tissue from further damage. Fibrosis or scar tissue, can lead to abnormal ventricular architecture that inevitably leads to the disruption of its function. At this stage of disease, chronic myocarditis progresses to dilated cardiomyopathy and can eventually lead to congestive heart failure (Esfandiarei et al. 2008).

The exact cause for myocarditis is still unknown though pathogen infections, hypersensitivity reactions, and systemic and autoimmune diseases are all likely contributing factors. It is suggested that acute viral myocarditis that progresses to chronic disease mirrors the clinical pathology observed with dilated cardiomyopathy patients and is likely the result of an inappropriate immune response after virus infection that leads to chronic inflammation and virus persistence (Escher et al. 2011).

Tracking the incidence of myocarditis is challenging. It is difficult to determine the potential disease burden to populations since there is a range in clinical symptoms associated with disease and endomyocardial biopsy to diagnose disease is rarely practised (Blauwet et al. 2010). Though mounting evidence of viral genomes recovered from chronic dilated cardiomyopathy patients provides some insight in to the potential burden of this devastating cardiac disease (Kuhl et al. 2005). Viral-induced myocarditis and dilated cardiomyopathy leads to a worse prognosis than other possible myocarditis/dilated cardiomyopathy etiological agents. Isolation of enteroviral RNA from endocardial biopsies of myocarditis and dilated cardiomyopathy patients renders these patients six times more susceptible to death after two years from diagnosis compared to virus-negative patients (Why et al. 1994). Sex is another contributing factor to disease susceptibility. Initially, it was

reported that men develop myocarditis twice as often as women and recent clinical work as supported this notion though the incidence is not quite as high as claimed in 1980 (Woodruff 1980; Mason et al. 1995; Kuhl et al. 2003; Cooper 2009).

Diagnosis and treatment of viral myocarditis is challenging due to the lack in specific clinical features and signature serological markers known for the acute phase of disease. The standard Dallas criteria that were typically used for diagnosis of myocarditis are not appropriate for determining viral or autoimmune myocarditis since they avoid the identification of inflammation or viral genome in the heart (Aretz 1987; Aretz et al. 1987). A more appropriate set of guidelines was established in 1995 by the WHO, with a classification of cardiomyopathies requiring endomyocardial biopsy, histological Dallas criteria, immunohistochemistry and viral PCR amongst the criteria for diagnosis (Richardson et al. 1996). Four stages of clinical disease in humans have been described since the implication of the 1995 WHO criteria: fulminant, subacute, chronic active and chronic persistent myocarditis. The first three stages involve mild to moderate dysfunction of the left ventricle, with the fourth, chronic persistent stage, characterized by normal ventricular function. Both chronic stages have ongoing inflammation with the development of scar tissue from myocardial damage. It is only during the chronic persistent stage that viral genome is detected from endomyocardial biopsy tissue (Lieberman et al. 1991; Olsen 1993). The incidence of viral infection, specifically coxsackievirus B infection, in human myocarditis, originates from seroepidemiologic and molecular studies between the 1950s and 1990s and from observations of viral genomes in cardiac tissue more prevalently in dilated cardiomyopathy patients compared to valvular or ischemic cardiomyopathies.

The true etiology and molecular pathogenesis responsible for viral myocarditis in humans remains unclear. Serological studies and endomyocardial biopsies from myocarditis patients have associated over 20 different viruses including coxsackieviruses, adenoviruses, cytomegaloviruses, parvoviruses, influenza viruses, and even human immunodeficiency viruses with the disease (Yajima et al. 2009; Blauwet et al. 2010). Of all the viruses implicated, it is the enteroviruses, specifically coxsackievirus B, which show the most likely contribution.

Coxsackievirus A and B are enteroviruses that are a part of the *Picornaviridae* family (Baboonian et al. 1997). They are human pathogens transmitted fecal-orally that cause enteric diseases (coxsackievirus A) as well as severe disease in the heart, pancreas, and central nervous system (coxsackievirus B) (Baboonian et al. 1997). Coxsackievirus A has 23 different serotypes, whereas coxsackievirus B has six. The six coxsackievirus B serotypes can instigate a variety of diseases including two very important autoimmune diseases: myocarditis and type I diabetes (Richer et al. 2009). It is also important to note that all coxsackievirus B serotypes are capable of triggering systemic disease in infants that can devastating lead to death (Esfandiarei et al. 2008). Relevant to myocarditis is the coxsackievirus B serotype 3 (coxsackievirus B3). Coxsackievirus B3 has been linked to approximately 30% of new dilated cardiomyopathy cases per annum though data establishing a direct link between coxsackievirus B3 pathogenesis and the onset of myocarditis in patients is lacking (Huber et al. 1998). The B3 serotype has a 7.4-kb single-stranded positive-sense RNA genome containing a VPg (3B) protein at the 5' end. The 7-methyl guanosine-like cap influences replication and translation following virus entry (Flanagan et al. 1977). To gain entry in to a cell, coxsackieviruses interact with both coxsackievirus and adenovirus receptor (CAR) and decay accelerating factor (DAF) located both in the host cell membrane (Figure 1) (Pelletier et al. 1988).

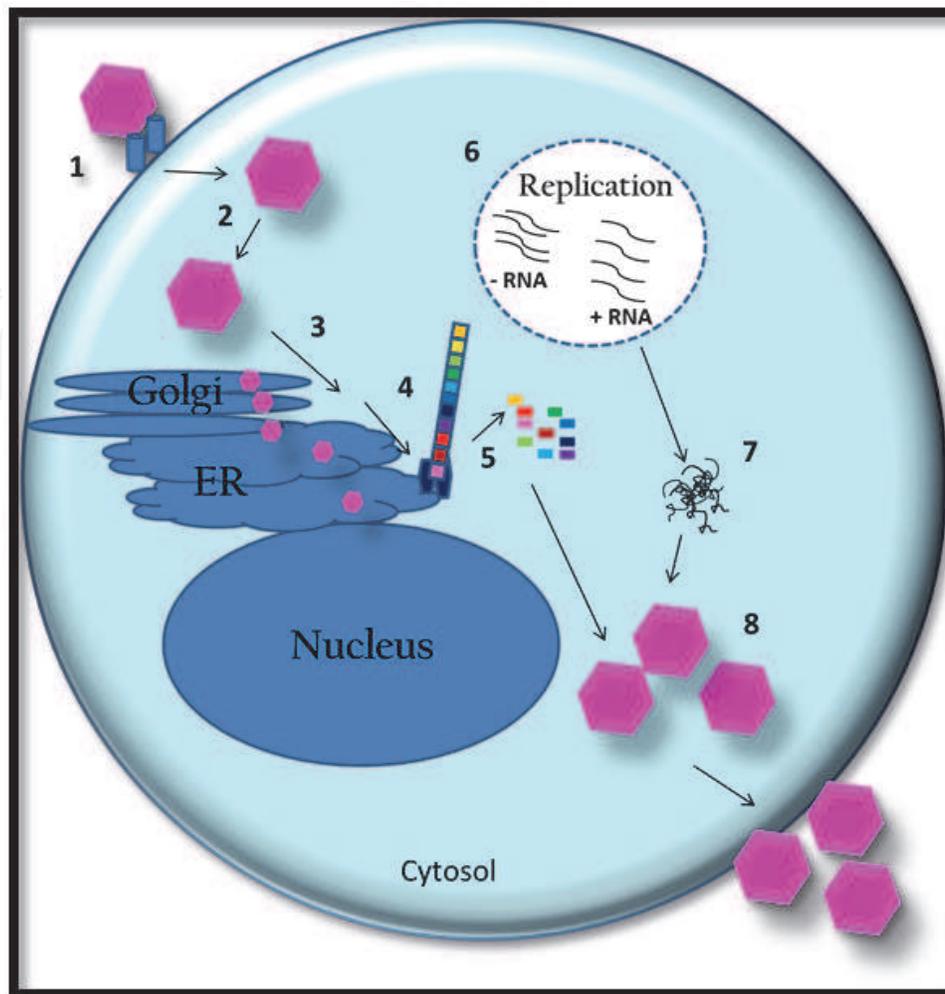


Fig. 1. Tentative coxsackievirus life cycle. The general host and virus components suggested to contribute to production of new coxsackievirus: 1) Viral entry through binding coxsackievirus and adenovirus receptor (CAR) and decay accelerating factor (DAF), 2) Internalization and transport of viral particles to the Golgi and endoplasmic reticulum (ER) 3) viral uncoating, 4) release of viral RNA, translation of RNA by ribosomes on the rough endoplasmic reticulum (ER) into viral polyprotein, 5) autocleavage of polyprotein into viral structural and functions proteins, 6) positive and negative strand RNA transcription to replicate viral genome, 7) release of viral genome in to the cytosol to encapsidate with structural proteins, 8) formation and release of viral progeny.

