The role of antigen presenting cells in coxsackieviral-induced autoimmune diseases

by

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Abstract

Susceptibility to autoimmune diseases is dictated by the interplay of genetic determinants and environmental factors including diet, toxins and infections. Viral infections have long been suspected to play a role in the etiology of several autoimmune disorders. In particular, coxsackieviruses are common human pathogens that have been linked to autoimmune myocarditis and type 1 diabetes (T1D). Evidence suggests that interactions between a pathogen and components of the innate immune system may influence the generation of a dysregulated adaptive response ultimately resulting in autoimmune disease development. Early recognition of viral infection is mediated by pattern recognition receptors (PRRs) expressed by a variety of cells including antigen presenting cells (APCs). PRR-mediated recognition of an invading pathogen results in wide ranging functional consequences that serve to trigger innate antiviral mechanisms as well as the maturation of APCs and the activation of adaptive immune responses. As such, innate interactions between viruses and APCs likely represent a potential risk factor for the development of autoimmunity following infection.

Here, I demonstrate that early protection from coxsackievirus infection is critically dependent on Toll-like receptor (TLR) 3 signaling on CD11b+CD11c- APCs. Interestingly, my work demonstrates that this same subset of APCs is central to the acceleration of T1D and that manipulation of the maturation and inflammatory status of CD11b+CD11c- APCs is sufficient to protect from coxsackievirus-induced autoimmune myocarditis and T1D. Protection from T1D is dependent on the reduction of costimulatory molecule expression, particularly CD40, on the surface of CD11b+CD11c- APCs which in turn increases the capacity of these APCs to induce protective regulatory T cells (Tregs) in the pancreas. Protection from autoimmune myocarditis is not dependent on Tregs and can be circumvented by activation of the TLR4 signaling pathway.

Taken together, this work illustrates an important role for a particular subset of APCs that is critical for both early protection of the host as well as the induction of autoimmunity following infection with coxsackieviruses. This strongly suggests that CD11b+CD11c- APCs represent a potential therapeutic target for the prevention of viral-induced autoimmunity.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ALT</td>
<td>alanine transaminase</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BBDR</td>
<td>Bio-breeding diabetes resistant rat</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackie and adenovirus receptor</td>
</tr>
<tr>
<td>CB3</td>
<td>coxsackievirus B3</td>
</tr>
<tr>
<td>CB4</td>
<td>coxsackievirus B4</td>
</tr>
<tr>
<td>CBV</td>
<td>coxsackievirus group B</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>CTn1</td>
<td>cardiac troponin 1</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCM</td>
<td>dilated cardiomyopathy</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EAM</td>
<td>experimental autoimmune myocarditis</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMCV</td>
<td>encephalomyocarditis virus</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box p3</td>
</tr>
<tr>
<td>GA</td>
<td>glatiramer acetate</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>Idd</td>
<td>insulin-dependent diabetes</td>
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IFN interferon
Ig immunoglobulin
IL interleukin
IMDM Iscove’s modified Dulbecco’s medium
iNOS inducible nitric oxide synthase
IP-10 interferon-inducible protein 10
IPEX immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IRF4 interferon regulatory factor 4
KO knock out
KRV Kilham rat virus
LCMV lymphocytic choriomeningitis virus
LPS lipopolysaccharide
M1 type I macrophages
M2 type II macrophages
MAVS mitochondrial antiviral signaling adaptor
MCMV mouse cytomegalovirus
MCP-1 monocyte chemoattractant protein 1
MDA-5 melanoma differentiation-associated gene-5
MHC major histocompatibility complex
MIG monokine induced by IFN-gamma
MIP-1α macrophage inflammatory protein 1
MMR macrophage mannose receptor
MS multiple sclerosis
MSR-A macrophage scavenger receptor
MyD88 myeloid differentiation primary response gene 88
MZB marginal zone B cell
NK natural killer cell
NKT natural killer T cell
NO nitric oxide
NOD non-obese diabetic
NOR non-obese resistant
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>nsSNP</td>
<td>non-synonymous single nucleotide polymorphisms</td>
</tr>
<tr>
<td>OAS</td>
<td>oligoadenylate cyclase synthase 1b</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PD-L1</td>
<td>programmed death ligand 1</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PI</td>
<td>post-infection</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLN</td>
<td>pancreatic lymph node</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PTPn22</td>
<td>protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic-acid-inducible-gene I</td>
</tr>
<tr>
<td>RLH</td>
<td>RIG-like helicase</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptors</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
</tr>
<tr>
<td>sem</td>
<td>standard error on the mean</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>Tbet</td>
<td>T-box expressed in T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler’s murine encephalitis virus</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
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Acknowledgements

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Dedication

To my wonderful wife Stephanie for her constant love and support
Co-authorship statement

Research design, data analysis and manuscript preparation for each experimental chapter were completed by the author under the guidance of Dr. Marc Horwitz. The author performed all experiments presented within this thesis except for the following exceptions where assistance was provided as described:

Chapter 2: D.J. Lavallée performed the plaque assay procedure for Figure 2.1B

Chapter 3: D. Fang performed some of the ELISA for Figure 3.2 and I. Shanina performed the plaque assay procedure for Figure 3.3

Chapter 4: N. Straka and I. Shanina performed the plaque assay procedure for Figure 4.13
Chapter 1

Introduction¹

¹ Sections of this chapter have been published:


1.1 Viruses and autoimmunity

Autoimmunity results from an inappropriate immune response directed at self-antigens. This can cause tissue destruction leading to systemic or organ-specific disorders. Although the adaptive immune system, in the form of autoreactive T cells or antibodies, is ultimately responsible for tissue destruction, mounting evidence points to an important role for dysregulated innate immune responses in the etiology of autoimmune diseases, particularly following a pathogenic insult. Susceptibility to autoimmune diseases is partially dictated by genetic determinants but several studies have demonstrated that predisposition to some of these diseases can be modified by a variety of environmental factors including diet, toxins and microbes (commensals and pathogens). Among these factors, virus infections have been linked to the induction or exacerbation of both systemic and organ-specific autoimmune diseases including systemic lupus erythematosus (1), myasthenia gravis (2), multiple sclerosis (MS) (3), autoimmune myocarditis (4) and type 1 diabetes (T1D) (5). In recent years, accumulating data has indicated that, more specifically, the interaction of viruses with components of the innate immune system may be responsible for the break in peripheral tolerance that ultimately leads to autoimmunity following infection (6-8).

1.1.1 Coxsackievirus-induced autoimmunity

Coxsackieviruses are small, single-stranded RNA viruses classified within the enterovirus genus of the *Picornaviridae* family (9). Coxsackievirus infections are typically transmitted in an oral-fecal manner and virus can disseminate to several organs including the pancreas and the heart (9, 10). In mouse models, where the virus has been adapted to replicate, viral levels typically peak 2 to 3 days post-infection (PI) in the pancreas and 5 days PI in the heart. By 15 days PI, replicating viruses are not usually detectable, suggesting complete viral clearance (11). Of particular interest, the group B coxsackievirus (CBV, serotypes 1 through 6) are common human pathogens that are not only associated with mild acute infections but also with more severe acute conditions such as meningitis, encephalitis and pericarditis as well as chronic conditions such as chronic myositis, chronic autoimmune myocarditis, dilated cardiomyopathy (DCM) and T1D (9). These viruses have been the
subject of intense investigation in order to elucidate the link between viral infection and autoimmunity in both humans and mice.

1.1.2 Autoimmune myocarditis susceptibility

Myocarditis is defined as inflammation of the heart muscle and has been well documented to be a leading cause of death among young adults (12). Several viral pathogens, including coxsackievirus, adenovirus and parvovirus have been associated with the induction of myocarditis (13). Myocarditis is considered to be a clinical precursor to DCM, a serious medical condition that usually requires heart transplantation (14). A large proportion of DCM cases reported in the United States have been associated with coxsackievirus infection (15).

In both patients and mice, susceptibility to the development of autoimmune myocarditis is dictated by the interplay between environmental factors and genetic determinants. Studies of patients suffering from DCM have demonstrated an association of certain major histocompatibility complex (MHC) class II alleles and disease development (16-18). Similarly, mouse model studies have suggested a strong link between MHC class II alleles and susceptibility to chronic autoimmune myocarditis (19). Further evidence suggests that non-MHC genes also play an important role in the overall susceptibility to the development of chronic autoimmune myocarditis. Studies in experimental autoimmune myocarditis models (EAM, a model of chronic autoimmune myocarditis in which disease is induced by injection of cardiac myosin emulsified in complete Freund’s adjuvant that allows for the study of the chronic phase of disease while removing the complications associated with the acute phase of viral infection) have identified susceptibility-conferring loci on chromosomes 1 and 6 termed Eam1 and Eam2 (20). Interestingly, these loci coincide with well known susceptibility regions for T1D and autoimmune thyroid disease suggesting a degree of overlap in the susceptibility to several autoimmune diseases (20). Further characterization of the Eam1 region has demonstrated that genes within this region lead to an increased resistance of lymphocytes, particularly CD4 T cells, to antigen-induced apoptosis (21). This suggests that susceptible hosts may have a decreased capacity to curtail adaptive immune responses following pathogen challenges leading to prolonged immune activation, inflammation and, ultimately, the development of autoimmunity. Loci conferring
susceptibility to acute myocarditis following viral infection have also been identified and these may ultimately be involved in dictating susceptibility to chronic autoimmune myocarditis. Of particular interest, one of the loci identified included a gene coding for a critical antiviral effector molecule, oligoadenylate cyclase synthase 1b (OAS) suggesting that early innate control of viral infection may play an important role in reducing the susceptibility to autoimmune disease development (22). Taken together, this suggests that susceptibility to both acute and chronic myocarditis is determined by a complex interplay of a number of genetic determinants with environmental factors.

1.1.3 Coxsackievirus-induced autoimmune myocarditis

Mouse models of myocarditis have been developed to study the pathogenesis of both acute viral-mediated myocarditis and chronic autoimmune myocarditis. Induction of myocarditis following coxsackievirus B3 (CB3) infection has been extensively characterized since heart disease progression following infection with this virus in mice closely resembles disease progression in humans (23). Heart disease induced following CB3 infection can be separated into two phases with an acute viral-mediated phase in the majority of infected hosts followed by a chronic autoimmune phase in genetically susceptible hosts. In mice, acute myocarditis is usually observed 7 to 14 days PI due to direct infection and destruction of cardiac tissue by viral particles (24). Inflammation typically resolves with clearance of the virus by day 15 and normal cardiac function is resumed with little long-term damage occurring. However, in genetically susceptible hosts such as A/J, Balb/c and non-obese diabetic (NOD) mice, viral clearance is followed by a chronic phase of disease characterized by immune cell infiltration leading to tissue destruction, scarring and deposition of fibrosis (4). This eventually results in the loss of contractile function, enlargement of the heart and development of DCM (23). Adoptive transfer experiments have clearly demonstrated that heart damage observed during the chronic phase of disease is immune-mediated and not a direct consequence of viral infection (25, 26). Despite the absence of replicative virus at this stage of disease, some groups have reported the presence of viral RNA (27, 28) or non-cytolytic viral variants (29) long after viral clearance and have suggested that viral persistence may contribute to disease pathogenesis. However, in another model of viral-
induced myocarditis, viral genomes have also been recovered at this later stage in mice that are resistant to the development of autoimmune myocarditis suggesting that viral persistence is not a major contributor to disease progression (23). Instead, disease susceptibility seems to be directed by several genetic factors leading to a dysregulated immune response following coxsackievirus infection.

1.1.4 Type 1 diabetes susceptibility

T1D results from the destruction of the insulin producing beta cells in the islets of Langerhans of the pancreas by autoreactive T cells. Disease onset occurs after a large majority of beta cells have been destroyed and the autoimmune response is initiated years before appearance of clinical symptoms (30). Progression of the autoimmune response can be, in part, followed by measuring levels of beta cell antigen specific autoantibodies which appear long before the onset of clinical symptoms (31). If left untreated, T1D can lead to several life threatening symptoms and the only treatments currently available involve the direct injection of insulin or islet transplantation (32). Several factors combine to determine overall susceptibility to T1D, with genetic determinants playing a prominent role. The human leukocyte antigen (HLA) region is the primary genetic determinant of susceptibility (33) and several other genetic loci, including the insulin promoter (34), protein tyrosine phosphatase, non-receptor type 22 (PTPn22) (35) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (36-38), also combine to increase susceptibility. Interestingly, recent reports have demonstrated a link between polymorphisms in an important innate detector of viruses, melanoma differentiation-associated gene-5 (MDA-5) and susceptibility to T1D, providing compelling evidence that the interplay of genetic and environmental factors is responsible for determining T1D susceptibility (39-41). Similarly, several genetic regions contribute to confer susceptibility to diabetes development in the NOD mouse, a commonly studied animal model of T1D, where spontaneous disease development is controlled by at least 20 genetic loci including the MHC region (42).

In humans, the incidence of T1D is increasing worldwide at a rapid rate (43-46) that cannot be explained by changes in the genetic makeup of the population alone, particularly as the incidence of disease has been on the rise in populations with HLA alleles generally
associated with reduced susceptibility (47). T1D often presents with geographic distribution patterns that do not correlate with genetic differences. For example, there is a large discrepancy in the incidence of T1D between Finland and neighboring regions of Russia despite similar prevalence of HLA alleles associated with susceptibility to disease (48). Furthermore, although certain populations are associated with lower incidences of T1D, immigration to areas associated with higher risk correlates with an increase of T1D within these normally protected populations. In particular, although Asian populations have one of the lowest T1D incidences in the world, prevalence of T1D in Asian populations living the United Kingdom is almost identical to the higher prevalence that occurs within the Caucasian population (49). Most convincingly, the concordance rate of T1D incidence in monozygotic twins is approximately only 40%, clearly indicating that genetic factors are insufficient to determine predisposition to T1D and that environmental factors influence the likelihood of developing disease (50). These factors are likely to include changes in diet, exposure to beta cell toxins or pathogens such as viruses (51).