Once the virus enters the cytosol and uncoats, its positive-sense genome is released in the cytosol for translation and later, transcription. As a polyprotein comprised of the virus proteins VP4, VP3, VP2, VP, 2A, 2B, 2C, 3A, 3B, 3C, and 3D emerges from translation at the rough endoplasmic reticulum (ER), it is cleaved into its respective structural and functional proteins. The virally encoded 3D<sup>pol</sup> is a RNA-dependent RNA polymerase that transcribes viral positive-sense RNA in to negative-sense RNA strands that serve as intermediates for the transcription of multiple positive-sense RNA strands needed for new progeny virions. After synthesis, the newly generated positive-RNA strands are packaged in new virus particles formed by the newly generated structural and functional virus proteins. The new progeny viruses are then released via plasma membrane by a mechanism likely mediated by viral

protein 2B (van Kuppeveld et al. 1997; Esfandiarei et al. 2008). Though many groups have identified key virus and host interactions throughout the coxsackievirus B life cycle, there still remains many stages unsolved. Nevertheless, the role of coxsackievirus B in the onset of myocarditis has been extensively studied thus far in animal models and cell culture systems. Here, we will discuss clinical and mouse studies that have investigated coxsackievirus-induced myocarditis and the role of key immune players in disease pathogenesis.

## 2. *In vivo* experimental systems

Mice provide a model system to distinguish characteristics of disease between autoimmune myocarditis and viral myocarditis. Mice are advantageous models because they share similar genetics to humans, they are cost-effective in handling and breeding, many transgenic strains are available and they are responsive to cardiotropic viruses (Cunningham 2001; Fairweather et al. 2001; Esfandiarei et al. 2008). Coxsackievirus B3 has been detected in 30-50% of dilated cardiomyopathy patients, providing support for a coxsackievirus B3-induced myocarditis mouse model (Escher et al. 2011). Following a single dose of coxsackievirus B3, acute myocarditis, encephalomeningitis, hepatitis, and even pancreatitis can ensue. Severe systemic pathogenicity observed with coxsackievirus B3 infection has also been related to the presence of sarcoma (Src) family kinase Lck (p56<sup>lck</sup>) (Liu et al. 2000). With coxsackievirus B3 infection, mice tend to either develop chronic dilated cardiomyopathy mirroring clinical disease after recovering from viral infection or mice die from severe cytopathic effects, thus making coxsackievirus B3-induced myocarditis an excellent yet challenging model to study (Liu et al. 2000).

Studies with mice have demonstrated the significant contribution of the Th1 immune response to disease severity even though coxsackievirus B3 directly targets and destroys the myocardium. The contribution of a Th1 response to viral myocarditis pathogenesis is supported by studies modulating and inhibiting immune components and improving cardiac damage and function (Jiang et al. 2008).

Interestingly and in parallel with human myocarditis, male mice infected with coxsackievirus B3 experience acute myocarditis with greater severity compared to females. Greater disease severity in males has been linked to enhanced cardiac and splenic mast cell and macrophage TLR-4 expression at 12 hours post-infection (Frisancho-Kiss et al. 2006; Frisancho-Kiss et al. 2007). In males virally infected, signalling through cardiac TLR-4 enhances the production of cardiac IL-1 $\beta$  and IL-18 and promotes a Th1 skewed immune response (Fairweather et al. 2003). Also, male mice infected with coxsackievirus B3 harbour macrophages of a different phenotype and have more severe disease compared to infected female mice though viral replication is at similar levels in the heart (Frisancho-Kiss et al. 2006; Frisancho-Kiss et al. 2007). Moreover, genes relating to cholesterol metabolism in macrophages and to androgen receptor, which are known predictors for myocarditis, dilated cardiomyopathy and heart failure in male mice and humans, are upregulated considerably in spleens of male mice (Onyimba et al. 2011). This not only affords support for a gender difference in the susceptibility to viral-myocarditis, but critically implicates the innate response in disease severity. Furthermore, to bring male mice to the same immunological plane as female mice during myocarditis, Frisancho-Kiss et al removed the gonads from BALB/c mice and observed an increase in IL-4 production, the activation of macrophages and induction of regulatory T cells in the heart (Frisancho-Kiss et al. 2009).

Myocarditis can be induced in female mice with TNF- $\alpha$  treatment on days 1 and 3 post coxsackievirus B3 infection (Huber 2010). The lack of disease susceptibility in females is

attributed to low mRNA and protein levels of TNF- $\alpha$  and IL-1 $\beta$  as well as reduced CD1d expression on splenic lymphocytes. CD1d is an important non-classical major histocompatibility complex antigen that can be regulated by TNF- $\alpha$  to induce myocarditis susceptibility in female mice (Huber 2010).

Not only is susceptibility to developing myocarditis dictating by sex, but development of chronic disease depends on the mouse strain. A.BY/SnJ & SWR/J are susceptible mouse strains that can develop ongoing myocarditis, where viral RNA is detected within the myocardium. C57BL/6J & DBA/1J mice are resistant strains capable of eliminating virus just after the early acute phase of disease. Tomioka and colleagues investigated neutralizing antibodies and their role in virus-induced myocarditis and B-cell-mediated immunity using BALB/c mice (Esfandiarei et al. 2008). NK-deficient mice have been used to look at the role of natural killer (NK) cells in killing virus-infected cardiomyocytes (Godeny et al. 1986). Perforin knockout mice inoculated with coxsackievirus B3 have also been used to describe the interplay of virus infection and lymphocyte infiltration in the myocytes and their effect on disease outcome (Godeny et al. 1987). Interestingly, with coxsackievirus B3 infection in suckling, weaning and adolescent mice, coxsackievirus B3 replicates in the heart, pancreas, spleen, and brain and causes human disease-like symptoms. In fact, following IP injection, three distinct immunovirological phases of disease have been observed.

In mice, coxsackievirus B3 can induce two forms of inflammatory heart disease, acute only or acute and chronic (biphasic) autoimmune disease (Horwitz et al. 2000; Cunningham 2001; Fairweather et al. 2001). Interestingly, coxsackievirus B3 replication is mainly observed in the pancreas and to a lesser extent in the heart. In genetically susceptible mice, such as A/J2, Balb/c and NOD mice, chronic autoimmune myocarditis after coxsackievirus B3 infection is observed. Autoimmune myocarditis in mice presents as early as day 7 pi with inflammatory cell infiltration in the heart and the formation of multifocal inflammatory lesions. At this stage, autoantibodies (autoAbs) against heart antigens, like cardiac myosin (cardiac myosin), are seen. In NOD mice, isotype switching ensues after 2 to 3 weeks, with the autoAbs switching from IgM to IgG subclasses (Kaya et al. 2001; Kaya et al. 2002). It is particularly remarkable to note that the chronic autoimmune heart disease induced by coxsackievirus B3 in mice resembles inflammatory heart disease seen with myocarditis and dilated cardiomyopathy in humans. There are still many complications with the full characterization and study of chronic virus and/or autoimmune induced myocarditis in mice and humans. One such complication is the acute infection that precedes chronic disease.

### 3. Experimental autoimmune myocarditis

As described in the previous section, mouse models greatly aid the analysis of autoimmune diseases. To set apart the autoimmune phase of disease from acute infection an experimental induced autoimmune myocarditis model was developed (Blyszczuk et al. 2008). Experimental induced autoimmune myocarditis mimics the typical chronic phase of disease observed in genetically susceptible mice infected with coxsackievirus B and different stages of disease severity observed with experimental induced autoimmune myocarditis models are graded according to the extent of inflammatory infiltrates at the peak of inflammation. Autoimmune myocarditis can be induced by the injection of cardiac myosin with complete Freund's adjuvant and pertussis toxin. Mice injected with this combination of self-antigen and adjuvants are able to generate cardiac myosin-specific autoantibodies and present with

heart pathology similar to coxsackievirus B3-induced disease 3 weeks post-injection (Figure 2) (Kodama et al. 1992).

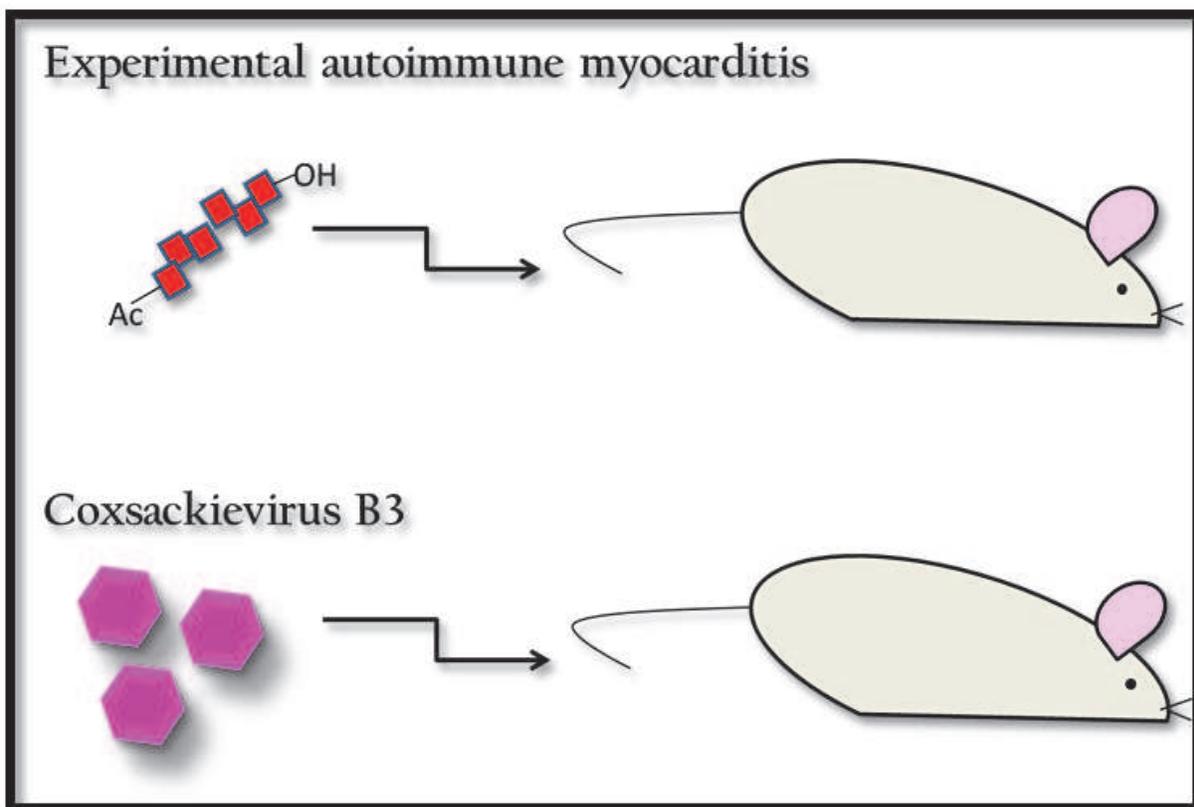


Fig. 2. Experimental autoimmune myocarditis versus coxsackievirus B3 induced myocarditis. Experimental autoimmune myocarditis involves the induction of autoimmunity by the injection of self-protein such as cardiac myosin or troponin I with adjuvants or without or injections of other cardiomyogenic peptides to induce chronic myocarditis as seen with coxsackievirus B3 infection in susceptible mice.

Neu et al suggest that autoimmune myocarditis is induced indirectly by viral infection and that one causative factor may be an autoimmune response to the cardiac myosin released or exposed after the virus-mediated myocyte damage (Neu et al. 1987). If this is the case, autoimmune disease should be induced by immunization with cardiac myosin alone and susceptibility to coxsackievirus B3-induced autoimmune myocarditis should be as likely as susceptibility to the myosin-induced autoimmune disease (Neu et al. 1987). Troponin I has also been used as an autoantigen for induced autoimmune myocarditis (Leuschner et al. 2009). Programmed cell death-1 receptor deficient mice develop cardiomyopathy with production of high-titered autoantibodies against cardiac troponin I (Okazaki et al. 2003). Cardiac troponin I induces a robust autoimmune response that encompasses both humoral and cellular responses that leads to severe inflammation and fibrosis in the myocardium of mice. Mice induced with cardiac troponin I have a genetic and sex biased susceptibility for myocardial inflammation compared to other autoimmune disease models (Leuschner et al. 2009). The key to reproducing ideal autoimmune disease conditions for studying myocarditis is to use a defined immunogen, ie cardiac myosin, troponin I etc. obtained from the same species as the study's model. Moreover, genetically defined inbred strains of susceptible and resistant mice allow

results to be reproducible and facilitate detailed analysis of the autoimmune disease cellular and molecular components (Neu et al. 1987).

Other species such as rats and Guinea pigs have also greatly contributed to our understanding of myocarditis. Immunization of different animal models with heart homogenates derived from various species has not been, unfortunately, successful at inducing myocarditis, where the results were often controversial (Neu et al. 1987). In most of those models, mild lesions appeared in the heart. However, out of the differing species models, 2 reports: the guinea pig model by Hosenpud et al and the murine model by Neu et al, showed enlargement of the heart with autoimmune myocarditis (Hosenpud et al. 1985; Neu et al. 1987). The authors also noted discoloration of cardiac surfaces, pericardial effusion and lethal clinical course that were conditions not previously observed in experimental induced autoimmune myocarditis. Kodama et al were also able to produce such pathological and clinical conditions using the Lewis rat model (Kodama et al. 1992) though the cardiac myosin immunization murine model seems to have had the greatest impact on our understanding of autoimmune myocarditis.

There are many ways in which autoimmune myocarditis can be induced in animal models. Immunization of susceptible mice with cardiac myosin or with a myocardiogenic peptide derived from the alpha cardiac heavy chain emulsified in complete Freund's adjuvant induces myocarditis in mice with a peak of inflammation in the heart around day 21. This inflammation observed with induced autoimmune disease is similar to that seen in the coxsackievirus B3 induced autoimmune myocarditis model during the chronic phase, however in this case, does not include the earlier complication of a pathogen infection. The immunization with cardiac myosin is associated with production of cardiac myosin-specific autoantibodies and cardiac myosin-specific T cells (Kodama et al. 1992; Godsel et al. 2001; Leuschner et al. 2009; Rose 2010). It has been demonstrated that the induction of disease by immunization with cardiac myosin can only be successful in genetically susceptible mice (Neu et al. 1987). We suggest that the complex genetics of the host therefore determines whether an infection will resolve or proceed to an adverse autoimmune outcome (Poffenberger et al. 2010). Identifying particular traits that favour susceptibility or resistance to an autoimmune incidence helps us understand how infectious diseases culminate in autoimmune disease. Other methods used to induce autoimmune myocarditis include administration of 2 early, critical proinflammatory cytokines, IL1b and TNF- $\alpha$  and injection of mice with myosin in combination with CFA and additional lipopolysaccharide. Some groups have reported the induction of autoimmune myocarditis using immunization with porcine cardiac myosin (Wang et al. 1999). Interestingly, Wittner et al. produced myosin autoantibodies and myocarditis in rabbits using immunization with bovine heart myosin (Wittner et al. 1983). Daniels et al reported the development of a recombinant model of experimental induced autoimmune myocarditis, induced by immunization with a 68kDa fragment of cardiac myosin referred to as Myo4 (Daniels et al. 2008). Myo4 induces severe autoimmune myocarditis in A/J mice 21 days post immunization. The immune response to Myo4 immunization is characterized by Th1 and Th17 features. Myo4-experimental induced autoimmune myocarditis has advantages over other models in terms of immunogen production and the ability to measure antigen-specific functional immunity in *ex vivo* assays. Myo4 and other immunoantigen experimental induced autoimmune myocarditis provide a means to investigate epitope spreading and the effect on disease pathology as well as prophylactic and therapeutic treatment studies aimed at developing therapeutics to alleviate acute and chronic autoimmune myocarditis (Daniels et al. 2008).

Experimental induced autoimmune myocarditis is mediated by a CD4<sup>+</sup> T cell immune response. Homing of cardiac myosin-specific CD4<sup>+</sup> T cells into the myocardium is the first pathologic event observed with experimental induced autoimmune myocarditis. Subsequently, neurohumoral factors such as cytokines and chemokines are released in the myocardium. This then recruits various bystander inflammatory cells to cross vascular endothelial cell walls and enter the myocardium. In effect, blocking the recruitment of inflammatory mediators to the myocardium may present as a critical target for myocarditis therapy (Tanaka et al. 2011). Experimental induced autoimmune myocarditis development is also typically associated with elevated titers of heart-specific autoantibodies, though in rats and susceptible mouse strains the response depends on the induction and expansion of heart specific, autoreactive CD4<sup>+</sup> T cells. In BALB/c mice, CD4<sup>+</sup> T cells belong to a specific subset of IL-17 producing helper cells, i.e. Th17 cells (Chang et al. 2008). With experimental induced autoimmune myocarditis, heart-infiltrating alpha-myosin-specific CD4<sup>+</sup> Th17 cells are pathogenic and cause ongoing inflammation in the myocardium (Blyszczuk et al. 2008). Notably, autoimmune myocarditis induced by myosin can be reiterated in mice adoptively transferred with pathogenic CD4<sup>+</sup> T lymphocytes (Sukumaran et al. 2011). In addition to T cells, passive administration of antimyosin monoclonal antibodies induces myocarditis in DBA/2 but not in BALB/c mice. DBA/2 mice are susceptible to passively induced disease due to the presence of myosin or a myosin like protein in their extracellular matrix. In addition to antibodies and T cells contributing to the pathogenesis of inflammatory myocardial lesions (Leuschner et al. 2009), injection of activated bone marrow (BM)-derived dendritic cells loaded with heart-specific self peptide can instigate disease pathogenesis (Kania et al. 2009).

Autoimmune myocarditis can be induced experimentally by several means as is the case for protection from induced disease. Amelioration of disease has been demonstrated with oral administration of specific antigens in several autoimmune models (Gonnella et al. 2009). Protection from experimental induced autoimmune myocarditis has also been observed with many studies from Godsel et al (Godsel et al. 2001). They successfully administered syngeneic splenocytes covalently coupled with ethylene carbodiimide (ECDI) by intravenous injections to prevent and treat a number of autoimmune diseases in animal models. Initial application of this approach in humans has been fortunately successful. Godsel et al essentially demonstrate in animal models that coupled-cell tolerance is an effective approach for the prevention of myocarditis and may present as a useful antigen-specific immunotherapy for treating myocarditis in humans (Godsel et al. 2001). Antigen-specific peripheral tolerance induction also represents a powerful tool for dissecting the mechanisms involved in cardiac autoimmunity. Although myosin-specific tolerization is commonly used to prevent experimental induced autoimmune myocarditis, it is also important to note that tissue homogenates are equally useful. This approach has yet to be tested in other models of myocarditis including coxsackievirus myocarditis (Godsel et al. 2001). Horwitz et al's investigation in to cardiac myosin tolerance and protection from experimental induced autoimmune myocarditis conflicted with results presented in the Godsel et al studies (Horwitz et al. 2005). Since cardiac myosin is a major autoantigen in virus-induced myocarditis the question was asked whether inhibition of this autoantibody response to cardiac myosin could prevent destructive autoimmunity. In NOD mice, cardiac myosin-specific antibodies develop following both coxsackievirus B3 infection and experimental induced autoimmune myocarditis induction. To ask whether the induction of peripheral tolerance to a single self-antigen could be used to prevent coxsackievirus-induced

autoimmune myocarditis, a disease likely directed at multiple self-antigens, NOD mice were tolerized to cardiac myosin using a covalently coupled antigen approach and subsequently challenged with coxsackievirus B3. Cardiac myosin tolerance to effectively prevent coxsackievirus B3-mediated autoimmune myocarditis was not observed, possibly reprimanding cardiac myosin as a single autoantigen player in viral-mediated disease (Horwitz et al. 2005).