Viruses were first recognized as potential T1D inducing agents when it was observed that disease onset sometimes follows acute viral infections. This was further supported with epidemiological data indicating that T1D onset follows seasonality in both hemispheres in a manner that is strongly correlated with the seasonality of a number of viral infections (52, 53). Several instances of local epidemics of T1D resembling sudden infectious epidemics have also been reported (54-57). Furthermore, seasonal onset is often observed in less genetically predisposed populations, suggesting the contribution of risk factors other than genetic determinants in those cases (58). This may also explain why broad human studies, which are usually carried out on the most genetically susceptible populations, have commonly failed to clearly identify any candidate viruses. Most convincingly, enteroviruses have been isolated directly from the pancreas of patients that succumbed following acute onset of T1D. Upon adaptation of these strains to allow them to replicate in mice, infection with these isolated viral strains induced hyperglycemia and a diabetes-like disease in infected animals, clearly demonstrating that viruses can lead to T1D (59). One of the strains isolated from an acute onset T1D patient was identified as coxsackievirus B4 (CB4) (59) and has become a commonly used tool to study the induction of T1D by viruses. The diabetogenic properties of CB4 have since been clearly demonstrated in genetically susceptible mouse
models (60-62). Accumulating evidence points to viral infections representing at least one of the environmental stimuli responsible for the rapid increase of T1D observed worldwide in the last few decades.

1.1.5 Coxsackievirus-induced type 1 diabetes

Since the first report of a link between coxsackieviral infections and T1D in the late 1960s (63), several reports, including some from large-scale epidemiological studies, have demonstrated that recent-onset diabetic patients harbor increased levels of coxsackievirus-specific antibodies or coxsackieviral RNA compared to control populations (64-73). Interestingly, this correlation was also observed in a Cuban study where the incidence of T1D is relatively low and coxsackievirus infections are very prevalent (74). Most convincingly, CB4 has been directly isolated from the pancreas of deceased recent-onset T1D patients (59, 75). Following isolation, this virus was passaged in murine pancreatic islets and this mouse-adapted strain of CB4 was shown to induce a diabetes-like disease in susceptible mice (59). Subsequent studies have clearly demonstrated the diabetogenic properties of this viral strain in two mouse models on the NOD genetic background (60, 61). Therefore, the currently available data strongly suggests that coxsackievirus infections represent an important factor in the etiology of T1D.

Although the mechanisms through which coxsackievirus infection leads to the induction of T1D remains poorly characterized in humans, animal model studies have contributed significantly to our understanding. Studies with the BDC2.5 T-cell receptor (TCR) transgenic model (a NOD derived transgenic model that does not spontaneously develop T1D) have demonstrated that induction of disease following CB4 infections is not due to viral-mediated destruction of pancreatic islets or to the induction of virus-specific effector T cells that cross-react with pancreatic antigen (molecular mimicry) (60). Instead, while viral infection of the beta cells causes minimal cell death, it induces cellular stress leading to the engulfment of the beta cells by resident antigen presenting cells (APCs) (76). This, in turn, leads to the presentation of previously sequestered antigens to a pre-existing population of beta cell antigen-specific autoreactive T cells in the inflammatory context of a viral infection. Activation of this autoreactive T cell population leads to the autoimmune
destruction of pancreatic beta cells and subsequently, the development of T1D. The induction of T1D following infection in these transgenic mice and in the wild-type (WT) NOD model appears to be critically dependent on the presence of a sufficient number of autoreactive T cells (Figure 1.1) (61, 77). A similar mechanism likely occurs in humans as infection of human pancreatic islet cells with enteroviruses has been demonstrated to affect islet function without significant induction of cell death (78). As such, enteroviral infection of human islets could lead to increased cellular stress, engulfment of beta cells and presentation of previously sequestered antigens in an inflammatory context. Taken together, this suggests that coxsackievirus infection is able to precipitate an already ongoing autoimmune reaction in susceptible hosts and as such may represent the last step in disease progression.

1.2 Innate recognition of viruses

Recognition of viral pathogens depends on a series of germ-line encoded pattern-recognition receptors (PRRs) expressed on cells of the innate immune system such as macrophages and dendritic cells (DCs). Several pathogen associated molecular patterns (PAMPs) associated with viruses can be recognized by PRRs. Among these, viral nucleic acids are sensed by two well-characterized families of PRRs, the Toll-like receptors (TLRs) and the retinoic-acid-inducible-gene I (RIG-I)-like receptors (RLRs) (79). A subset of TLRs located within cellular endosomes that includes TLR3 (double stranded RNA), TLR7 (single stranded RNA), TLR8 (single stranded RNA, in humans) and TLR9 (unmethylated CpG motifs of DNA) are activated following recognition of common intermediates of viral replication (79). Similarly, the RLRs (RIG-I, 5’ triphosphatase moiety on single stranded RNA and MDA-5, double stranded RNA) recognize intermediates of viral replication directly within the cytoplasm of cells (79). Additionally, several studies have demonstrated that viral proteins can also serve as PAMPs and these are typically recognized by TLRs expressed on the cell surface, particularly TLR2 and TLR4 (79). The importance of PRRs in the immune response to viruses is now well documented and it is becoming increasingly clear that they are not simply redundant receptors. Instead, they likely represent an evolutionarily conserved mechanism to recognize the molecular signatures associated to
specific viruses as an essential part of a large arsenal of immune defense mechanisms that are tailored for specific invading pathogens.

Interaction of a PRR with its specific ligand activates signaling cascades (Figure 1.2) with important functional consequences for the developing immune response. Among these is the production of several pro-inflammatory cytokines including type I interferons (IFNs). Type I IFNs are pleiotropic cytokines that play a crucial role in the early immune response to viruses. Signaling by type I IFNs leads to the establishment of an antiviral state by stimulating the transcription of several IFN-inducible genes that allow for partial control of viral spread through the inhibition of cellular processes that are important for viral replication (80). Production of type I IFNs can also contribute to the activation of natural killer (NK) cells and the maturation of APCs (81). The production of several other cytokines and chemokines following PRR signaling also serves to enhance innate immune function and the generation of a viral-specific adaptive immune response (82). Furthermore, TLR signaling directly contributes to the development of the adaptive immune response by inducing the maturation of APCs that subsequently migrate to the lymph nodes to activate antigen-specific T and B cells that are ultimately responsible for clearance of the virus (82). Recent evidence using myeloid differentiation primary response gene 88 (MyD88, an important signaling adaptor required for signaling from every TLR with the exception of TLR3) or TLR2-deficient mice has indicated that TLR signaling may also directly influence effector T cell and regulatory T cell (Treg) function (83, 84). Therefore, PRR recognition of viral pathogens is not only crucial for early protection of the host by the innate immune response but also exerts a critical influence on the developing adaptive immune response.

Although PRR signaling is clearly important for host defense against viral pathogens, several lines of evidence have also demonstrated that, under certain circumstances, this may be detrimental to the host. In particular, TLR3 signaling increases the pathogenesis of West Nile virus infection in mice by enhancing the production of cytokines that cause permeabilization of the blood-brain barrier leading to the establishment of potentially fatal encephalitis (84). Similarly, TLR3 deficiency in mice reduces the pathological effects of both influenza virus and Punta Toro virus infections (85, 86). Further, TLR3 signaling has been linked to the induction of autoimmunity following viral infection (87). Taken together, these
reports demonstrate that inappropriate activation of the innate immune system can have detrimental consequences for the host.

1.2.1 Innate immune responses to coxsackievirus

The innate immune system appears to be critical for the host response to coxsackieviruses. This is best illustrated by experiments demonstrating that severe combined immunodeficient (SCID) mice lacking both T and B cells are able to survive for several weeks following CB4 infection (88). *In vitro* experiments have demonstrated that coxsackieviral proteins can be recognized by TLR4 (89) while nucleic acids from these viruses can stimulate cytokine production by signaling through both TLR7 and TLR8 (90). The relative contribution of these signaling events to the immune response to coxsackievirus remains unclear however, as our recent data have demonstrated that mice lacking MyD88 (and therefore unable to signal from any TLR except TLR3) do not harbor increased viral titers or present with increased pathology or mortality at early time points following CB4 infection (91). In addition, MyD88 deficient mice have significantly reduced mortality compared to WT mice following CB3 infection (92). This suggests that MyD88-dependent pathways are not only dispensable for the innate immune response to coxsackieviruses; they may actually be detrimental to the host. Taken together, this indicates that although MyD88-dependent TLRs can recognize coxsackieviruses, MyD88-dependent signaling pathways are not necessary for the immune response to these viruses. Conversely, we demonstrated that TLR3-deficient mice are unable to control viral replication following CB4 infection leading to an increase in cardiac pathology and mortality compared to WT mice (91). This was correlated with decreased production of pro-inflammatory mediators, particularly tumor necrosis factor (TNF)-α and RANTES (regulated on activation normal T cell expressed and secreted, CCL5) suggesting an important role for these molecules in the immune response to coxsackieviruses (91). These results were confirmed in a separate study demonstrating an important role for TLR3 following CB3 infection although in that model, increased mortality was linked to the inability of TLR3 deficient mice to produce type II IFNs (93). The importance of RLRs in the detection of CB4 remains unclear although MDA-5 has been shown to recognize other coxsackievirus strains as well as other picornaviruses and may be
of particular importance for the type I IFN response (94, 95). However, results from TLR3 deficient mice indicate that even if the RLR signaling pathway is activated following coxsackievirus infection, it is not sufficient to compensate for the lack of TLR3 signals and protect the host (91, 93). Taken together, this indicates that coxsackieviruses can be recognized by several PRRs that likely contribute to the generation of an appropriate immune response with TLR3 playing a critical non-redundant role.

Several innate effector cell types including NK cells, macrophages and DCs have been demonstrated to be involved in the immune response to coxsackieviruses (96). Macrophages, however, seem to play a particularly crucial role in controlling viral replication and reducing tissue pathology following infection. To this effect, it has been demonstrated that inducible nitric oxide synthase (iNOS) expressing macrophages migrate to infected tissues following CB3 infection and that inhibition of iNOS results in increased viral titers (97). This suggests that nitric oxide (NO) production by macrophages is critical for the early control of viral replication. Furthermore, we recently demonstrated that TLR3 signaling on a population composed largely of macrophages is sufficient to protect TLR3 deficient mice following infection with CB4 by reducing cardiac pathology (91). Interestingly, TLR3 deficient mice produce lower levels of TNF-α, an important transcriptional activator of iNOS (91, 97). This suggests that TNF-α production following TLR3 signaling may, in part, protect the host from viral infection by inducing the production of NO from macrophages. Taken together, this indicates that macrophages are central to the innate immune response to coxsackievirus infection.

1.3 Innate immune mechanisms in coxsackievirus-induced autoimmunity

The innate response to viral infection plays a pivotal role in directing the ensuing adaptive immune response (98). As such, the induction of innate immune responses can influence the development of autoreactive adaptive immune responses with the potential for autoimmune pathology. The involvement of innate responses in the development of autoimmunity has been the subject of intense investigation in recent years.
1.3.1 Pattern recognition receptors and the induction of autoimmune myocarditis by coxsackieviruses

Several lines of evidence have demonstrated the importance of PRRs, particularly TLRs, in the pathogenesis of coxsackievirus-induced autoimmune myocarditis. MyD88 deficient mice are unable to signal from the majority of TLRs and are protected from both viral and adjuvant-induced myocarditis (EAM) (92, 99). The results of these experiments demonstrated that MyD88 signaling might be involved in several aspects of the pathogenesis of chronic autoimmune myocarditis. MyD88 deficiency dampened inflammation at the sites of infection with a significant reduction in cardiac expression of several cytokines including interleukin (IL)-1β and TNF-α (92), two cytokines that have long been associated with the induction of autoimmune myocarditis (discussed below and (100, 101)). Additionally, MyD88 signaling leads to the upregulation of the coxsackievirus receptor (coxsackie and adenovirus receptor, CAR) on cardiac tissue, suggesting that TLR signaling may facilitate cardiac infection (92). Surprisingly, MyD88 deficient mice produced higher levels of IFN-β following infection leading to faster viral clearance suggesting that MyD88-dependent signaling pathways are negative regulators of type I IFNs (92). The importance of MyD88 signaling in the chronic phase of myocarditis was further confirmed as MyD88-deficient mice were protected from the induction of EAM due to the inability of MyD88-deficient APCs to activate autoreactive T cells (99). A role for MyD88 signaling has also been suggested in humans as DCM patients harbor increased levels of MyD88 mRNA compared to a control population (102). Combined with the demonstration that MyD88-independent pathways are critical for host protection while MyD88-dependent pathways may be dispensable (91), this suggests that therapies aimed at dampening the effects of MyD88 signaling may prove beneficial to patients suffering from coxsackievirus-induced autoimmune myocarditis.