Another possible player in viral-mediated disease as demonstrated with experimental induced autoimmune myocarditis may be multinucleated giant cells. Wang et al showed that the cardiac lesions in experimental induced autoimmune myocarditis are histologically similar to human myocarditis, with myocyte swelling, necrosis, and fibrosis that are accompanied by mononuclear cell infiltration consisting of granulocytes, macrophages, CD4+, CD8+ T cells, B cells and multinucleated giant cells (Wang et al. 1999; Blyszczuk et al. 2008; Daniels et al. 2008). Also, the histological features observed in myocarditis induced by cardiac myosin are very similar to the description of active giant-cell myocarditis in humans (Wang et al. 1999; Blyszczuk et al. 2008). Therefore, it has been inferred that macrophage-derived multinucleated giant cells, together with other inflammatory cells, are important mediators of myocyte destruction. The presence of a heterogeneous population of cellular components in the cardiac infiltrate also implies the existence of a complex, cytokine-rich microenvironment that may contribute to the pathogenesis of autoimmune myocarditis. TNF- $\alpha$ , IL-1 $\beta$ , IL-2, IFN- $\gamma$ , IL-4 and IL-10 are cytokines that have been detected in culture supernatants of splenocytes derived from mice at the peak of myocardial disease. Their detected levels were higher in mice that developed myocarditis than mice immunized but not developing disease. Furthermore, it has been previously suggested that TNF- $\alpha$  contributes to myocarditis pathogenesis by either causing direct injury of cardiomyocytes or, together with IL1b, triggering the production of nitric oxide. The expression of E-selectin, VCAM-1 and ICAM-1 has also been found to be markedly increased in inflamed hearts during induced disease. The increased expression of these adhesion molecules on the vascular endothelium may contribute to the extravasation and accumulation of inflammatory cells observed in the myocardium (Wang et al. 1999; Blyszczuk et al. 2008).

There are limitations of using experimental induced autoimmune myocarditis as a model to separate the mechanisms of biphasic (acute infection and chronic) viral disease, where the model relies too heavily on the inoculation of a single antigen. There are undoubtedly other key immune players that contribute to the onset of autoimmune disease following virus infection with the innate immune system being one of the major contributors having a dual role: controlling and perpetuating disease. Despite the fact that the experimental induced autoimmune myocarditis models are rather artificial, they offer the advantage of studying disease pathogenesis and autoimmune mechanisms *in vivo* in the absence of an infective agent. Immunization with myosin peptide-loaded activated dendritic cells offers a useful tool to dissect the role of antigen-presenting cells (APCs) and effector cells while studying disease mechanisms. From the experimental induced autoimmune myocarditis model we can not only learn about autoimmune mechanisms contributing to disease development, but we can study the pathophysiology of inflammatory heart disease and design novel immunomodulating treatment strategies. In addition, the experimental induced autoimmune myocarditis model might offer a potential tool to improve the diagnostic accuracy of currently available and future imaging technologies (Blyszczuk et al. 2008).

Work from our lab using the experimental induced autoimmune myocarditis model has helped focus our attention to the role of innate immunity in response to an infectious agent

and as a driving force in the development of autoimmune disease. The innate immune system includes many key players like pathogen recognition receptors that recognize highly conserved pathogen-associated molecular patterns on microbial invaders. These receptors include the Toll-like receptors and expression of these receptors on antigen presenting cells such as macrophages and dendritic cells determine not only innate immunity but the subsequent adaptive immune response.

#### **4. Key players in immunity**

##### **4.1 Pathogen recognition receptors**

Cardiotropic viruses can be cytopathic, killing off host cells, yet their viral RNA is detected and tends to persist in cardiac muscle. Viral persistence in the myocardium can then lead to chronic inflammatory cardiomyopathy. Pathogens such as viruses are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors of the innate immune response. The recognition of a pathogenic insult releases proinflammatory cytokines that serve as protectors from infection and perpetrators of chronic inflammatory disease (Lane et al. 1993; Kawai et al. 2006). Activation of Toll-like receptors by pathogen associated molecular patterns and the subsequent production of proinflammatory cytokines can lead to protection as well as the exacerbation of an autoimmune response.

Pathogen-associated molecular patterns from viruses like double-stranded RNA are sensed by Toll-like receptor 3 (TLR3) (Schnare et al. 2001; Pasare et al. 2003). Infections with coxsackievirus B in TLR3 knock out mice have demonstrated an important role for TLR3 in host defense. The innate antiviral response is mediated, at least in part, by nucleic acid-sensing receptors such as TLR3, retinoic acid inducible gene I (RIG-I), and melanoma differentiation-associated protein-5 (MDA-5). The activation of RIG-I/MDA5 receptor pathways is thought to evoke type I IFN responses. TLR3, which recognizes double stranded RNA, is critical in the antiviral immune response against coxsackievirus B3. TLR3-deficient mice are highly susceptible to coxsackievirus B3, where impaired antiviral responses and acute myocarditis ensue. Increased disease in TLR knock out mice is associated with decreased production of IL-12p40, IFN $\gamma$  and IL-1 $\beta$  post infection. Mice deficient in the TLR3 adaptor protein, Trif, have a similar disease course as TLR3 knock out mice suggesting that the TLR3-Trif pathway is also important in the host response to coxsackievirus B3 infection. Research from Negishi et al reveals a critical cooperation between the RIG-I/MDA5-type I IFN and TLR3-type II IFN signaling axes for efficient innate antiviral immune responses (Negishi et al. 2008). Importantly, a rare TLR3 variant has been identified in patients diagnosed with enteroviral myocarditis (Gorbea et al. 2010). These patients also held a greatly increased incidence of a common polymorphism. Gorbea et al also demonstrated that induction of the TLR3 variant or the TLR3 possessing the common polymorphism with synthetic double stranded RNA hindered proper TLR3-mediated signaling. Also, with coxsackievirus B3 infected cell lines, mutated TLR3 impaired type I IFN signalling and production and failed to control viral replication (Gorbea et al. 2010). The Gorbea et al study thus suggests that individuals who possess these particular TLR3 variants may have an ineffective innate anti-enteroviral response that fails to clear the virus and in turn, elevates the associative risk for cardiac disease. Interestingly, human cardiac myosin pathogenic epitopes can directly stimulate other human Toll-like receptors such as Toll-like receptors 2 and 8 (TLR2, TLR8). Stimulation of these receptors allows for the production of proinflammatory cytokines from human monocytes. TLR8, found within

endosomes, detects single stranded RNA, such as the coxsackievirus B3 genome and instigates inflammation (Triantafilou et al. 2005; Zhang et al. 2009).

Signalling through another critical receptor, Toll-like receptor 4 (TLR4) also leads to the expression of proinflammatory cytokines, but has been implicated as a cardiomyopathy etiological factor. Satoh et al have suggested that myocardial expression of TLR4 is linked to coxsackievirus B3 replication in human cardiomyopathy and that TLR4 may be directly involved in the pathogenesis of disease (Satoh et al. 2004). Viral proteins have actually been found to co-localize with TLR4 in infected cardiac tissue. In coxsackievirus B infected mice, TLR4 deficiency reduces viral pathogenesis and the production of several cytokines including IL-1 $\beta$  and IL-18 (Pasare et al. 2003; Pasare et al. 2004).