The role of individual TLRs in the induction of autoimmune myocarditis has also been partially elucidated. DCM patients present with elevated mRNA levels for both TLR4 and TLR8 (102, 103), two TLRs that have been demonstrated to signal in the presence of coxsackievirus (89, 90). Accumulating evidence suggests that TLR4 signaling may be central to the induction of autoimmune myocarditis. The detection of TLR4 mRNA in DCM patients
was shown to correlate with enteroviral infection and viral proteins were observed to colocalize with TLR4 in infected cardiac tissue (103). TLR4 deficiency in mice results in reduced pathogenesis following coxsackievirus infection and this correlates with a reduction in the levels of important cytokines such as IL-1β and IL-18 (104). It has also been suggested that TLR4 signaling increases the capacity of virus to replicate as TLR4-deficient mice harbored lower levels of virus than their WT counterparts at day 12 PI (104). This is potentially explained by the increased CAR expression mediated by MyD88 signaling (92). TLR4 has further been linked to the overall susceptibility to disease. In both humans and mice, myocarditis is more common in males compared to females despite similar prevalence of coxsackievirus infection between genders (105). In mice, this correlates with increased cardiac expression of TLR4 in males following coxsackievirus infection suggesting that increased signaling through TLR4 can have detrimental effects on the host (105). Similarly, it has long been described that treatment with lipopolysaccharide (LPS, a TLR4 ligand) at the time of coxsackievirus infection is sufficient to break genetic resistance (100). We have further demonstrated that ligands that signal through TLR4 but not those that signal through TLR2 are also sufficient to break resistance conferred by transgenic expression of transforming growth factor (TGF)-β (106). This suggests that signaling through TLR4 exerts an influence on the susceptibility to autoimmune myocarditis and that differences leading to increased expression or increased signaling from this receptor could represent a significant risk factor for patients. The role of other TLRs and PRRs in the progression of coxsackievirus-induced autoimmune myocarditis remains poorly documented and warrants further investigation.

1.3.2 Pattern recognition receptors and the induction of type 1 diabetes by coxsackieviruses

The role of TLR signaling in the pathogenesis of coxsackievirus-induced T1D is not currently well defined. Recent evidence however, suggests that TLR signaling may be involved in the development of spontaneous T1D. Patients suffering from T1D have been observed to harbor increased levels of both TLR2 and TLR4 compared to non-diabetic control populations suggesting that increased TLR signaling could contribute to pancreatic
inflammation (107). In mice, one of the insulin-dependent diabetes (Idd) susceptibility loci, Idd6, has been demonstrated to control the expression of TLR1 and mice congenic for this region expressed lower levels of TLR1 and were protected from T1D development, thus suggesting a role for TLR1 in disease susceptibility (108). Recent data have demonstrated that both the TLR2 and MyD88 signaling pathways are also involved in the development of T1D in mice (109, 110). TLR2 has been proposed to act as a sensor of beta cell death leading to the maturation of APCs and resulting in the activation of self-reactive T cells (109). As such, deficiency of TLR2 in NOD mice significantly reduced the incidence of spontaneous T1D (109). Furthermore, TLR2 has been demonstrated to signal directly within Tregs leading to increased proliferation concomitant with decreased suppressive capacity (84). This suggests that the TLR2-dependent recognition of secondary necrotic beta cell may result in decreased immune regulation. This potentially allows further activation of autoreactive T cells and destruction of pancreatic islet cells. MyD88 deficiency was also observed to reduce incidence of T1D although this was attributed to changes in the natural microbial flora of the gut that, in turn, affected the development of autoreactive T cells (110). The role of other TLRs in spontaneous development of T1D has also been investigated in mice and it was observed that the TLR9 signaling pathway is involved in disease pathogenesis while both TLR3 and TLR4 do not appear to dictate disease development (109, 111). Although there is currently little data linking TLRs with coxsackievirus-induced T1D, data from other viral-driven models of T1D suggest that TLRs are likely to be involved. For example, infection of normally resistant biobreeding diabetes resistant (BBDR) rats with the parvovirus Kilham rat virus (KRV) results in diabetes induction (112, 113). The capacity of KRV to induce disease in these rats is dependent in part on interactions with TLR9 (114) and TLR3 signaling can synergize with KRV infection to increase susceptibility to disease induction (87). Based on the importance of TLR3-mediated signals in the immune response to coxsackieviruses (91), it is possible to speculate that this receptor will also be involved in the induction of T1D following coxsackievirus-infection. Taken together, this suggests that TLR signaling is involved in the progression of spontaneous T1D and is likely to be involved in the pathogenesis of viral-induced T1D. Whether the same TLRs will be involved in both disease models warrants further investigation.
1.3.3 Antigen presenting cells and the induction of autoimmune myocarditis by coxsackieviruses

One important functional consequence of TLR signaling is the activation and maturation of both macrophages and DCs. This allows for the induction of antiviral mechanisms such as the production of NO from macrophages and, more importantly, it leads to the activation of the adaptive immune system. As such, APCs are likely to be central players in the induction of autoimmunity following infection.

Several lines of evidence point to an important role of APCs in the development of autoimmune myocarditis and DCM. Patients with end-stage heart disease often present with elevated numbers of DCs in the periphery (115). Furthermore, patients suffering from cardiac inflammation harbor increased numbers of CD11c+ (a DC marker) cells that constitutively express cardiac antigens suggesting that these DCs may serve to activate autoimmune responses in patients (116, 117). The importance of DCs in the pathogenesis of autoimmune myocarditis was clearly supported in a mouse model in which adoptive transfer of DCs loaded with a cardiac myosin peptide was sufficient to induce disease in genetically susceptible hosts (118). Differences in both the function and the number of DCs have been associated with disease susceptibility in mice. A comparison of the effects of coxsackievirus infection on genetically resistant C57BL/6 mice and genetically susceptible A.BY/SnJ mice has revealed that, in response to infection, DCs from susceptible mice produced lower levels of cytokines and chemokines, particularly interferon-inducible 10 (IP-10) a chemokine with cardioprotective properties (119). Furthermore, it was observed that resistant mice harbored significantly more of a particular subset of cross-priming DCs than genetically susceptible mice (119). This was correlated with lower virus titers in cardiac tissue of resistant mice suggesting an important role for this cross-priming DC subset in the control of coxsackievirus infection. These results suggest that changes in APC subsets and DC functionality are linked to susceptibility to coxsackievirus-induced myocarditis.

Macrophages play a critical role in the immune response to coxsackievirus and data suggest that they are involved in the pathogenesis of coxsackievirus-induced autoimmune myocarditis. Macrophages are broadly characterized into two groups, type I or type II based on their activation status and cytokine production profile. Alternatively activated or type II
macrophages (M2) are typically less inflammatory than classically activated or type I macrophages (M1) (120). Type II macrophages have been ascribed an important role in the healing of cardiac tissue following acute myocardial infarction (121). Mice deficient for the cytokine IL-13 develop greatly enhanced autoimmune pathology following CB3 infection. This was correlated with a decreased number of alternatively activated macrophages/monocytes compared to WT mice (122). The importance of macrophages was further demonstrated by our studies in a mouse model in which transgenic expression of TGF-β protects from the induction of autoimmune myocarditis following CB3 infection (106, 123). In this model, protection was correlated with reduced maturation of macrophages suggesting that reducing the inflammatory properties of macrophages may represent a way to prevent disease induction (106). Interestingly, decreased plasma levels of TGF-β have been linked to susceptibility to peripartum cardiomyopathy in patients (124) suggesting that increased maturation of macrophages due to lower levels of TGF-β may contribute to heart disease pathogenesis in humans. Taken together, this suggests that the balance between the inflammatory macrophages necessary for the immune response to viruses and alternatively activated macrophages involved in tissue healing and the resolution of the immune response may be critical to the development of an appropriate anti-viral immune response that does not degenerate into autoimmunity.

1.3.4 Antigen presenting cells and the induction of type 1 diabetes by coxsackieviruses

APCs are also involved in the development and pathogenesis of T1D. In both humans and mice, macrophages and DCs are among the first cell types to infiltrate the pancreatic islets (125, 126). In NOD mice, depletion of macrophages significantly reduces incidence of spontaneous T1D suggesting an important role for APCs in disease progression (127). Macrophages have also been proposed to directly contribute to the destruction of pancreatic beta cells as NOD/Rag knock-out mice (lacking T and B cells) transgenically expressing the chemokine CCL2 (also known as monocyte chemoattractant protein (MCP)-1) still develop diabetes (128). This was linked to an increased influx of CCR2+ (the CCL2 receptor) macrophages/monocytes into the pancreas (128). The role of APCs in T1D induced following viral infection has been well documented using the BDC2.5 model. In this
transgenic mouse model, viral infection leads to the engulfment of pancreatic beta cells by APCs and purified APCs from infected mice are sufficient to induce proliferation of autoreactive BDC2.5 T cells in vitro and to induce disease in uninfected BDC2.5 recipients following adoptive transfer (76). This suggests that viral infection induces the presentation of previously sequestered antigens leading to the activation of autoreactive T cells and the subsequent destruction of pancreatic beta cells (Figure 1.1). Taken together, the evidence currently available indicates that macrophages are likely involved in the induction of T1D following coxsackievirus infection.

Further evidence suggests that changes in macrophage function may also contribute to disease pathogenesis. To this effect, it has recently been demonstrated that monocytes/macrophages are involved in the maintenance of peripheral tolerance and changes within this population may be linked to the development of T1D. Disease progression, in both humans and mice, has been associated with a decline in the capacity of Tregs to maintain peripheral tolerance (129). Anderson and colleagues recently demonstrated that CD11b+CD11c- APCs (a population containing a large proportion of monocytes and macrophages) from NOD mice are deficient in the maintenance of the suppressive capacity of Tregs and that this is linked to disease progression (130). This suggested that deficiencies within the APC compartment rather than Treg intrinsic defect are associated with susceptibility to T1D. Interestingly, we recently demonstrated that CB4 infection in the context of TGF-β expression is sufficient to protect NOD mice from T1D (131). This was correlated with changes in the maturation status of this same CD11b+CD11c- APC population and increased levels of Tregs within the pancreas of protected mice (131). Taken together, this suggests that APCs play a central role in the induction of T1D following viral infection and that therapies aimed at specific APC subsets may be sufficient to prevent autoimmunity following infection and offer an alternative to current therapeutic approaches. To this effect, it was recently shown that glatiramer acetate, a drug commonly used to treat MS patients, protects mice from MS symptoms by reducing APC maturation levels and production of inflammatory cytokines which, in turn, leads to the induction of protective Tregs (132). This further validates APCs as a target for therapies aimed at preventing autoimmune diseases.
1.3.5 Cytokines and chemokines in the induction of autoimmune myocarditis by coxsackieviruses

Another important functional consequence of viral detection by the innate immune system is the production of cytokines. Although cytokines are clearly important for the immune response to viruses there is accumulating evidence that they are also involved in the progression and pathogenesis of autoimmune diseases.

Studies using transgenic mice expressing cytokines specifically within the pancreas have demonstrated an important role for the cytokine milieu at the primary site of infection in the progression towards autoimmunity. NOD mice expressing either TGF-β or IFN-γ under the control of an insulin promoter are protected from the chronic autoimmune phase of myocarditis following CB3 infection without a concomitant protection from the acute phase of disease (123, 133). This suggested that the cytokine milieu at the primary site of infection exerts a critical influence on the decision between tolerance and autoimmunity following viral challenge. Cytokine production has also been linked to disease susceptibility. Genetic resistance to autoimmune myocarditis can be overcome by the induction of TNF-α and IL-1β following LPS treatment at the time of CB3 infection (100). Similarly, direct addition of either one of these cytokines at the time of infection is sufficient to break genetic resistance (101). Production of TNF-α is also critical for disease progression in genetically susceptible hosts as illustrated by the inability of CB3 strain H310A1 to induce autoimmune myocarditis (134). This viral strain is capable of infecting and replicating within cardiac tissues but does not induce the TNF-α response normally observed following infection (134). Convincingly, addition of exogenous TNF-α at the time of infection with this attenuated strain is sufficient to induce autoimmune myocarditis (134). Taken together, this suggests that TNF-α plays a critical role in the pathogenesis of coxsackievirus-induced autoimmune myocarditis. The effect of TNF-α production on disease progression is likely due to its capacity to induce several downstream molecules. Pro-inflammatory chemokine production can be upregulated by TNF-α and two of these, MCP-1 (CCL2) and macrophage inflammatory protein (MIP)-1α are induced following coxsackievirus infection and have been linked to heart disease. MCP-1 has been found to be elevated in patients suffering from myocarditis and expression levels correlates with disease severity (135). Similarly, elevated levels of MCP-1 in mice
have been associated with increased disease severity due to enhanced migration of CCR2+ monocytes to the heart (136). Mice deficient for MIP-1α do not develop chronic autoimmunity following CB3 infection suggesting an important role for this chemokine in disease progression (137). Although TNF-α plays a critical role in the development of autoimmunity, any therapies aimed at targeting this molecule will have to take into account the importance of this cytokine in the immune response to viral infection.

The production of other cytokines has been associated with disease progression and studies on their contribution has highlighted that both timing and duration of cytokine expression can exert a critical influence on the development of autoimmunity. One such example is the pleiotropic cytokine IL-6 that is typically induced following coxsackievirus infection. IL-6 expression has been linked to increased severity of myocarditis and poor prognosis for patients (138). Data from experiments on encephalomyocarditis virus (EMCV)-infected mice has revealed that early expression of IL-6 can serve to reduce cardiac damage (139) while more persistent expression of IL-6 actually leads to increased disease severity (140). Similarly, data from our laboratory indicates that the absence of IL-6 results in a dramatic increase in disease severity following CB3 infection and that this correlates with the increased production of inflammatory mediators linked to the progression of myocarditis including TNF-α and MCP-1 (141). This suggests that early production of IL-6 is critical to dampen the immune response and protect from the induction of autoimmunity. These results illustrate that the production of cytokines following infection must be tightly regulated at several levels in order to allow for the proper development of a viral-specific immune response without concomitant induction of autoimmunity.

1.3.6 Cytokines and chemokines in the induction of type 1 diabetes by coxsackieviruses

Although the role of specific cytokines and chemokines in the induction of T1D following coxsackievirus infection is currently not well understood, several lines of evidence suggest that production of inflammatory mediators is involved in disease pathogenesis. Similar to the heart disease model, the cytokine milieu at the primary site of viral infection exerts an influence on disease progression. We have recently demonstrated that, in mice,
CB4 infection in the context of TGF-β leads to protection from the induction of T1D (131). Interestingly, the presence of TGF-β did not affect the capacity of the host to clear the viral infection suggesting that it may be possible to develop therapies that allow for the proper clearance of an invading pathogen while preventing the development of autoimmunity (131). The production of other cytokines, specifically IFN-γ and IL-4, has also been associated with the capacity of coxsackievirus to accelerate T1D onset in NOD mice (62). The production of TNF-α following coxsackievirus infection is also likely involved in the pathogenesis of T1D. In another model of viral-induced T1D, it has been demonstrated that early production of TNF-α enhances the incidence of T1D (142). Interestingly, it was further demonstrated that when production of TNF-α was induced later in the course of infection, this was sufficient to protect from viral-induced diabetes (142). This highlights that the timing of cytokine production may be critical for the induction of autoimmunity following viral infection.

Chemokines are also likely involved in the induction of T1D following viral infection. MCP-1 is produced at high levels following coxsackievirus infection and transgenic expression of this chemokine directly within the pancreas significantly increases disease incidence due to its capacity to attract macrophages (128). This suggests that MCP-1 is likely to be involved in the pathogenesis of coxsackievirus-induced T1D. Cytokines and chemokines induced following viral infection are potentially critical to the induction of T1D by viruses. Research aimed at gaining a better understanding of the role of these molecules in the immune response to viruses and the progression to autoimmunity may yield new potential therapeutic targets that would allow for the prevention of autoimmune diabetes while maintaining the capacity of the host to respond to viral infections.

1.3.7 Cellular innate effectors and the induction of autoimmunity following coxsackievirus infection

Several cellular effectors are involved in the induction and pathogenesis of autoimmune diseases following coxsackievirus infection. A particular subset of CD1d-restricted γδ-T cells partially controls viral infection by directly killing infected cells (143). This same subset of innate T cells also contributes to the induction of autoimmunity by activating CD4 helper T cells that subsequently activate autoreactive CD8 T cells (143). NK
cells are typically involved in the innate immune response to viruses and they have been observed as part of the immune infiltrate in the heart following coxsackievirus infection. Differences in the NK-gene complex between resistant and susceptible strains of mice have been characterized further linking this cell type to the susceptibility to chronic autoimmune myocarditis (23). Finally, the complement cascade may be protective against the induction of autoimmunity following viral infection as mice lacking complement receptor 1 and 2 showed faster disease progression and greater disease severity following CB3 infection (144). Several innate cellular effectors are also likely to be involved in the progression to T1D following infection although this is not currently well characterized. Both NK and NK T cells have been demonstrated to be involved in the development of spontaneous diabetes in NOD mice and they are likely to contribute to disease pathogenesis following viral infection (145, 146). Research aimed at defining the role of these innate effector cell types in the induction of autoimmune diseases following viral infection should provide important information that, in turn, could lead to the design of better therapeutics.

1.4 Regulatory T cells, TGF-β and autoimmunity

1.4.1 Regulatory T cells

Tregs are a well-described T cell subset involved in the maintenance of peripheral tolerance. Sakaguchi and colleagues originally demonstrated that the ablation of a small population of CD4 T cells constitutively expressing the high affinity IL-2 receptor alpha chain (IL-2Rα, CD25) resulted in the induction of widespread autoimmunity suggesting a critical role for these cells in the maintenance of peripheral tolerance (147, 148). Further studies demonstrated that Tregs specifically express the transcription factor Foxp3 and that mutations within this transcription factor were responsible for the induction of autoimmunity in the scurvy mouse and in patients suffering from IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) (149-153). Foxp3 is a member of the forkhead-winged helix family of transcription factors and is directly or indirectly responsible for the activation and repression of several hundred genes that are associated with Treg function (154-158). Retroviral transduction of Foxp3 in naïve T cells is sufficient to confer
suppressive capacity to these cells, highlighting the central role of Foxp3 in Treg function (151, 152). Foxp3 expression is required throughout the lifespan of Tregs in order to maintain suppressive capacity and prevent autoimmunity (159) and even a partial reduction in Foxp3 expression results in loss of suppressive capacity and development of lymphoproliferative disorders (160). In some cases, Foxp3 expression has been demonstrated to be unstable and loss of Foxp3 expression yields highly inflammatory cell types that could contribute to autoimmune disease such as T1D (161). CD4^+CD25^{high}Foxp3^+ Treg populations with suppressive capacities have also been identified in humans although, as opposed to mice, Foxp3 expression is not exclusive to Tregs but can also be expressed transiently by activated T cells rendering Treg analysis in human populations inherently more complicated (162). Several autoimmune diseases have been proposed to be associated with reduced Treg function and Treg immunotherapy may represent an interesting therapeutic avenue for some of these diseases.

It is now well established that Tregs can be broadly classified in two groups based on their origins. Natural Tregs are derived from the thymus and make up approximately 1-10% of peripheral CD4 T cell populations. These Tregs possess a broadly varied TCR repertoire that is largely distinct from the TCR repertoire of conventional T cells (163) suggesting a diverging developmental pathway. Thymic development of Tregs is strongly dependent on costimulatory signals from CD28 (164, 165) and on γ common chain cytokines, particularly IL-2 (166-168). Adaptive Tregs represent a collection of T cells with suppressive capacity that are induced in the periphery from conventional T cells, these include Foxp3 expressing cell types as well as non-Foxp3 expressing subsets such as Il-10 producing Tr1 cells and TGF-β dependent Th3 cells (169). Generation of adaptive Tregs has been described in vitro and in vivo and appears to be largely dependent on the cytokine milieu and antigen dose during T cell stimulation (169). Similarly to natural Tregs the generation of adaptive Tregs is strongly dependent on the presence of IL-2 (170). TGF-β is integral to the generation of adaptive Tregs and this will be discussed in more detail in the following section (171). The lack of markers specific to adaptive Tregs has complicated the analysis of their relative contribution to the maintenance of peripheral tolerance but in vitro converted Tregs clearly possess suppressive capacity and are able to suppress T cell responses and prevent autoimmunity in vivo (171).
The mechanisms of Treg activation remain poorly defined and are the subject of intense investigation. In particular, several groups have attempted to determine whether Treg activation and/or suppression occurs in an antigen-specific manner. Antigen-specific Tregs have been identified and in the NOD mouse it has been demonstrated that antigen-specific Tregs have a greater capacity to prevent T1D than polyclonal Tregs (172, 173). It was further demonstrated that Tregs specific for pancreatic antigen require stimulation with their cognate antigen in order to suppress T1D onset (174). This is supported by in vitro experiments demonstrating that TCR stimulation is required to activate the suppressive capacity of Tregs (174, 175). Interestingly, in vivo and in vitro data have demonstrated that following activation with their cognate antigen, Tregs can suppress conventional T cells with a different antigenic specificity (174). Although this remains controversial, this suggests that, in vivo, Tregs require antigen-specific interactions for their activation but possess the capacity to maintain peripheral tolerance in an antigen-independent manner. Interestingly, recent reports have suggested that Tregs may be specialized for the subsets of T cells they suppress. For example, Tregs lacking the Th2 transcription factor IRF4 were unable to suppress Th2 responses while Tregs lacking the Th1 transcription factor Tbet were unable to suppress Th1 responses (176, 177). This suggests that Treg-mediated suppression is tightly regulated by several factors.

Similarly, the exact mechanisms through which Tregs ultimately maintain peripheral tolerance remain unclear. Tregs can inhibit the proliferation and/or activation of several cell types including T cells, DCs, macrophages, B cells, NK and NK T cells (178). Tregs have been proposed to contribute to the suppression of these cell types through a variety of mechanisms that include direct cell contact-dependent inhibition, inhibition through the secretion of immunosuppressive cytokines as well as target cell killing (Figure 1.3) (178). In vivo studies have demonstrated that deficiency of single molecules proposed to play a role in Treg function is usually not enough to completely abrogate Treg function and induce autoimmunity suggesting that Tregs possess many complementary mechanisms in which they maintain tolerance (178). The contribution of each of these mechanisms to the maintenance of peripheral tolerance is not currently well understood and warrants further investigation.
1.4.2 TGF-β and regulatory T cells

TGF-β is a pleiotropic cytokine involved in several aspects of the immune response (Figure 1.4). TGF-β is a member of the TGF-β superfamily and there are three isoforms in mammals (TGF-β1, TGF-β2 and TGF-β3) (179). TGF-β1 is the main isoform involved in immune responses although all three isoforms have been demonstrated to have similar effects in vitro (179). TGF-β is typically expressed as a latent complex comprised of a TGF-β homodimer noncovalently associated with the latency associated complex (180). Removal of the latency associated complex, through a currently poorly understood mechanism, results in the release of active TGF-β (180). Studies of animals deficient for TGF-β or lacking some of the critical TGF-β signaling components have demonstrated an important dual role for TGF-β which includes coordination of early immune responses (181, 182) and, perhaps more importantly, a critical role in resolution of immune responses and maintenance of tolerance (179). This was convincingly demonstrated by the study of TGF-β1 deficient mice which developed a predominantly T cell mediated multi-organ inflammatory disease characterized by an increase in inflammatory cytokine production (183-186). This phenotype was also observed in mice in which T cells are unable to respond to TGF-β signaling, confirming the importance of TGF-β in maintaining T cell tolerance at steady-state (187, 188). In addition to its critical effect on T cells, TGF-β signaling can also directly modify the function of several other cell types including macrophages and DCs (Figure 1.4) (179). TGF-β signaling on APCs results in reduced maturation including lower surface expression of MHC class II as well as reduced production of inflammatory mediators, ultimately lowering the capacity of TGF-β treated APCs to present antigens and generate inflammatory responses (189-196).

This mechanism may be of particular importance for the capacity of macrophages to maintain tissue homeostasis without inducing inflammation as engulfment of apoptotic debris by macrophages induces the production of TGF-β which in turn prevents macrophage maturation (197, 198). Taken together, the evidence available demonstrates that TGF-β is a critical mediator for the maintenance of peripheral tolerance.

In addition to its direct effect on immune cells, TGF-β is also indirectly involved in the maintenance of tolerance due to its capacity to affect T cell polarization. Studies have
long demonstrated that TGF-β signaling can prevent the differentiation of T cells towards a Th1 or Th2 phenotype by inhibiting the expression of transcription factors required for T cell polarization (199-202). More recently, studies have determined that together with IL-6, TGF-β is critical for the generation of IL-17 producing Th17 cells, a T cell subset that has been ascribed a pathogenic role in several autoimmune diseases (203-206). Of particular interest, TGF-β has also been implicated in the generation and maintenance of Tregs. In vitro studies have clearly demonstrated that TCR stimulation of naïve T cells in the presence of TGF-β results in the induction of Foxp3 expression and the acquisition of suppressive capacities (171, 207). Similarly, TGF-β has been linked to the induction and/or expansion of Tregs in vivo (208). Importantly, induction of Tregs in the presence of TGF-β is critically dependent on the presence of APCs (171). Immature APCs appear to be particularly efficient at inducing Tregs suggesting that the capacity of TGF-β to prevent APC maturation is central to the induction of Tregs (209). Furthermore, the capacity of TGF-β to induce Tregs can be potentiated by other factors including nutrients. To this effect, the vitamin A derivative retinoic acid enhances the capacity of gut-derived DCs to induce the conversion of naïve T cells to a regulatory phenotype potentially by increasing the surface expression of the TGF-β receptor on target cells (210-213). Taken together, this suggests that TGF-β is critical to the maintenance of peripheral tolerance, in part by modulating APC function leading to the induction of adaptive Tregs.

Interestingly, TGF-β1 deficient mice present with reduced numbers of Tregs in the periphery despite normal numbers in the thymus (179, 214). Furthermore, Tregs from TGF-β deficient mice expressed lower levels of Foxp3 and reduced suppressive function (214). This suggests that TGF-β plays a critical role in the peripheral homeostasis of Tregs and in the maintenance of suppressive capacity. Additionally, TGF-β has been implicated in the suppressive mechanism of Tregs. Tregs express both latent and active TGF-β on their surface and reports have suggested that this may contribute significantly to the capacity of Tregs to mediate contact-dependent suppression (215-217). To this effect, treatment with an antibody specific for TGF-β or genetic ablation of TGF-β was sufficient to abrogate the protective effect of Tregs in mouse models of inflammatory bowel disease and colitis (218, 219). Conversely, Tregs derived from TGF-β1 deficient mice still retain some suppressive activity suggesting that TGF-β represents only one of the many mechanisms through which Tregs
maintain tolerance (219-221). The available data highlight a critical role for TGF-β in the generation, maintenance and function of Tregs in the periphery.

1.4.3 Regulatory T cells and viral infections

In addition to their role in maintaining tolerance during homeostasis, Tregs have recently been demonstrated to play an important role during immune responses to pathogens including viruses. Over the course of some infections, Tregs are critical to dampen overactive immune responses and protect the host from immune-mediated damage. For example, Tregs are required to protect against the development of inflammatory lesions following ocular infections with herpes simplex virus (HSV) (222). Intriguingly, Tregs may also coordinate early immune responses to certain viral infections through an as yet undetermined mechanism (223). For several other viral pathogens however, the presence of Tregs may be detrimental to the host particularly by allowing the establishment of chronic infections. Several studies have demonstrated that Tregs may limit the capacity of T cells to control human immunodeficiency virus (HIV) infections and that Treg depletion results in increased anti-HIV T cell responses ex vivo (224-227). Similarly, depletion of Tregs greatly enhanced control of viral replication in a humanized mouse model of HIV infection (228). Conversely, increased Treg presence has been linked to better disease prognosis and reduced overall immune activation suggesting that Tregs may play a critical role in reducing immunopathology in the later stages of HIV infection (226, 229, 230). Patients chronically infected with hepatitis B (231-235) or hepatitis C virus (HCV) (236-239) typically present with higher levels of Tregs in the periphery and in the liver confirming a potential role for Tregs in the establishment of chronic viral infections. Similarly to what has been described for HIV infected patients, Tregs may also play a beneficial role after infection is established as HCV patients demonstrate an inverse correlation between Treg accumulation in the liver and liver damage (237, 240). Taken together, this suggests that Tregs may hinder viral control at early stages of infection but may play a crucial role in preventing immune-mediated damage at later stages of infection. Based on these observations, any therapies aimed at manipulating Tregs in order to prevent autoimmunity following viral infection will
have to take into account both the antigenic specificity of Tregs and the timing of therapy in order to avoid suppressing the immune response directed at the invading pathogen.