Another major player in host defense is the critical adaptor protein for TLR signaling myeloid differentiation primary response gene (MyD88). MyD88 signaling has been associated with several aspects of the pathogenesis of chronic autoimmune myocarditis. MyD88 activates self-antigen presenting cells and promotes autoreactive CD4<sup>+</sup> T-cell expansion in experimental induced autoimmune myocarditis. To determine the role of MyD88 in the progression of acute myocarditis to an end-stage heart failure, Blyszczuk et al used alpha-myosin heavy chain peptide (MyHC-alpha)-loaded activated dendritic cells (Blyszczuk et al. 2008). They induced myocarditis in wild-type and MyD88 knock out mice and observed comparable heart-infiltrating cell subsets and CD4<sup>+</sup> T-cell responses. Injection of complete Freund's adjuvant or MyHC-alpha/complete Freund's adjuvant into diseased mice caused cardiac fibrosis, ventricular dilation, and disrupted heart function in wild-type but not MyD88 knock out mice (Pasare et al. 2003; Marty et al. 2006; Blyszczuk et al. 2008). The protection of MyD88 knock out mice from the induction of experimental induced autoimmune myocarditis is likely from the impairment of other key players of autoimmunity such as antigen presenting cells. The role of MyD88 in cardiac fibrosis has been demonstrated with chimeric mice, where the origin of fibroblasts that replace inflammatory infiltrates was determined to be from the bone marrow. MyD88 has thus been suggested to be critical for the development of cardiac fibrosis during progression to heart failure (Pasare et al. 2003; Marty et al. 2006). Fuse et al observed elevated MyD88 cardiac protein levels in the hearts of wild-type mice after exposure to coxsackievirus B3 and MyD88 knock out mice have a greater survival rate (86%) compared to wild type mice (35%) after coxsackievirus B3 exposure (Fuse et al. 2005). MyD88 is implicated not only in cardiac inflammation and mediating cytokine production, but is also associated with skewing the Th1/Th2 cytokine balance, increasing the expression of coxsackie-adenoviral receptor important for virus entry and viral titers after coxsackievirus B3 incidence. In the absence of MyD88, protection from virus infection and disease is observed and is suggested to be associated with IRF-3 and IFN- $\beta$  activation (Fuse et al. 2005). From the above mentioned MyD88 work, it is fair to infer that MyD88 could be a useful target for preventative heart-specific autoimmunity and cardiomyopathy treatments (Marty et al. 2006). TLR signalling may be a major contributor to the initiation and progression of autoimmune myocarditis though there are many additional players such as the cells that express viral and self antigen sensors (antigen presenting cells) that remain poorly understood.

#### **4.2 Antigen presenting cells (APCs)**

Following viral infection, a cellular immune response is needed to completely clear the virus. However these same cells can drive chronic inflammation and autoimmune responses. Antigen presenting cells and other cell types critical in activating the cellular

response to viral infection such as CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells,  $\gamma\delta$  T cells, B cells, macrophages, mast cells, neutrophils, NK cells and DC cells are all detected in the hearts of mice post coxsackievirus B3 infection and with experimental induced autoimmune myocarditis induction (Afanasyeva et al. 2004; Cooper 2009; Kemball et al. 2010).

Antigen presenting cells play a pivotal role in the stimulation of acquired immunity and can be manipulated by cytokines and environmental factors. The manipulation of antigen presenting cells modulates the T cell response and results in changes in tolerance to specific antigens. Antigen presenting cells influence lymphocyte responses by 1) promoting helper T-cell 1 (Th1), Th2 or Th17 responses, 2) inducing peripheral tolerance, and 3) activating regulatory T cells (Chatenoud et al. 2005; Ait-Oufella et al. 2006; Blyszczuk et al. 2008). From autoimmunity studies it has been curiously determined that regulatory T cells and Th17 cells have opposing functions during autoimmunity (Langrish et al. 2005). Th17 cells are an important pro-inflammatory T cell lineage during heightened tissue inflammation and autoimmunity, whereas regulatory T cells function to suppress immune responses (Richer et al. 2008; Korn et al. 2009; Marchant et al. 2010; Wing et al. 2010; Zou et al. 2010). Antigen presenting cells trigger changes in regulatory T cells and other T cell populations, which alters disease outcome and may be a promising therapeutic avenue to further investigate in viral-induced autoimmune diseases such as viral myocarditis.

Damage to the myocardium and the onset of coxsackievirus B3-induced acute myocarditis in mice is attributable to many immune factors including activated antigen-specific T cell activity. Two signals are required for activating T cells: first through the T-cell receptor engaging with antigen loaded MHC on antigen presenting cells and next through costimulatory molecules on antigen presenting cells such as CD40 and B7. CD40L on T cells engages CD40 on antigen presenting cells activating them to secrete cytokines and express adhesion molecules. Signalling from both the T cell receptor and costimulatory molecules promotes the proliferation of the antigen-specific T cells and stimulates an anti-antigen immune response. Early work with CD40/CD40L revealed enhanced CD40 expression on cardiac myocytes of coxsackievirus B3-infected mice and reduced myocardial inflammation with anti-CD40L/B7-1 monoclonal antibody treatment (Seko et al. 1998). Increased expression of CD40 and the B7 family of costimulatory molecules has also been observed in myocardial tissue from patients with dilated cardiomyopathy and acute myocarditis (Seko et al. 1998). Recently, CD40-Ig treatment was used post-coxsackievirus B3 infection to block the interaction between CD40/CD40L in male Balb/c mice and notably, this treatment reduced inflammation and coxsackievirus B3 transcription. CD40-Ig treatment also skewed the Th1/Th2 response in favour of Th2 cytokines rather than Th1 (Bo et al. 2010). This work and studies with myocardial tissue from patients has important implications not only for the role of CD40, but also for potential therapeutic options that downregulate the inflammatory Th1 response in coxsackievirus B3-mediated acute myocarditis.

Dendritic cells are highly specialized antigen presenting cells that upon encountering a pathogen undergo maturation. This process involves antigen processing, upregulation of major histocompatibility class (MHC) class II molecules, induction of costimulatory activity and migration to lymph nodes, where they prime antigen-specific T cells. With their antigen processing capability, dendritic cells can trigger activation of autoreactive T cells (Eriksson et al. 2003). Dendritic cells may also be involved in both host defense and maintenance of peripheral tolerance. Dendritic cells may also play an important role in autoimmune myocarditis (Marty et al. 2006). Dendritic cells from infected susceptible mice produce lower levels of cytokines and chemokines, particularly IP-10, a chemokine

with cardioprotective properties. In the hearts of healthy wild type mice, tissue-resident dendritic cells take up and present endogenous heart-specific peptides (Eriksson et al. 2003). Activated and self-antigen loaded dendritic cells induce myocarditis and heart failure in genetically susceptible mice. The mechanism by which dendritic cells instigate damage to the myocardium is likely a combined effect from tissue damage and innate immunity activation that causes dendritic cells to activate autoreactive T cells and target the myocardium (Marty et al. 2006). This proposed mechanism was supported by Eriksson et al who have shown that injection of dendritic cells loaded with cardiac myosin peptide induces CD4<sup>+</sup> T-cell-mediated autoimmune myocarditis (Eriksson et al. 2003). Interestingly, the dendritic cell-induced autoimmunity observed by Eriksson et al resulted only with TLR and CD40 stimulation. They demonstrated how TLR signalling following the onset and resolution of acute myocarditis instigates the reoccurrence of inflammatory infiltrates in the heart and the onset of autoimmunity. TLR signalling activation was also important for myocarditis induction in mice injected with damaged, immune stimulating cardiomyocytes (Eriksson et al. 2003). These few studies provide an insight into the possible role of dendritic cells in the induction of myocarditis and they offer an alternative, complete Freund's adjuvant-free method of inducing experimental induced autoimmune myocarditis (Afanasyeva et al. 2004).

The work done by Eriksson et al suggests targetting TLR signalling pathways may be needed as a therapeutic avenue to protect from heart-specific autoimmunity. In a scenario where microbial infections are acting concurrently with myocardial damage, such as with coxsackievirus B3 infection and myocarditis onset, self peptide-loaded dendritic cells might respond to the various pathogen associated molecular patterns in the environment that stimulate different TLRs and induce tolerance rather than act in antigenic mimicry. The end result may not be antigenic mimicry to instigate autoimmunity, but downregulation of autoreactive T cells and induction of tolerance (Eriksson et al. 2003; Blyszczuk et al. 2008). With this in mind, innate activation pathways such as TLR signaling, may be attractive targets for autoimmune myocarditis therapy.

Macrophages, another type of antigen presenting cell, play a critical role in the immune response to coxsackievirus B3 infection and have been implicated in the pathogenesis of coxsackievirus B3-induced autoimmune myocarditis. There are two groups of macrophages, type I or type II that are defined by their activation markers and cytokine production. Type II macrophages have been linked to the cardiac repair stage following acute myocarditis (Nahrendorf et al. 2007). The significance of macrophages in coxsackievirus B3 myocarditis was demonstrated in previous work by Richer et al and Horwitz et al, where a transgenic TGF- $\beta$  mouse model demonstrated a protective role for TGF- $\beta$  against autoimmune disease. This protection coincided with a reduction in macrophage maturation suggesting the important involvement of macrophage inflammatory properties (Horwitz et al. 2006; Richer et al. 2006). It has been suggested therefore, that a balance between the inflammatory macrophages that are necessary for defense against viruses and the macrophages necessary for the resolution of an immune response and tissue healing is critical for an appropriate antiviral immune response that avoids autoimmunity (Heath et al. 2004). Interestingly, macrophage phenotype can differ between male and female mice with coxsackievirus B3-induced myocarditis. Since coxsackievirus B3 infection induces severe myocarditis only in male mice it is possible that myocardial infiltrating macrophages detected in female mice will have a distinct functional phenotype that contributes to their protection from coxsackievirus B3-induced myocarditis. Li et al observed myocardial infiltrating

macrophages from coxsackievirus B3-infected male mice expressing high levels of classically activated macrophages (type I) markers, such as inducible nitric oxide synthase, IL-12, TNF- $\alpha$ , and CD16/32, whereas macrophages from females had increased expression of arginase 1, IL-10, macrophage mannose receptor and macrophage galactose type C-type lectin that are typically associated with alternatively activated macrophages (type II) (Li et al. 2009). Li et al also demonstrated a distinct myocardial-derived cytokine signature that is sex-biased and contributes to differential macrophage polarization after coxsackievirus B3 infection. With adoptive transfer experiments using *ex vivo* programmed M1 macrophages, Li et al observed significantly increased myocarditis in both male and female mice. However, the transfer of M2 macrophages into susceptible male mice protected mice from myocardial inflammation (Li et al. 2009). This protection was postulated to be the result of a modulated local cytokine profile that contributed to the promotion of peripheral regulatory T cells differentiation. This work has helped our understanding of a possible mechanism that underlies the gender bias in coxsackievirus B3 myocarditis susceptibility. Developing therapeutic strategies that manipulate macrophage polarization may be a promising avenue for the treatment of inflammatory heart diseases.