1.4.4 Regulatory T cells and autoimmune myocarditis

The role of Tregs in the progression of autoimmune myocarditis following coxsackievirus infection is currently poorly understood. The evidence currently available suggests that although Tregs are not sufficient to prevent autoimmune targeting of cardiac tissue, they may function to reduce pathology. For example, in the EAM model, reduced cardiac presence of CD4+CD25+ T cells correlates with increased disease severity (241). Similarly, IL-13 deficiency resulted in greatly enhanced cardiac pathology following both coxsackievirus infection and EAM induction and has been correlated with reduced Treg levels in the spleen suggesting that Treg presence in the periphery can modulate autoimmune responses within cardiac tissue (122). Furthermore, increased susceptibility of females to coxsackievirus-induced autoimmune myocarditis during specific phases of the ovarian cycle has been linked with a sex-hormone mediated reduction in overall Treg levels (242). Taken together, this suggests that Tregs are important in minimizing cardiac pathology and that decreased Treg function may be linked to the susceptibility of developing severe autoimmune myocarditis following coxsackieviral infection.

1.4.5 Regulatory T cells and type 1 diabetes

It is well documented that patients that eventually suffer from T1D develop antibodies directed at pancreatic antigens long before clinical symptoms of the disease arise. Similarly, in NOD mice, islet infiltration (or insulitis) starts at approximately 2 to 4 weeks of age although clinical disease is not typically observed until approximately 14 or 15 weeks of age (42). This indicates that mechanisms of peripheral tolerance are able to maintain partial protection and keep the autoimmune T cell response in check over a certain period of time. Among these mechanisms of peripheral tolerance, the role of Tregs in the progression of T1D has been the subject of intense investigation.
Several lines of evidence have established that Tregs are a major player in the natural progression of T1D. In particular, NOD mice with reduced numbers of Tregs due either to genetic deficiency of CD28, antibody depletion or cyclophosphamide treatment, develop T1D with dramatically enhanced kinetics compared to WT or untreated mice (42). Of clinical relevance, patients with a deficiency in Foxp3 develop a multi-organ autoimmune syndrome called IPEX that is typically associated with the development of T1D (243). Additionally, T cell responses specific for a pancreatic islet antigen associated with T1D, glutamic acid decarboxylase, are enhanced when T cells purified from healthy patients are depleted of Tregs supporting the hypothesis that Tregs can maintain tolerance to pancreatic antigens in healthy individuals (244). Taken together this clearly indicates that Tregs are integral to maintaining at least partial tolerance to pancreatic antigens leading to a delay in onset of disease in genetically susceptible hosts.

As Tregs are critical for the maintenance of peripheral tolerance to pancreatic antigens, several groups have investigated whether a decrease in either numbers or function of Tregs can explain the eventual onset of disease. Although, at present, this remains a controversial topic, the majority of studies in both humans and mice have determined that the numbers of Tregs do not decrease in susceptible hosts with progression towards clinical T1D (129). However, several lines of evidence have indicated that Treg function diminishes over time and that this correlates with the onset of overt T1D. You and colleagues have demonstrated that the capacity of Tregs from older NOD mice to suppress effector T cells is significantly impaired compared to Tregs isolated from younger NOD mice (245). These findings have been confirmed by several subsequent studies (129). More importantly, Tregs from certain T1D patients exhibit reduced suppressive capacity when compared to Tregs from a control population (129). Further contributing to disease progression, effector T cells from both NOD mice and T1D patients become progressively less sensitive to suppression by Tregs (Figure 1.5) (129). The mechanism explaining the progressive loss of Treg function in T1D is currently not well understood although recent studies have associated this with variations in the gene coding for IL-2 (246, 247). Moreover, recent data in NOD mice have suggested that this defect is not intrinsic to Tregs but rather to a reduced capacity of APCs to properly maintain the suppressive function of Tregs (130). The capacity of APCs to properly direct activation of Tregs is controlled by the same genetic region containing the IL-2 gene
Similarly, APCs from T1D patients have recently been demonstrated to be deficient in the capacity to maintain Treg function and to present with an inflammatory phenotype that may favor the development of pathogenic Th17 cells (248, 249). The consensus emerging from recent literature is that onset of T1D is, at least in part, determined by a decrease in function of Tregs and that this in turn may be dictated by a defect in the capacity of APCs to properly maintain Treg function. This strongly suggests that therapies aimed at correcting the inability of APCs to maintain peripheral tolerance may represent a powerful therapeutic avenue to reestablish Treg function and provide long-term protection from the development of autoimmunity.

1.5 Objectives, hypothesis and aims

The overall objective of this thesis was to determine the link between innate signals, APCs and the development of autoimmunity following coxsackievirus infection. As such, I hypothesized that TLR-mediated signals and their functional consequences, including APC maturation and cytokine production, are responsible for a breakdown in peripheral tolerance and the induction of autoimmunity following coxsackievirus B infection. Further, I hypothesized that a specific subset of APCs is responsible for the induction of autoimmunity. Finally, I hypothesized that modulation of APCs will influence T cell responses and ultimately determine disease outcome by affecting the decision between induction of autoimmunity and maintenance of tolerance.

Experiments were designed to determine the innate signals involved in viral recognition and whether the functional consequences of this detection can be linked to the induction of autoimmunity. To address this, I studied a mouse model lacking the dsRNA sensor TLR3 and determined its importance in the immune response to viruses. I further asked whether TLR ligands could be used to circumvent established protection and allow for the development of autoimmunity in a mouse model of coxsackievirus-induced autoimmune myocarditis. Additionally, experiments were designed to address the role of specific APC populations in the induction of autoimmunity following viral infection and whether these populations can be manipulated in a way to induce protection rather than autoimmunity. To
this effect, we studied a previously described mouse model derived from the NOD mouse which expresses TGF-β directly within the beta cells of the pancreas (250).

The data accumulated over the course of these studies have demonstrated that CD11b+CD11c- APCs are critical for both the immune response to coxsackieviral infection and the ensuing development of autoimmune diseases such as autoimmune myocarditis and T1D. I further demonstrated that it is possible to modulate the function of these APCs in such a way to prevent the development of autoimmunity while maintaining a proper immune response to the virus.
Figure 1.1: Schematic representation T1D induction following CB4 infection

Tropism for pancreatic beta cells is one of the critical requirements for the capacity of viruses to induce and/or accelerate T1D. Infection of pancreatic beta cells leads to the presentation of previously sequestered antigens in the context of an inflammatory milieu generated by infection. This leads to the activation of autoreactive T cells recognizing pancreatic antigen and, if sufficient autoreactive T cells are present, destruction of beta cells and eventual development of T1D. Reprinted from Autoimmunity Reviews, 8(7), Richer MJ and Horwitz MS, Coxsackievirus infection as an environmental factor in the etiology of type 1 diabetes, 611-5, © 2009, with permission from Elsevier.
Figure 1.2
**Figure 1.2. Toll-like receptor signaling pathways**

Interaction of a TLR with its cognate ligand activates a signaling pathway involving several signaling adaptor molecules. Of particular importance, MyD88 is a critical adaptor molecule used by all the TLR signaling pathways with the exception of TLR3 which signals through TRIF. Activation of the TLR signaling pathways result in several functional consequences including the production of inflammatory cytokines. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Liew, FY, Xu, D, Brint, EK and O’Neill, LAJ, Negative Regulation of Toll-like receptor-mediated immune responses, © 2005.
Figure 1.3: Mechanisms of regulatory T cell mediated suppression

Tregs can mediate suppression and maintain tolerance through a variety of mechanisms. These include A) the secretion of inhibitory cytokines including TGF-β, B) direct lysis of effector T cells, C) disruption of effector T cell metabolism (eg. cytokine deprivation) and D) the inhibition of the maturation and function of dendritic cells which in turn prevents the activation of effector T cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Vignali DAA, Collison, LW and Workman CJ, How regulatory T cells work, © 2008.
Figure 1.4: TGF-β is a pleiotropic cytokine with effects on a broad range of immune cells

Schematic representation of positive (arrow pointing up) or negative (arrow pointing down) effects of TGF-β on various immune cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Rubtsov YP, Rudensky AY, TGFbeta signalling in control of T-cell-mediated self-reactivity. © 2007.
Figure 1.5: Schematic representation of T1D progression in NOD mice

Top panels: Stages of pancreatic pathology associated with T1D progression. Leukocytes progressively invade islet of Langerhans ultimately resulting in beta cell destruction and T1D development. Bottom panel: T1D progression has been associated with a progressive decline in Treg function that occurs concomitantly with increased resistance of autoreactive T cells to Treg mediated suppression.
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Chapter 2

Toll-like receptor 3 signaling on macrophages is required for survival following coxsackievirus B4 infection²

2.1 Introduction

Protective immunity to viral infection is often critically dependent on early recognition of the pathogen. This first-line of defense against viral infection is mediated, in part, by a series of pattern-recognition receptors that include Toll-like receptors (TLRs) and Rig-like helicases (RLH). TLRs act as sensors that can recognize several molecular patterns associated with viruses including surface glycoproteins (TLR1, 2 and 4) (1-3) and nucleic acids (TLR3, 7, 8 and 9) (4-7). Recognition of a cognate ligand induces a downstream signaling cascade culminating in the release of pro-inflammatory cytokines and maturation of antigen presenting cells (APCs). As such, TLR signaling contributes to the establishment of an anti-viral state and, by activating APCs, to the establishment of an adaptive anti-viral response.

TLR3 has long been proposed to play an important role in the innate response to viruses due to its capacity to recognize double stranded (ds) RNA, a common intermediate of viral replication (4). Binding of dsRNA to TLR3 initiates a unique signaling cascade that, unlike other TLR ligands (with the exception of some TLR4 ligands that can initiate two separate signaling pathways) does not depend on the signaling adaptor MyD88 but rather utilizes the molecular adapter TRIF (or TICAM-1) (8-10). Despite the well documented capacity of Poly I:C, a synthetic mimic of dsRNA, to increase antiviral immunity in a TLR3 dependent manner (11-16), the protective role of TLR3 in vivo remains controversial. For instance, a protective role for TLR3 has been demonstrated in a model of viral induced myocarditis. Following encephalomyocarditis virus (EMCV) challenge, TLR3 deficiency resulted in heightened mortality that correlated with increased viral replication and viral mediated cardiac damage. However, in this study TLR3 signaling was only partially protective as EMCV infection of WT mice still resulted in over 75% mortality (17). TLR3 was also shown to play a minor role in the immune response to mouse cytomegalovirus (MCMV) (18) and murine norovirus (19) as TLR3 deficient mice were observed to harbor increased viral titers following infection although this did not affect their capacity to survive viral infection. In contrast, TLR9 deficient mice rapidly succumbed to infection by MCMV (18). Furthermore, a dominant negative TLR3 allele was found in patients suffering from Herpes Simplex encephalitis implying a role for this receptor in protection from neurotropic
viruses (20). TLR3 signaling may be of particular importance for the immune responses to viruses that do not directly infect dendritic cells as it was demonstrated to promote cross-priming of CD8 T cells in response to dsRNA (21). Conversely, Edelmann and colleagues have reported that in the absence of TLR3 signaling, mice can still mount a protective immune response following challenge with various viruses, including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), MCMV, and reovirus, resulting in unaffected viral clearance and no increase in viral pathogenesis (22). Further, it was reported that TLR3 does not play a major role in the immune response of astrocytes to Theiler's murine encephalomyelitis virus (TMEV) (23). Intriguingly, TLR3 has been observed to play a deleterious role towards the host following pathogen challenge in several models. TLR3 deficiency has been reported to confer a survival advantage following influenza A challenge due to a reduction in inflammation (24) and was also determined to protect mice following challenge with lethal doses of Punta Toro Virus by reducing viral-induced liver pathogenesis (25). Furthermore, TLR3 deficiency was shown to be beneficial following West Nile virus infection. It was demonstrated that while the absence of TLR3 signaling resulted in increased peripheral viral load it also served to reduced neural inflammation leading to a decrease in both viral loads and pathology in the brain (26). Conversely, a recent report has suggested that TLR3 may act to protect from West Nile virus infection directly within neurons (27). Taken together, these data demonstrate that TLR3 may only be required for the response to a specific subset of viruses. Further, multiple reports suggest that TLR3 signaling is either dispensable or even harmful following infection with other RNA viruses.

Coxsackievirus B4 (CB4) is a small single stranded + sense RNA picornavirus with tropism for the pancreas, where it has been associated with induction of pancreatitis and autoimmune diabetes (reviewed in (28)), and the heart where it can cause both acute and chronic myocarditis (29). It is estimated that coxsackieviral infections are responsible for nearly a third of all new cases of dilated cardiomyopathy a disease responsible for the majority of heart transplantations (26, 30). Additionally, CB4 was originally isolated from a patient suffering from Type 1 diabetes (31) and has been demonstrated to accelerate onset of diabetes in non-obese diabetic (NOD) mice (32, 33). The immune response to this virus may be strongly dependent on innate mechanism as it has been previously demonstrated that
SCID mice are capable of surviving infection even at high infectious doses (34). It was previously reported that CB4 can signal through TLR4 on pancreatic islet cells (3) and that a closely related virus, CB3, signals through TLR8 on cardiomyocytes (5). The generation of dsRNA, the ligand for TLR3, is a common by-product of the replication cycle of single stranded RNA viruses including picornaviruses like coxsackievirus and arenaviruses like LCMV. The importance of TLRs and particularly TLR3 following CB4 infection in vivo remains to be elucidated.

Here, we demonstrate that the early immune response to CB4 is solely dependent on TLR3 signaling as infection of TLR3KO but not MyD88KO mice had fatal consequences. We have further demonstrated that this increased mortality is due to the inability of the host to control viral replication rapidly leading to severe cardiac damage. Interestingly, TLR3 deficiency did not affect the maturation of APCs or the activation of the adaptive immune response. Rather, TLR3 deficiency decreases the production of the pro-inflammatory mediators TNF-α and CCL5 resulting in uncontrolled viral replication that outpaces the developing adaptive immune response. Most importantly, we demonstrate that adoptive transfer of WT macrophages is sufficient to protect TLR3KO mice from succumbing to CB4 infection indicating that TLR3 signaling on macrophages is critical for the host response to this RNA virus.

2.2 Results

2.2.1 TLR3 plays a critical role for survival following CB4 infection

In order to elucidate the role of TLR3 in the innate immune response to coxsackievirus, NOD mice deficient for TLR3 (TLR3KO) were infected with sublethal doses of CB4 and were monitored for survival. As a control, NOD mice lacking MyD88 (MyD88KO) (and therefore unable to signal from the remaining TLRs) were also infected with CB4. We observed a significant increase in mortality following CB4 challenge in TLR3KO mice with nearly 60% of TLR3KO mice dying by day 7 compared to less than 15% of wild-type (WT) NOD and MyD88KO mice (Figure 2.1A). For comparison to other studies
and to ensure that the phenotype observed was not simply dependent on mouse strain differences, mice were challenged with LCMV as TLR3 was previously demonstrated to be dispensable for the immune response to this RNA virus (22). As previously reported, deficiency of TLR3 did not result in mortality following LCMV infection confirming that TLR3 is not necessary for the response to this virus (Figure 2.1A) (22). Taken together, this suggests that while TLR3 plays a critical role in the response to some RNA viruses like CB4, it is not necessary for the control of other RNA viruses like LCMV. Importantly, the MyD88-dependent TLR signaling pathway is not required for survival following CB4 challenge, nor can it compensate for the lack of TLR3 signaling.

### 2.2.2 Mortality is associated with increased viral replication and increased cardiac damage

In order to determine whether increased mortality in TLR3 deficient mice resulted from an inability to control viral replication, viral titers in the pancreas, liver and heart were determined by plaque assay at day 3 and 7 PI with CB4. No significant differences were observed at day 3 PI (data not shown). Conversely, we observed a significant increase in viral replication in the heart and liver of TLR3KO mice compared to both WT NOD and MyD88KO mice at day 7 PI (Figure 2.1B). We also observed increased viral replication in the pancreas of TLR3KO mice although this difference did not reach statistical significance (Figure 2.1B). Most notably, at day 7 PI the viral titers in the hearts of TLR3KO were approximately 100 and 1000 fold higher than that observed in WT NOD and MyD88KO mice respectively (Figure 2.1B). We next determined whether increased viral replication resulted in heightened tissue damage that might explain the rapid death observed in TLR3KO mice. Histological examination of pancreatic tissue revealed extensive damage in TLR3KO mice that was comparable to WT NOD mice (Figure 2.2A). Additionally, blood glucose levels were comparable in all 3 strains at day 3 and 7 PI (data not shown) suggesting that pancreatic dysfunction is unlikely to explain the increased mortality observed. Analysis of TLR3KO livers did not reveal any obvious differences in pathology compared to either WT NOD or MyD88KO mice following CB4 infection (Figure 2.2A). Liver damage was further analyzed at day 3 and 7 PI using a quantitative assay based on the levels of Alanine...
transaminase (ALT) in the serum (Figure 2.2B). We observed increased ALT levels in the serum of TLR3KO mice compared to WT NOD mice at day 3 and 7 PI although this difference never reached statistical significance suggesting that liver damage is not a major contributor to the observed increased mortality in TLR3KO mice. Finally, we measured the extent of cardiac damage. Histological analysis revealed an atypical increase in the number and severity of cardiac lesions at day 7 PI in TLR3KO mice compared to both WT NOD and MyD88KO mice (Figure 2.2A). These observations were confirmed by a significant increase in the presence of cardiac Troponin I (cTnI), a marker for myocardial injury (35), in the serum of TLR3KO compared to both WT NOD and MyD88KO mice at day 7 PI (Figure 2.2C). Taken together, these results demonstrate that the absence of signaling from TLR3 but not other TLRs leads to uncontrolled viral replication in several organs including the heart where heightened damage is most likely responsible for the observed increase in mortality.

2.2.3 TLR3 deficiency does not affect activation of innate or adaptive effectors following CB4 infection

TLR mediated signals are important for the activation of several innate effectors including NK cells and APCs. TLR signaling can also affect the activation of the adaptive immune response either directly or through effects on the APC population. As such we sought to determine the activation status of several important antiviral effectors following CB4 infection in TLR3KO mice. First, we determined the activation of NK (panNK+TCRβ-) and NK T (panNK+TCRβ+) cells by measuring surface expression of the activation marker CD69 by flow cytometry. At 48 hours post-infection, we observed that both NK and NKT cells from TLR3KO mice upregulated CD69 to levels comparable with WT NOD mice suggesting that these cells can respond normally to viral infection despite the absence of TLR3 signaling (Figure 2.3A, B). Interestingly, NK and NK T cell activation was completely abrogated in MyD88KO (Figure 2.4A,B). This highlights an important role for this pathway and other TLRs in the activation of NK and NK T cells although it also suggests that activation of these cells is not necessary for survival following CB4 challenge. We next determined the activation status of APCs by measuring surface expression of the costimulatory molecules CD80 (Figure 2.3) and CD86 (data not shown) on macrophages.
(CD11b+CD11c-) and DCs (CD11c+) at day 4 PI. We observed comparable upregulation of costimulatory molecules on macrophages and DCs in both WT NOD and TLR3KO mice demonstrating that TLR3 signaling is not necessary for the maturation of APCs and further suggest that a defect in APC maturation is not responsible for the increased mortality observed in TLR3KO mice (Figure 2.3C,D). Following infection of MyD88KO mice, we observed reduced maturation of both macrophages and DCs compared to WT mice again suggesting a role for the MyD88 pathway in the maturation of APCs (Figure 2.4C,D). These results suggest that the maturation of APCs is not necessary for survival following CB4 infection. As a control WT NOD and TLR3KO mice were also infected with LCMV and as expected, no differences in APC maturation were observed (Figure 2.5).

To determine whether TLR3 deficiency results in changes in the activation of the adaptive immune system, the activation status of B cells and T cells was monitored by flow cytometry. At day 4 PI we observed that B cells (CD19+) from TLR3KO mice responded to viral infection by upregulating surface expression of the activation marker CD69 and the costimulatory molecule CD86 to levels comparable to that observed on B cells from WT NOD mice (Figure 2.6). As for innate effectors, we observed reduced activation of B cells in MyD88KO mice following CB4 challenge although as opposed to what we observed for NK cells, activation was not completely abrogated (Figure 2.4E). Similarly, the activation status of T cells from TLR3KO mice was comparable to WT NOD mice at day 4 PI with either CB4 (Figure 2.7) or LCMV (Figure 2.8) as measured by upregulation of CD69 and downregulation of CD62L and at day 7 (Figure 2.9) as measured by upregulation of CD44. Consistent with the limited maturation of APCs observed in MyD88KO mice following CB4 infection, T cell activation was also greatly reduced (Figure 2.4F, G). These data suggest that the maturation and activation of both innate and adaptive effectors following CB4 infection is dependent on the MyD88 signaling pathway and independent of TLR3. This further suggests that activation of cellular effectors is not critical for survival following CB4 infection.
2.2.4 TLR3 deficiency results in lower production of inflammatory mediators following infection

One important functional consequence of TLR ligation with a cognate ligand is the production of pro-inflammatory cytokines and chemokines. Accordingly, we determined the serum levels of cytokines following infection. At 48 hours following infection we measured the levels of IFNα in the serum of CB4 infected NOD mice (Figure 2.10), this production was abrogated in both TLR3KO and MyD88KO mice suggesting that a deficiency in type 1 interferon is unlikely to explain the increased mortality and viral replication observed in TLR3KO mice. At day 4 following CB4 infection of WT NOD mice, we observed significant increases compared to uninfected controls in the levels of both TNF-α (Figure 2.11A) and IL-6 (Figure 2.11B) and to a lesser extent IFN-γ (Figure 2.11C) Little to no production of IL-4, IL-5, IL-10, IL-12p70 was observed following infection (data not shown). TLR3KO mice produced significantly reduced levels of TNF-α compared to both WT NOD and MyD88KO mice while the levels of all other cytokines measured were similar to WT NOD mice (Figure 2.11). This suggests that production of TNF-α following CB4 infection is strongly dependent on TLR3 and independent of MyD88. Further, this suggests that TNF-α production is critical to the early immune response following infection. Interestingly, we observed a significantly reduced level of IL-6 and IFN-γ following infection of MyD88KO mice compared to WT NOD indicating that these cytokines are produced following ligation of TLRs other than TLR3 (Figure 2.11B,C). Further, these data suggest that IL-6 and IFN-γ do not play a critical role in the survival of mice following CB4 challenge as MyD88KO mice survived infection and controlled viral replication despite reduced levels of both of these cytokines. Following LCMV infection and consistent with prior data (36), we observed a strong cytokine response dominated by the production of IFN-γ (Figure 2.11C). It was previously reported that cytokine production following LCMV infection is strongly dependent on the MyD88 pathway (37, 38). Similarly, we observed a significant reduction of IFN-γ and TNF-α production following infection of MyD88KO compared to WT NOD mice (Figure 2.11A,B). TLR3KO mice produced comparable levels of IFN-γ following LCMV challenge (Figure 2.11C) confirming that this receptor does not play a central role in the response to LCMV.
Serum levels of chemokines were investigated at day 4 PI with CB4 and LCMV. Following infection significant increases of CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP1-β), CCL5 (RANTES) and CXCL9 (MIG) were observed in WT NOD mice compared to uninfected controls (Figure 2.12). Following CB4 challenge, TLR3KO mice produced similar levels of CCL2 (Figure 2.12A) and CXCL9 (Figure 2.12E) while levels of CCL3 (Figure 2.12B) and CCL4 (Figure 2.12C) were slightly reduced but were not significantly different from either WT NOD controls or MyD88KO mice suggesting that these chemokines are not critical for survival following infection (Figure 2.12). Importantly, levels of CCL5 were significantly reduced in TLR3KO mice compared to both WT NOD and MyD88KO mice (Figure 2.12D) suggesting that production of this chemokine following CB4 challenge is strongly dependent on TLR3 and that production of this chemokine is central to the capacity of the host to control CB4 viral replication. No significant differences in chemokine production were observed between WT NOD and MyD88KO mice following infection further suggesting that the early immune response to CB4 is MyD88 independent (Figure 2.12). As we observed in the case of the cytokine response, the production of chemokines following LCMV infection was primarily dependent on the MyD88 pathways with MyD88KO mice producing significantly reduced levels of CCL2 and CCL5 compared to WT NOD mice. Taken together, these data demonstrate that the production of some inflammatory mediators following CB4 infection is independent of the MyD88 signaling pathway and strongly dependent on TLR3 signaling. Further, the production of TNF-α and CCL5 appear to be critical for the control of viral replication and the survival of the host following CB4 challenge.

2.2.5 TLR3 signaling on macrophages plays an important role in the immune response to CB4

Several cell types including macrophages and DCs are responsible for production of cytokines and chemokines following TLR ligation. In order to determine whether presence of TLR3 on macrophages or DCs was sufficient to decrease the susceptibility of a TLR3 deficient host, adoptive transfer experiments were performed. Splenocytes from WT NOD or TLR3KO mice were purified by flow cytometry in order to obtain two separate populations.
based on CD11c and CD11b expression. TLR3KO mice were adoptively transferred with a population containing either predominantly macrophages (CD11b+CD11c-) or a population containing DCs (CD11c+) and challenged with CB4 at 24 hours post-transfer. We observed that while adoptive transfer of WT DCs did not confer a survival advantage (data not shown), transfer of WT macrophages resulted in greater survival with the median survival time extended to 20 days compared to 9 days for mock treated and nearly 25% more mice surviving to 21 days PI (Figure 2.13A). Further, adoptive transfer of WT macrophages conferred a significant survival advantage compared to mice adoptively transferred with macrophages purified from TLR3KO donors with the median survival time extended to 20 days compared to 7.5 days and greater than 35% more mice surviving to day 21 (Figure 2.13A) confirming that simply increasing the number of macrophages available to respond to virus is not sufficient to confer protection. Instead, TLR3 signaling on macrophages is required to establish protection and ensure survival following CB4 infection. In addition, this suggests that TLR3 signaling on DCs is not sufficient to confer survival following CB4 infection. Importantly, after transfer of WT macrophages, we observed a significant reduction in serum levels of cTn1 (Figure 2.13B) as well as the number and severity of cardiac lesions in infected mice (Figure 2.13C) compared to infected mice adoptively transferred with macrophages from TLR3KO mice. This demonstrates that TLR3 signaling on macrophages is critical to prevent viral-mediated cardiac pathology. Furthermore, we observed that protection following adoptive transfer of WT macrophages did not correlate with increased serum levels of TNFα or CCL5 (data not shown). These results suggest that macrophages contribute to protection independent of significant increases of these mediators in the periphery and, as expected, macrophages contribute to the antiviral response in a number of ways both globally and locally within the infected tissue.