#### 4.3 Cytokines and chemokines

Cytokines and chemokines also play critical roles in the detection of pathogens and the response by the innate immune system. Unfortunately, they are also actively involved in the pathogenesis and progression of viral myocarditis. Transgenic mouse models expressing cytokines have facilitated our understanding of interplay between cytokines at the sites of infection and the development of autoimmune disease.

During experimental induced autoimmune myocarditis, it has been thought that CD4<sup>+</sup> Th cells differentiate into IL-2- and IFN $\gamma$ -producing Th1 and IL-4-, IL-10- and IL-13-producing Th2 cell subsets and that the balance in T-helper cytokines can influence susceptibility and outcome of myocarditis (Horwitz et al. 2000). In recent research, it has been revealed that IL-1, IL-6, and IL-23 promote the differentiation of a distinct CD4<sup>+</sup> T cell population that produces IL-17 and develops independently of Th1 and Th2 lineages (Blyszczuk et al. 2008). This new population denoted Th17, plays an important role for various models of immune-mediated tissue injury, including organ-specific autoimmunity diseases like myocarditis (Horwitz et al. 2000).

Work from our laboratory has established a critical link between IL-6 and disease severity. Work done by Poffenberger and colleagues has shown a significant increase in disease severity with the absence of IL-6 after coxsackievirus B3 infection in mice. An increase in inflammatory mediators associated with the progression of myocarditis such as TNF- $\alpha$  and MCP1 was observed in concordance with the increase in disease severity (Poffenberger et al. 2009). Without IL-6 to regulate the early immune response after infection, the early inflammatory response leads to increased chronic myocarditis severity as the disease progresses (Poffenberger et al. 2009).

An important factor affecting the immune response to the virus and viral clearance is the pro-inflammatory cytokine interferon- $\gamma$  (IFN $\gamma$ ). Coxsackievirus B3 infection in mice deficient in IFN $\gamma$  results in increased disease severity and increased viral replication in the heart (Eriksson et al. 2001; Fairweather et al. 2005). Expression of IFN $\gamma$  in the pancreas can control viral replication as well as the virus-mediated damage in the heart and ensuing autoimmune disease (Horwitz et al. 2000). This cytokine also controls disease severity in an adjuvant induction disease model. In essence, IFN $\gamma$  likely limits myocarditis pathology by

decreasing viral replication and virus-mediated damage. Resolution of inflammation and progressive remodeling are associated with high levels of another cytokine transforming growth factor- $\beta$  (TGF- $\beta$ ) in the myocardium.

TGF- $\beta$  is a pleiotropic, immunomodulating cytokine that greatly contributes to myocardial repair and remodelling (Khan et al. 2006; Rubtsov et al. 2007). Cardiac fibroblasts are the predominant source of secreted TGF- $\beta$  within the heart. Secretion of TGF- $\beta$  drives differentiation of cardiac fibroblasts into their more active myofibroblast form (Lijnen et al. 2002). It is with this active connective tissue cell form and stimulation by TGF- $\beta$  that copious amounts of collagen can be secreted (Petrov et al. 2002). Fibrillar collagen is a leading contributor to extensive fibrosis. With disproportionate amounts of secreted collagen, ventricles tighten, restricting proper diastolic function (Kania et al. 2009). TGF- $\beta$  contributes to the secretion of collagen via the TGF- $\beta$ -Smad pathway that promotes collagen gene activation and translation (Khan et al. 2006). TGF- $\beta$  also enhances the production of adhesion molecules that in turn, promote the longevity of myofibroblasts (Vaughan et al. 2000). Macrophages also secrete TGF- $\beta$  in the heart (Riemann et al. 1994). They colocalize with myofibroblasts in fibrotic heart tissue and act as either initiating or supplemental sources of TGF- $\beta$ 1 (Hinglais et al. 1994; Kuwahara et al. 2004).

Coxsackievirus B3 first targets and replicates in the pancreas before reaching the heart. To inhibit coxsackievirus B3 spread to the heart and initiation of chronic disease, Horwitz et al developed a transgenic TGF- $\beta$  mouse model where TGF- $\beta$  is overexpressed in the pancreas. The expression of TGF- $\beta$  in the pancreatic beta cells recruited macrophages into the pancreas, reduced viral replication, and inhibited the onset of coxsackievirus B3-induced autoimmune myocarditis. This study also demonstrated that the protective effect was strictly attributed to TGF- $\beta$  and not IL-4, which has been linked to both autoimmunity suppression and antigen-presenting cell activation (Horwitz et al. 2006). Later on, Richer et al demonstrated that LPS from *Salmonella minnesota* and signalling through TLR-4 was capable of bypassing the protective effect provided by TGF- $\beta$  in coxsackievirus B3-mediated autoimmune myocarditis (Richer et al. 2006). The authors also showed that neither antibody isotype switching, the extent of viral replication, nor the expression of CD40 was modulated with LPS induced TLR-4 signalling, rather the circumventing effect was due to failed APC expression of CD40 and inherent TLR-4 signalling effects such as the production of pro-inflammatory cytokines (Richer et al. 2006).

Though over-expression of TGF- $\beta$  in the pancreas can protect from coxsackievirus B3-induced autoimmune myocarditis, there is still evidence that increased levels of TGF- $\beta$ 1 in the heart enhance, rather than protect from chronic disease. Elevated levels of TGF- $\beta$  have also been tied to dilated, ischemic and hypertrophic cardiomyopathies (Khan et al. 2006). As mentioned previously, TGF- $\beta$  can enhance collagen secretion and thus promote extensive tissue fibrosis. A recent study examined the effect of astragaloside IV, a Chinese medical herb that has anti-myocardial injury and immunoregulatory properties, to inhibit myocardial fibrosis in Balb/c mice inoculated with coxsackievirus B3 (Chen et al. 2011). Interestingly, astragaloside IV exhibited a protective effect alike TGF- $\beta$  against myocardial fibrosis and significantly ameliorated survival in coxsackievirus B3-infected mice that developed dilated cardiomyopathy. The authors also suggest that the protective role exerted by astragaloside IV is likely due to its ability to interfere with TGF- $\beta$ -Smad signalling through the direct downregulation of Smad2/3 and Smad 4 (Chen et al. 2011).

Lipopolysaccharide injection at the time of coxsackievirus B3 infection helps overcome genetic resistance in susceptible mice. This investigation also identified interleukin (IL)-1 as

the mediator responsible for causing the change in disease course. Injection of IL-1 alone overcomes the genetic resistance to induced myocarditis similarly to lipopolysaccharide treatment. The change in disease susceptibility is likely due to increased IL-1 production in the heart. Production of IL-1 begins during the acute stage of disease but persists into the chronic phase of disease. Interestingly, the levels of IL-1 in the heart correlate with the degree of fibrotic lesions during disease. Eriksson et al demonstrated that injection of an IL-1 receptor agonist prior to infection sufficiently decreases viral titres in the heart and reduced chances of mortality. They also showed that IL-1 receptor stimulation is required for efficient dendritic cell activation, the subsequent induction of autoreactive CD4<sup>+</sup> T cells, and resulting autoimmune disease (Eriksson et al. 2003).

Another important immunomodulating cytokine that contributes to viral-induced myocarditis and controls macrophage activation is IL-10. IL-10 is produced in the myocardium during both the acute and chronic stages of virus-induced myocarditis. Chronic coxsackievirus B3-induced myocarditis features viral RNA persistence and chronic inflammation that is primarily mediated by macrophages and T cells therefore, cytokines like IL-10 that control these critical immune cells are important to investigate. IL-10 gene-deficient mice have been used to confirm the regulatory role of IL-10 in the outcome of coxsackievirus B3 myocarditis. Mice deficient in IL-10 have uncontrolled nitric oxide synthase production, which likely contributes to their ongoing myocardial injury (Szalay et al. 2006). IL-10 in experimental induced autoimmune myocarditis mice hearts is mainly detected in non-cardiomyocytic non-inflammatory cells (ie. fibroblasts, smooth muscle cells, and endothelial cells) and IL-10-targeting cells. The IL-10-targeting cells, which express both IL-10 receptors 1 and 2, are mainly T cells expressing  $\alpha\beta$ T cell antigen receptors ( $\alpha\beta$ T cells) and CD11b<sup>+</sup> cells such as macrophages, dendritic cells, and granulocytes. Several studies have demonstrated a therapeutic effect for IL-10 in autoimmune and inflammatory diseases. In myocarditis models, IL-10 has been shown to inhibit the secretion of proinflammatory cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , iNOS, IL-2 and IL-12 and has displayed major effects on immune cells (Horwitz et al. 2000). These few studies suggest that the role of IL-10 in disease development could be predominantly protective.