2.3 Discussion

In this study, we demonstrate that TLR3 signaling on macrophages is required for survival following CB4 infection. Mice lacking TLR3 succumb rapidly to viral infection due to uncontrolled viral replication leading to a rapid increase in cardiac damage. Conversely, we observed that MyD88 deficiency did not result in increased mortality suggesting that
signaling from other TLRs is not necessary during the early stages of CB4 infection. Our data further highlight the important role of pro-inflammatory mediators, specifically TNF-α and CCL5, in controlling viral replication in the host.

In light of the redundancy of innate receptors capable of recognizing virus-associated patterns, it is intriguing that TLR3 plays such a critical role in the early response to CB4 infection. Despite the fact that coxsackieviruses have previously been described to signal through other TLRs (3, 5), our results suggest that the other TLRs as well as the other innate viral sensors such as RLHs are unable to compensate for the loss of TLR3. Additionally, our results suggest that not only are other innate viral sensors unable to compensate for the loss of TLR3 they are in fact dispensable for the early response to CB4. This is supported by a report describing that deficiency of MyD88 provided a survival advantage compared to WT mice following challenge with high infectious doses of coxsackievirus B3 (39). These intriguing observations can be explained by several non-exclusive mechanisms. One likely explanation is that CB4 is recognized by TLRs other than TLR3 but the functional consequences of this interaction are not critical for survival. To this effect, we observed that IL-6 production was significantly reduced following CB4 infection in MyD88KO but not TLR3KO mice, suggesting that CB4 must signal through at least one MyD88-dependent pathway and that the resulting production of IL-6 is not critical for the early control of viral replication. Subcellular localization may explain why the RLHs, in particular MDA-5 which has previously been demonstrated to play an important role in the recognition of other viruses including murine norovirus and the picornavirus, EMCV (19, 40), are unable to compensate for the lack of TLR3. As opposed to TLR3 which is usually localized within endosomal compartments (21), MDA-5 is localized in the cytoplasm (41). CB4 enters the cell by receptor-mediated endocytosis and therefore would be localized within the endosome rapidly after infection. However, as CB4 is a single stranded virus it seems unlikely that dsRNA intermediates are generated prior to the start of viral replication in the cytoplasm. Alternatively, other picornaviruses, including poliovirus and hepatitis A virus, have been demonstrated to prevent MDA-5 signaling by cleaving either MDA-5 itself or the signaling adaptor molecule mitochondrial antiviral signaling adaptor (MAVS) (42, 43). Based on these reports, we are currently investigating the interaction between RLHs and CB4 and speculate
that this virus may be able to abrogate signaling from RLHs potentially explaining the critical role of TLR3 over the course of infection.

Here, we report that TLR3 deficiency does not affect the activation of several cellular effectors typically involved in the response to viruses including NK cells and APCs. Instead, the absence of TLR3 signaling resulted in significantly reduced levels of the cytokine TNF-α and the chemokine CCL5. This suggests that early production of these inflammatory mediators plays a critical role in controlling viral replication and that, in their absence, even the activation of innate effectors and the adaptive response are insufficient to reestablish control of the viral infection. To this effect, we observed that although MyD88 mice present with reduced immune cell activation following CB4 infection, they produce similar levels of TNF-α and CCL5 compared to WT mice. The survival of these mice following CB4 infection further highlights the importance of these pro-inflammatory mediators in the control viral replication at the earliest stages of infection. Similarly, it was previously reported that SCID mice, that lack T and B cells, are able to survive the early stages of infection with CB4 suggesting that these cell types are not critical for survival (34).

TNF-α is a pro-inflammatory cytokine produced by several cell types, including macrophages, that has been ascribed antiviral properties both in vitro and in vivo (reviewed in (44)). Wada and colleagues have reported that mice lacking TNF-α succumb rapidly to EMCV infection due to increased viral replication in the heart (45), an observation that was supported following EMCV infection of TLR3 deficient mice where reduced TNF-α production was observed and correlated with increased viral replication, cardiac damage and increased mortality (17). The antiviral effects of TNF-α may be, in part, mediated by its capacity to induce the production of nitric oxide from macrophages as this molecule has previously been demonstrated to play an important role in the control of coxsackieviral replication within the heart (46). Taken together with the data presented in this report, this suggests that the TLR3-dependent production of TNF-α plays a particularly important role in cardiac protection following infection with cardiotropic viruses.

CCL5 is a pro-inflammatory chemokine, with chemotactic properties towards both T cells and monocytes/macrophages, that is commonly associated with anti-viral responses. Blocking CCL5 activity has previously been demonstrated to result in increased viral antigen
in the central nervous system (CNS) of mice following TMEV challenge (47). Interestingly, normal T cell infiltration was also observed in these mice suggesting that T cells were still able to migrate to the CNS and that the antiviral activity of CCL5 is not simply due to recruitment of T cells (47). Based on the early mortality observed in our model, it seems unlikely that improper recruitment of T cells is responsible for this increased susceptibility to CB4 infection. Rather, we hypothesize that CCL5 deficiency results in a decrease recruitment of macrophages that may be important for the production of TNF-α and, potentially, nitric oxide. To this effect, we observed that the adoptive transfer of a population composed mostly of macrophages but not DCs from WT mice, was sufficient to reduce mortality induced by CB4 in TLR3KO mice by preventing viral-mediated cardiac pathology. These data suggest that TLR3 signaling on macrophages results in the production of TNF-α and CCL5 both of which act to control early viral replication which, in turn, likely allows for the adaptive immune response to activate and clear the viral infection.

Contrary to what has been reported for several other viruses including another picornavirus (22, 23), TLR3 signaling plays a role in the protection of the host from cardiotropic viruses such as coxsackievirus and EMCV(17). Susceptibility to viral-induced myocarditis is dependent on several genetic traits (48). Our observations suggest that genetic differences leading to changes in TLR3 function or expression could be linked to susceptibility to viral-induced myocarditis. To this effect, it was recently reported that differences in susceptibility to chronic viral-induced myocarditis between a C57Bl/6 and A.BY/SnJ mice was linked with reduced production of TNF-α and CCL5 following coxsackievirus B3 infection in a potentially TLR3-dependent manner (49). Similarly, patients with a TLR3 deficiency are highly susceptible to Herpes Simplex encephalitis (20). Taken together, these observations suggest that therapies aimed at reestablishing TLR3 signaling or its functional consequences may provide an effective means to protect susceptible patients from suffering the fatal consequences of viral myocarditis.

In summary, we provide evidence for an essential non-redundant role for TLR3 signaling on macrophages in the early stages of coxsackievirus B4 infection. Our results demonstrate that the production of important pro-inflammatory mediators such as TNF-α and CCL5 are dependent on the TLR3 signaling pathway and independent of the MyD88 pathway. Further, we demonstrate that mortality occurs despite the normal activation of the
adaptive immune response. This suggests that in the absence of macrophages recognizing the invading pathogen in a TLR3 dependent manner, viral replication cannot be controlled and is allowed to outpace the developing adaptive immune response. The differences in requirement for TLR3 signaling between RNA viruses (eg. LCMV and CB4) suggest that the wide variety of viral-specific pattern recognition receptors does not represent simple redundancy. Rather this system has been evolved to provide a first-line of defense tailored to the requirements of the host to protect itself against a specific virus. As such, gaining understanding of this complex interplay of viruses with the innate immune system will likely provide insight for the design of more effective antiviral therapeutics.

2.4 Materials and methods

Mice. NOD/ShiLtJ and TLR3KO mice were obtained from The Jackson Laboratory (Bar Harbor, USA). TLR3KO mice were backcrossed and maintained on the NOD mouse background. MyD88KO mice on the NOD background were a generous gift from Dr. A. Chervonsky (University of Chicago). All mice were bred and maintained in our rodent facility and monitored for blood glucose prior to infection to ensure that only pre-diabetic mice were analyzed. All performed procedures followed the guidelines of the institutional animal care committee.

Virus. Stocks of CB4 Edwards strain 2 was originally obtained from Dr. C. Gauntt (University of Texas-San Antonio) and prepared as described previously (50, 51). LCMV Armstrong strain 53b was originally obtained from Dr. M.B. Oldstone and propagated as described previously (52). 6-8 week old mice were infected intraperitoneally with sublethal doses (less than half LD$_{50}$) of 400 PFU of CB4 or 1X10$^5$ PFU of LCMV. Viral titers were measured following standard plaque assay procedures.

Flow cytometry. Single cell suspensions were generated from the spleen at the indicated time points, stained for the appropriate markers and analyzed by flow cytometry. Fluorescently conjugated antibodies directed against CD11b (clone M1/70), CD11c (clone HL3), CD4 (clone L3T4), CD8 (clone 53-6.7), CD19 (clone eBio 1D3), panNK (clone DX5) and CD69 (clone H1.2F3) were purchased from eBiosciences (San Diego, USA) while biotin
conjugated antibodies directed against CD44 (clone IM7), CD62L (clone Mel-14), CD69 (clone H1.2F3), CD80 (clone 16-10A1) and, CD86 (clone GL1) were purchased from BD Biosciences (Missisauga, Canada).

**Immunohistochemical staining.** Tissue sections were prepared as previously described (51) at day 7 post-infection. Staining was performed using standard procedures for hematoxylin and eosin (Wax-It, Vancouver, British Columbia). Tissue sections were graded for pathology.

**Quantitative measurement of liver damage.** Liver damage was quantified at days 3 and 7 PI by measuring serum levels of alanine transaminase (ALT) using commercially available Infinity™ ALT reagents (Thermo Scientific, Waltham, MA) according to manufacturer’s instructions.

**Quantitative measurement of heart damage.** Heart damage was quantified by measuring release of cardiac troponin 1 into the serum of mock-infected or infected mice at days 3 and 7 PI using a commercially available ELISA kit (Life Diagnostics, West Chester, PA) according to manufacturer’s instruction.

**Cytokine and chemokine analysis.** Serum cytokine and chemokine levels were measured at day 4 PI using a mouse inflammation CBA kit (BD Bioscience) allowing for simultaneous detection IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70 from a single sample or a chemokine flex set (BD Bioscience) customized to allow for simultaneous detection of CXCL9 (MIG), CCL3 (MIP1α), CCL4 (MIP1β) and CCL5 (RANTES) from a single sample. Samples were prepared according to manufacturer's instructions and analyzed on a BD-FacsArray equipped with FCAP software (BDBiosciences). Serum levels of IFNα were measured at 48 hours PI using a VeriKine™ Mouse interferon alpha kit (PBL interferon source, Piscataway, NJ) according to manufacturer’s instructions.

**Adoptive transfer experiments.** Splenic single cell suspension from uninfected WT NOD or TLR3KO mice were stained with fluorescently conjugated antibodies specific for CD11b and CD11c. Cells were sorted by FACS into two separate populations composed mainly of macrophages (CD11b+CD11c-) or mainly dendritic cells (CD11c+ with varying expression of CD11b). TLR3KO mice were adoptively transferred with 2X10^5 and a number of DCs corresponding to the natural ratio of these cells compared to the CD11b+CD11c- group.
(between 70,000 to 150,000) intraperitoneally and challenged with CB4 24 hours post-transfer.

**Statistical Analysis.** Kaplan-Meier analysis was used to compare survival curves and unpaired Student’s t-test was used for all other analyses. A P value of < 0.05 was considered significant. Stars denote statistical significance.
Figure 2.1: Deficiency of TLR3 results in increased mortality and increased viral replication following CB4 challenge

A) WT NOD (solid grey line), TLR3KO (solid black line) and MyD88KO mice (dashed black line) were challenged with 400 pfu of CB4 and mortality was monitored. TLR3KO mice were also challenged with 1X10^5 pfu of LCMV (dashed grey line) as an additional control. Significantly decreased survival was observed for TLR3KO mice challenged with CB4 compared to all other mouse strains and treatment tested. B) Mice were sacrificed at day 7 PI and viral load from the pancreas, liver and heart of WT NOD (black bars, n=8), TLR3KO (white bars, n=5) or MyD88KO (grey bars, n=6) were measured by standard plaque assay. The viral titers are presented as log10 pfu/g of tissue and represent the average values of duplicates from at least 4 mice in each group. Stars denote statistical significance.
Figure 2.2

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Figure 2.2: TLR3 deficiency results in increased cardiac damage during acute CB4 infection

A) Representative tissue section of pancreas (left column), liver (center column) or heart (right column) stained for H&E from mock-infected (top row) or CB4-infected WT NOD (second row), TLR3KO (third row) or MyD88KO (bottom row) mice at day 7 PI. B) Serum ALT levels of WT NOD (black bars), TLR3KO (white bars) or MyD88KO (grey bars) mice either mock-infected with DMEM or at day 3 and 7 PI with CB4. Pooled data from at least 2 independent experiments are presented as mean +/- s.e.m. C) Serum cardiac troponin 1 levels of WT NOD (black bars), TLR3KO (white bars) or MyD88KO (grey bars) mice either mock-infected with DMEM or at day 3 and 7 PI with CB4. Pooled data from at least 2 independent experiments are presented as mean +/- s.e.m. Stars denote statistical significance. Magnification:400X.
Figure 2.3
Figure 2.3: TLR3 deficiency does not affect the capacity of innate effectors to activate following CB4 infection