IL-12, another key cytokine, is comprised of p40 and p35 subunits. IL-12 signals through a heterodimeric receptor composed of two units, IL-12R $\beta$ 1 and IL-12R $\beta$ 2. p40 and IL-12R $\beta$ 1 deficient mice show less or no myocardial disease with adjuvant-induction and treatment of wild type mice with IL-12, exacerbates disease suggesting IL-12 as a driving force in disease development similar to the actions of IL-1 $\beta$  and TNF $\alpha$  (Eriksson et al. 2001; Afanasyeva et al. 2004). However, Fairweather et al have found that IL-12 deficiency does not prevent myocarditis, rather viral replication significantly increases, causing more myocardial tissue damage. A decrease in inflammatory infiltrates was also observed and corresponded to with reduced TNF- $\alpha$  and IFN- $\gamma$  levels in the heart. IL-12 and IFN $\gamma$  positively regulate each other and type I inflammatory responses. Type I inflammatory responses are believed to be responsible for tissue damage in autoimmune diseases. Eriksson et al further investigated the role of the IL-12/IFN- $\gamma$  (Th1) axis in the development of autoimmune myocarditis. They observed resistance to disease in IL-12p40-deficient mice that were bred on a susceptible background. In the absence of IL-12, they suggested that autospecific CD4<sup>+</sup> T cells proliferated poorly and exerted Th2 cytokine responses. IFN- $\gamma$ -deficient mice developed fatal autoimmune disease. Interestingly, blocking IL-4R signalling did not confer susceptibility to myocarditis in IL-12p40-deficient mice. This suggests that IL-12 triggers autoimmunity in a manner independent of the cytokines IFN- $\gamma$  and IL-4 (Eriksson et al. 2001).

IL-13 is another cytokine that can protect mice from both viral and adjuvant induced myocarditis. Cihakova et al demonstrated that IL-13 knock out BALB/c mice develop severe autoimmune myocarditis and their pathology is characterized by increased cardiac inflammation, increased total intracardiac CD45<sup>+</sup> leukocytes, elevated anti-cardiac myosin autoantibodies, and increased cardiac fibrosis, with impaired cardiac function and heart failure. Hearts of IL-13 knock out mice showed elevated levels of the proinflammatory and profibrotic cytokines including IL-1 $\beta$ , IL-18, IFN- $\gamma$ , TGF- $\beta$ , and IL-4. CD4<sup>+</sup> T cells were also highly increased in IL-13 knock out hearts. Splenic T cells from the knock out mice were greatly activated and with myosin stimulation, immensely proliferated. Regulatory T-cells harvested from spleens were also affected in knock out mice, where they showed a decrease in numbers compared to wild type mice. IL-13 knock out also reduced alternatively activated CD206(+) and CD204(+) macrophages and heightened levels of classically activated macrophages. Caspase-1 activation was increased, which then likely increased production of both IL-1 $\beta$  and IL-18. This study exemplified IL-13 as another important immunomodulating cytokine that protects against myocarditis by manipulating T cell and macrophage populations (Cihakova et al. 2008).

Examining T cells subsets is another avenue investigated to determine pathogenic or protective factors in myocarditis development. Mice deficient in T-bet, a T-box transcription factor required for Th1 cell differentiation and IFN- $\gamma$  production, develop severe autoimmune heart disease. T-bet can also regulate autoimmunity by controlling nonspecific CD8<sup>+</sup> T cell bystander functions in the inflamed target organ such as the heart. CD4<sup>+</sup> Th1 cells producing INF $\gamma$  are protective in experimental induced autoimmune myocarditis. This protection is likely attributed to regulation of IL-17 production by Th17 cells. Th17 cells produce IL-17, a proinflammatory cytokine that activates T cells and other immune cells to produce a variety of cytokines, chemokines and cell adhesion molecules. Rangachari et al have shown that Th17 cells are involved in acute viral myocarditis and enhance humoral responses (Rangachari et al. 2006). The relationship between Th17 cells and coxsackievirus B3 replication still remained unclear so they infected BALB/c mice with the virus and observed increased viral replication, expression of splenic Th17 cells, serum IL-17, and cardiac IL-17 mRNA that were all accompanied by progressive cardiac injury. Interestingly, Th1 and CD8<sup>+</sup> T cell expression was elevated and the neutralization of IL-17 further upregulated splenic Th1 and CD8<sup>+</sup> T cell numbers and levels of cardiac IFN- $\gamma$  mRNA. Cardiac pathology was improved after IL-17 neutralization and correlated with reduced viral replication and decreases in cardiac inflammatory cytokines IL-17, TNF- $\alpha$ , and IL-1 $\beta$ . This study implicates Th17 cells in contributing coxsackievirus B3 replication in viral myocarditis, and implicates IL-17 as a target for regulating antiviral immune responses (Yuan et al. 2010). Th17 cells are activated by IL-23, a cytokine likely produced by activated macrophages and dendritic cells, through receptor interactions made of IL-12R $\beta$ 1 and IL-23 receptor (Langrish et al. 2005). IL-23 is a heterodimeric cytokine like IL-12 that is composed of a p19 and p40 subunit similar to IL-12. IL-23-mediated immune responses have a different gene expression pattern than IL-12-driven T cell responses and IL-23 does not promote the development of IFN- $\gamma$ -producing Th1 cells as does IL-12. IL-23 is however, one of the many contributors to pathogenic CD4<sup>+</sup> T cell expansion. With its anti-IFN- $\gamma$  and Th1 cell activity, IL-23 helps establish and maintain organ-specific inflammatory autoimmune diseases such as myocarditis (Langrish et al. 2005). Understanding the molecular basis for the differential gene expression pattern observed with IL-23-dependent T cell populations and investigating IL-23's cellular mechanism of action in autoimmunity could provide additional therapeutic targets for the treatment of inflammatory autoimmune diseases.

Experimental and preliminary human studies have demonstrated that TNF- $\alpha$  plays a crucial role in viral-induced myocarditis. Calabrese et al investigated the expression of TNF- $\alpha$  and both its receptors (TNFR1 and TNFR2) in both viral and non viral myocarditis. Expression of TNF- $\alpha$  was significantly enhanced in viral myocarditis compared to non viral myocarditis. Importantly, cardiac myocytes express TNF $\alpha$  receptors TNFR1 (TNFRp55) and TNFR2 (TNFRp75), implicating the possible importance of enhance TNF- $\alpha$  in the myocardium. Histological analysis revealed that myocardial necrosis and cellular infiltration are more prominent in TNF- $\alpha$ -positive cases further supporting the notion that the expression of TNF- $\alpha$  significantly contributes to the pathogenesis of viral myocarditis and including the severity of cardiac dysfunction (Calabrese et al. 2004).

Chemokines act as chemotactic mediators in leukocyte trafficking to sites of infection (Groom et al. 2011). In addition to their chemo-attractant activity, chemokines can influence disease severity by modifying immune response strength and polarity. This immune modulating capability thus makes them attractive targets for viral myocarditis therapeutics. Chemokines apart of the CC chemokine family such as CCL2, CCL4, and CCL19 have been shown to mediate mononuclear cell migration to the heart in coxsackievirus B3-induced myocarditis (Chen et al. 2009). In fact, a recent gene therapy approach using a CCL2 mutant that lacked chemo-attractant activity in a Balb/c coxsackievirus B3-infection model impaired appropriate Th1 immune responses and significantly controlled myocardial disease (Yue et al. 2011). Blocking CCL2 with expression of the mutant did not assist in viral clearance. The potential therapeutic effect of blocking CCL2 lies in the CCL2 mutant's ability to weaken the pro-inflammatory Th1 immune response (Yue et al. 2011).

The CXC family of chemokines also contribute to myocarditis pathogenesis. CXC chemokines act on monoclear CXCR3 expressing cells and include such members as IFN-inducible protein 10 (IP10/CXCL10), monokine induced by IFN- $\gamma$  (Mig/CXCL9), and IFN-inducible T-cell a chemoattractant (I-TAC/CXCL11) (Groom et al. 2011). CXCL10 and its interaction with its receptor CXCR3 have been implicated in many virus disease models. CXCL10 is a key contributor in the innate immune response to viral infection. By interacting with its receptor CXCR3, CXCL10 manipulates natural killer cell trafficking and their production of IFN- $\gamma$ . Yuan et al recently found CXCL10 levels in the heart to be inversely related to viral titers after coxsackievirus B3 infection and a massive infiltration of CXCR3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells (Yuan et al. 2009). The production of associated inflammatory cytokines followed with the infiltration of leukocytes though the anti-viral response was not effective in clearing the virus or ensuring survival in coxsackievirus B3-infected CXCL10 transgenic mice (Yuan et al. 2009). CXCL10 did assist viral clearance and protect myocytes from damage in the early stages of infection by robustly attracting NK cells and enhancing IFN- $\gamma$  production to infection sites (Yuan et al. 2009). Though CXCL10 appears to improve cardiac pathology and reduce viral persistence during initial infection stages, the caveat with using this chemokine as a potential coxsackievirus B3-induced myocarditis therapy lies with its inherent actions as a Th1-type chemokine. Th1 immune responses escalate detrimental cardiac fibrosis during the reclamation stage of disease so enhancing important contributors to this repair process may acceleration rather than prevent the development of chronic disease.

Determining the effect of CXCL10 on viral replication and recruitment of innate immune cells in other organs than the heart such as the liver and pancreas must still be pursued. As seen with another immunomodulatory factor, TGF- $\beta$ , the immune responses in other organs than the heart can affect susceptibility to severe myocardial injury and the development of chronic disease.

Recent research has demonstrated that coxsackievirus B3-infected Balb/c mice have increased levels of cardiac CXCL10 and this level fluctuates in a time and dose-dependent manner (Yue et al. 2011). The same research group treated coxsackievirus B3-infected mice with a CXCL10 mutant that lacks the critical chemo-attractant part. This mutant effectively blocked endogenous CXCL10 activity and protected coxsackievirus B3-infected mice from developing myocarditis (Yue et al. 2011). The CXCL10 mutant expressing mice had greater survival, less changes in body weight, less inflammation and necrosis in the heart. Cardiac Th1 cytokines IFN- $\gamma$ , IL-12, TNF- $\alpha$ , were also found to be significantly reduced with CXCL10/CXCR3 signalling inhibition. This suggests that dampening the Th1 response to coxsackievirus B3 infection through blocking CXCL10 activity may present as an effective strategy to suppress immune inflammation and myocardial damage in coxsackievirus B3-mediated myocarditis (Yue et al. 2011).

The production of immunomodulating mediators such as cytokines and chemokines affects not only antigen presenting cell and T cell populations acting at the site of injury, but greatly influences the outcome of disease. The expression of particular cytokines and chemokines after a pathogenic insult, such as coxsackievirus B3 infection, influences the balance of effector versus regulatory responses that ensue. Once the balance between effector versus regulatory responses tips in a particular direction, chronic autoimmune-type disease or the prevention of disease will materialize (Rouse et al. 2010; Wing et al. 2010) (Figure 3). It is therefore critical to bear in mind the possibility of tipping the immunity balance when manipulating key immune players in coxsackievirus-induced myocarditis.

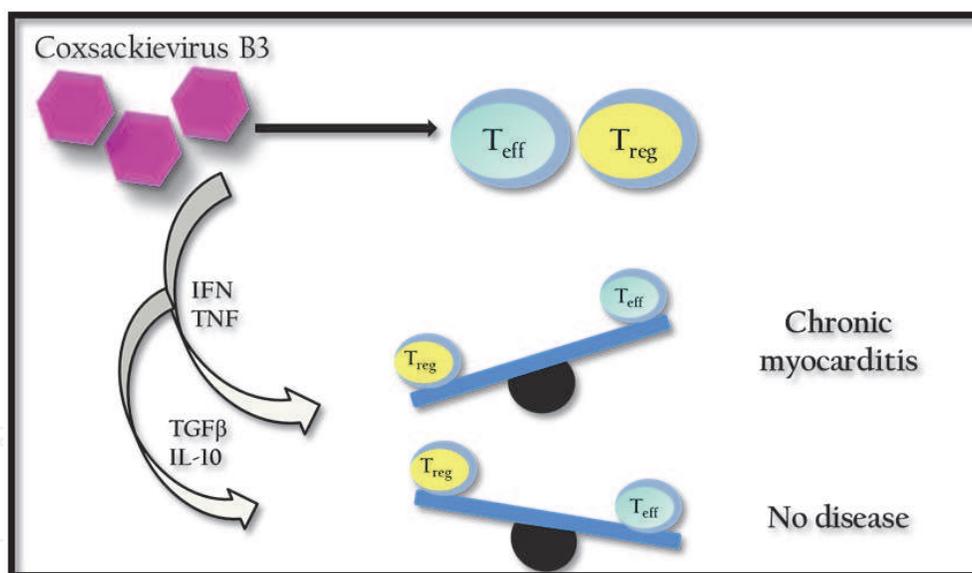


Fig. 3. Immunomodulation of the immune balance and the effects on disease outcome. With coxsackievirus B3 infection, populations of regulatory and effector cells are produced. It is with particular immunomodulating mediators such as cytokines interferon (IFN) and tumor necrosis factor (TNF) that enhanced production and proliferation of effector T cells ( $T_{eff}$ ) is promoted and leads to the persistent destruction of the myocardium and chronic myocarditis disease. Pro-regulatory cytokines transforming growth factor- $\beta$  (TGF- $\beta$ ) and interleukin-10 (IL-10) are examples of immune modulators that promote the enhanced production of regulatory T cells (regulatory T cells) and the prevention of autoimmune myocarditis disease.

## 5. Therapeutic challenges and future directions

Treatment for myocarditis mainly involves targeting the symptoms of heart dysfunction. If the disease progresses to dilated cardiomyopathy, heart transplantation is necessitated. Regrettably, for 50% of patients progressing to dilated cardiomyopathy, survival is limited to 5 years. Broad-spectrum antivirals tested in clinical trials with children and adults have been few and mildly successful. Specific therapies designed against viruses (enteroviruses) for prevention and/or management of viral myocarditis are still desperately needed. One challenge in designing specific antivirals or vaccines for viral myocarditis is the range of possible etiologies and immune mechanisms that may be responsible. Although enteroviral infection inducing or exacerbating an autoimmune response is a likely cause for viral myocarditis, supporting data defining a link between viral infection and the onset of acute and/or chronic myocarditis in humans must still be determined. The existence of many viral serotypes for promising etiological agents like coxsackievirus B also poses a problem in designing specific antiviral therapies.

Dampening the Th1 immune response in viral myocarditis is a therapeutic avenue consistently featured in past and recent studies. Many key innate immune players, such as cytokines and chemokines, are targets of potential Th1 skewing therapeutics. Cytokines presenting at the site of viral infection have a significant influence on whether a tolerant or autoimmune response is chosen. Targeting cytokines, chemokines and other immunomodulating factors for myocarditis therapy may depend on the timing and duration of the particular factor and the maintenance of immune balance to prevent the development of autoimmunity.

## 6. Conclusion

For quite some time scientists have investigated virus and host cellular and molecular events that underlie the pathogenesis of enteroviral-induced myocarditis. Our lab has demonstrated the significance of many key innate players that contribute to coxsackievirus-B3 induced myocarditis in mouse models. Horwitz et al first revealed the importance of IFN- $\gamma$  expression in the pancreas for the protection from coxsackievirus B3 infection and the induction of myocarditis (Horwitz et al. 2000). Later on, Horwitz et al again demonstrated a protective role against coxsackievirus B3-induced myocarditis with cytokine expression in the pancreas, this time, with transgenic expression of TGF- $\beta$  in the beta cells (Horwitz et al. 2006). This work was followed by Richer et al, who provided a role for TLR4 and stimulation by *Salmonella minnesota* lipopolysaccharide in bypassing the protective effect exerted by TGF- $\beta$  in coxsackievirus B3-induced myocarditis (Richer et al. 2006). Our lab has also investigated the role of IL-6 in disease severity. Poffenberger et al demonstrated that in the absence of IL-6, a greater early immune response occurred, instigating a severe chronic disease pathology (Poffenberger et al. 2009). Recently, Poffenberger et al described susceptibility loci on chromosome 17 that implicate the highly suggested genetic component as a factor dictating viral myocarditis pathogenesis (Poffenberger et al. 2010). Though work from our lab has contributed a great deal to the understanding of coxsackievirus B3-induced myocarditis, there remains many unanswered questions with regards to the true key immune players that dictate disease pathogenesis. It is likely that an interplay of genetic and environmental factors influence the susceptibility and severity of virus-induced myocarditis however, we must not discount the significance of the innate immune response in shaping the outcome of virus-induced disease. It is possible that this early immune response may

ultimately dictate whether an acute or chronic immune response ensues with enteroviral infection.

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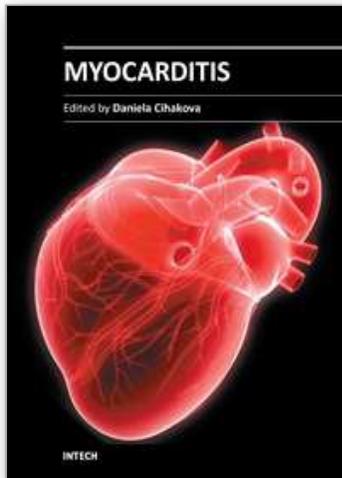
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## **Myocarditis**

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Myocarditis, the inflammation of the heart muscle, could be in some cases serious and potentially fatal disease. This book is a comprehensive compilation of studies from leading international experts on various aspects of myocarditis. The first section of the book provides a clinical perspective on the disease. It contains comprehensive reviews of the causes of myocarditis, its classification, diagnosis, and treatment. It also includes reviews of Perimyocarditis; Chagasâ€™ chronic myocarditis, and myocarditis in HIV-positive patients. The second section of the book focuses on the pathogenesis of myocarditis, discussing pathways and mechanisms activated during viral infection and host immune response during myocarditis. The third, and final, section discusses new findings in the pathogenesis that may lead to new directions for clinical diagnosis, including use of new biomarkers, and new treatments of myocarditis.

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