Representative histograms of CD69 expression on (A) NK (PanNK+, TCRβ-) or (B) NK T cells (PanNK+, TCRβ+) from WT NOD (left panels) and TLR3KO (right panels) mice at 48 hours post-infection with 400 pfu of CB4 (solid black lines) or mock-infection with DMEM (shaded histogram). Representative histograms of CD80 expression on the surface of (C) macrophages (CD11b+CD11c-) and (D) dendritic cells (CD11c+) from WT NOD (left panels) and TLR3KO (right panels) mice at 4 days post-infection with 400 pfu of CB4 (solid black lines) or mock-infection.
Figure 2.4: MyD88 deficiency decreases the capacity of cellular effectors to activate in response to CB4 infection

Representative histograms of expression of activation or costimulatory marker (as indicated) on the surface of A) NK cells (Pan-NK+, TCRβ-), B) NK T cells (Pan-NK+, TCRβ+), C) macrophages (CD11b+CD11c-), D) dendritic cells (CD11c+), E) B cells (CD19+), F) CD4+ T cells and G) CD8+ T cells from MyD88KO mice at day 4 post-infection with 400 pfu of CB4 (solid black lines) or mock-infection with DMEM (shaded histogram). Data are representative of at least 2 separate experiments.
Figure 2.5
Figure 2.5: TLR3 deficiency does not affect the capacity of APCs to mature following LCMV infection

Representative histograms of CD80 and CD86 expression on the surface of (A) macrophages (CD11b+CD11c-) and (B) dendritic cells (CD11c+) from WT NOD (left panels) and TLR3KO (right panels) mice at 4 days post-infection with 1X10^5 pfu of LCMV (solid black lines) or mock-infection with DMEM (shaded histogram). Data are representative of at least 2 separate experiments.
Figure 2.6: TLR3 deficiency does not affect the capacity of B cells to activate following CB4 infection

Representative histograms of CD69 and CD86 expression on the surface of B cells (CD19+) from WT NOD (left panels) and TLR3KO (right panels) mice at 4 days post-infection with 400 pfu of CB4 (solid black lines) or mock-infection with DMEM (shaded histogram). Data are representative of at least 2 separate experiments.
Figure 2.7

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Figure 2.7: TLR3 deficiency does not affect the capacity of T cells to activate following CB4 infection

Representative histograms of CD62L and CD69 expression on the surface of (A) CD4 and (B) CD8 T cells from WT NOD (left panels) and TLR3KO (right panels) mice at 4 days post-infection with 400 pfu of CB4 (solid black lines) or mock-infection with DMEM (shaded histogram). Data are representative of at least 2 separate experiments.
Figure 2.8

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Figure 2.8: TLR3 deficiency does not affect the capacity of T cells to activate following LCMV infection

Representative histograms of CD62L and CD69 expression on the surface of (A) CD4 and (B) CD8 T cells from WT NOD (left panels) and TLR3KO (right panels) mice at 4 days post-infection with 1X10^5 pfu of LCMV (solid black lines) or mock-infection with DMEM (shaded histogram). Data are representative of at least 2 separate experiments.
Figure 2.9
Figure 2.9: TLR3 deficiency does not affect T cell activation following LCMV or CB4 infection

Representative histograms of CD44 expression on the surface of (A, C) CD4 and (B, D) CD8 T cells from WT NOD (left panels) and TLR3KO (right panels) mice at 7 days post-infection with (A,B) 400 pfu of CB4 (solid black lines) or (C,D) 1X10⁵ pfu of LCMV (solid black lines) or mock-infection with DMEM (shaded histogram). Data are representative of at least 2 separate experiments.
Figure 2.10: Type 1 interferon production is reduced following CB4 infection in both TLR3KO and MyD88KO mice

Serum levels of IFNα from WT NOD (black bars), TLR3KO (white bars) and MyD88KO (grey bars) mice were measured with a VeriKine Elisa Kit at 48 hours following infection with 400 pfu of N= at least 7 for each group. Pooled data from at least 2 independent experiments are presented as mean +/- s.e.m.
Figure 2.11: TLR3 deficiency results in reduced production of TNF-α following CB4 infection

Serum levels of A) TNF-α, B) IL-6 and C) IFN-γ from WT NOD (black bars), TLR3KO (white bars) and MyD88KO (grey bars) mice were measured with a CBA inflammation kit 4 days following infection with 400pfu of CB4, 1X10^5 pfu of LCMV or mock-infection with DMEM. N= at least 3 for DMEM and LCMV treatments and n= at least 5 mice for CB4 treatments. Pooled data from at least 2 independent experiments are presented as mean +/- s.e.m. Stars denote statistical significance.
**Figure 2.12**

Figure 2.12: TLR3 deficiency results in reduced production of CCL5 following CB4 infection

Serum levels of A) CCL2, B) CCL3, C) CCL4, D) CCL5 and E) CXCL9 from WT NOD (black bars), TLR3KO (white bars) and MyD88KO (grey bars) mice were measured with a CBA chemokine flex set 4 days following infection with 400 pfu of CB4, 1X10^5 pfu of LCMV or mock-infection with DMEM. n= at least 3 for DMEM and LCMV treatments and n= at least 5 mice for CB4 treatments. Pooled data from at least 2 independent experiments are presented as mean +/- s.e.m. Stars denote statistical significance.
Figure 2.13: Adoptive transfer of WT macrophages rescues TLR3KO mice following CB4 challenge

A) TLR3KO mice were adoptively transferred with macrophages from WT NOD mice (dashed black line) or macrophages from TLR3KO mice (solid grey line) or mock-treated with DMEM (solid black line) and infected with 400 pfu of CB4 24 hours post-transfer. B) Serum cardiac troponin 1 levels of TLR3KO mice adoptively transferred with either WT NOD macrophages (black bars, n=5) or TLR3KO macrophages (white bars, n=3) at day 7 PI with CB4. Pooled data from at least 2 independent experiments are presented as mean +/- s.e.m. Stars denote statistical significance. C) Representative tissue section of day 7 PI hearts stained for H&E from CB4-infected TL3KO mice adoptively transferred with WT NOD macrophages (left panel) or TLR3KO macrophages (right panel). Magnification:400X.
2.5 References


47. Ure DR, Lane TE, Liu MT, Rodriguez M: Neutralization of chemokines RANTES and MIG increases virus antigen expression and spinal cord pathology during Theiler's virus infection. *Int Immunol* 17:569-579, 2005


Toll-like receptor 4-induced cytokine production circumvents protection conferred by TGF-β in coxsackievirus mediated autoimmune myocarditis³

3.1 Introduction

Cardiovascular disease is a leading cause of death in North America and is predicted to become one of the major causes of death worldwide by the end of the next decade (1). Autoimmune myocarditis, an inflammation of the heart responsible for a large proportion of sudden deaths in young adults, often progresses to dilated cardiomyopathy (DCM) leading to congestive heart failure (2). Coxsackie B virus (CBV) infections are estimated to be responsible for nearly a third of all new cases of DCM in the United States annually (3). A variety of viruses have been shown to cause acute myocarditis but only a few, including coxsackievirus B3 (CB3), are associated with the induction of chronic autoimmune myocarditis (3).

Several mouse models have been established to study autoimmune heart disease induction following CBV infection. In these models, myocarditis is followed by the development of DCM and closely resembles the profile of inflammatory heart disease observed in humans (4). CBV-induced autoimmune myocarditis follows a biphasic progression characterized by an initial acute viral-mediated phase followed by a chronic autoimmune phase. The virus first infects the pancreas but eventually replicates in the heart, initiating the acute response that typically resolves within 7 to 14 days corresponding to clearance of the virus from the host (5). Although viral replication causes some myocyte destruction, little to no long-term damage remains and normal cardiac function is maintained. In genetically susceptible strains such as A/J or nonobese diabetic (NOD) mice, disease progresses to a chronic phase that is characterized by immune cell infiltration and the development of inflammatory lesions (5). Adoptive transfer experiments have clearly demonstrated that damage incurred during the chronic phase is autoimmune in nature and cannot be attributed solely to direct viral damage (6, 7). Autoreactive antibodies directed to heart antigens such as cardiac myosin are commonly detected during this phase of the disease. Eventually, both the infiltration and lesions resolve, leaving large areas of fibrosis and scar tissue. The formation of scar tissue and the destruction of myocytes ultimately lead to loss of contractile function and ventricular enlargement.

We have previously demonstrated that local expression of transforming growth factor (TGF)-β as a transgene exclusively in the pancreas of genetically susceptible NOD mice was
sufficient to protect from CB3-induced autoimmune myocarditis (8). Although these mice (NODTGFβ) develop acute lesions and autoreactive antibodies, the lesions resolve with clearance of the virus and never progress to a chronic stage. In susceptible models, the progression to chronic autoimmune heart disease is generally associated with an isotype switch of the autoantibodies from IgM to IgG1, antibodies that are associated with increased disease pathogenesis (9, 10). Interestingly, in NODTGFβ mice, a heightened isotype switch towards IgG3 is observed after infection and IgG3 has been associated with reduced autoreactivity and pathogenicity (11). CB3 viral titers in the pancreas and heart of NODTGFβ mice were significantly lower, possibly explaining the protection observed. However, sufficient level of viral replication and damage still occur in the heart to induce autoreactive antibodies and pathology. Furthermore, NODTGFβ mice were susceptible to experimental autoimmune myocarditis (EAM) (8). This suggests that TGF-β mediated protection requires early inflammation in the pancreas to suppress disease.

It has previously been established that genetic resistance to CB3-induced autoimmune myocarditis can be overcome when mice are treated with lipopolysaccharide (LPS) from Salmonella minnesota in the context of viral infection (12). This effect is likely due to increased cytokine secretion since the major cytokines induced by LPS treatment, interleukin (IL)-1 and Tumor Necrosis Factor (TNF), were sufficient to overcome resistance (13). LPS is usually recognized by the pattern recognition receptor Toll-like receptor 4 (TLR4), which induces signaling cascades ultimately resulting in cytokine secretion and maturation of APCs (14). Eriksson and colleagues have demonstrated that APCs loaded with self-antigen are sufficient to induce autoimmune heart disease upon activation with LPS (Escherichia coli 0111:B4) coupled with CD40 ligation (15). This indicated the maturation state of APCs is a crucial element in the induction of autoimmunity. Furthermore, LPS (Salmonella typhimurium) has been associated with relapses of autoimmunity in models of EAE (16) and induction of Theiler’s virus-mediated demyelinating disease (17). Other bacterial components have also been shown to both induce and exacerbate autoimmune diseases (18).

In this report, we determined that APCs from NODTGFβ mice fail to upregulate the costimulatory molecule CD40 in response to infection, suggesting a link with the observed protection in this model. We further determined that signaling events leading to the production of pro-inflammatory cytokines following stimulation of TLR4 but not TLR2 are
sufficient to circumvent the protection conferred by pancreatic expression of TGF-β from coxsackievirus mediated autoimmune myocarditis.

3.2 Results

3.2.1 LPS treatment circumvents protection from CB3-mediated autoimmune myocarditis in NODTGFβ mice

Since a TLR4 agonist, LPS, has been previously demonstrated to induce disease in otherwise genetically resistant models when combined with viral infection (12), we asked whether LPS would also be sufficient to break the protection conferred by TGF-β expression. NODTGFβ mice and nontransgenic NOD littermates were infected with CB3 alone or in combination with either LPS from S. minnesota (TLR4 ligand, hereby referred to as LPS4) or LPS from P. gingivalis (TLR2 ligand, hereby referred to as LPS2). Following treatment, mice were fully characterized for myocarditis including heart pathology and autoantibody production.

As described previously, nontransgenic NOD mice developed chronic autoimmune myocarditis; immune cells infiltrated cardiac tissue as early as 7 days post infection (PI) (19) and these mice progressively developed cardiac lesions with large areas of fibrosis 28 days PI (Figure 3.1, Table 3.1). NODTGFβ mice are entirely protected from CB3-induced chronic autoimmune disease with the presentation of acute damage, but no development of long-term lesions (Figure 3.1, Table 3.1 and (8)). NODTGFβ mice treated with LPS4 at the time of CB3 infection, developed chronic myocarditis with a similar progression as NOD mice infected with CB3. Immune infiltration was observed as early as 7 days post-infection (data not shown) and large regions of fibrosis were observed at 28 days PI (Figure 3.1, Table 3.1). Interestingly, NODTGFβ mice treated with CB3/LPS2 remained protected. As described, for infection with CB3 alone, these mice developed only acute inflammation and did not progress to chronic myocarditis (Figure 3.1, Table 3.1). This result indicates that events specific to TLR4 signaling and not triggered by TLR2 signaling are necessary to overcome the protection conferred by TGF-β expression.
Since autoantibodies to cardiac myosin and heart extract are an important marker of disease, their production was measured following treatment of NODTGFβ mice and their nontransgenic NOD littermates. As described previously, following CB3 infection, NODTGFβ mice generate and produce reduced amounts of autoantibodies as compared to CB3 infected NOD mice (8). NODTGFβ mice co-treated with CB3 and LPS4 generated increased levels autoantibodies specific to cardiac myosin and whole heart extract as early as 14 days in quantities comparable to that observed in NOD mice also co-treated with LPS4 and CB3 (Figure 3.2). As expected, co-treatment with LPS2 and CB3 did not increase the quantity of autoantibodies produced (Figure 3.2). As described previously, a higher proportion of cardiac myosin specific IgG is IgG3 in NODTGFβ mice post-infection as compared to infected NOD mice (Figure 3.2B and (8)). Co-administration of LPS2 or LPS4 at the time of CB3 infection did not significantly alter the proportion of cardiac myosin IgG3 generated in NODTGFβ mice (Figure 3.2B). This suggests that LPS4 does not affect the relative proportion of IgG isotypes characteristic of NODTGFβ mice.

3.2.2 Increase in viral replication is not responsible for breaking protection

Since pancreatic expression of TGF-β significantly reduces CB3 replication in both the pancreas and the heart (8) we asked whether LPS circumvents protection simply by increasing viral replication, thereby increasing direct cardiac damage in the mouse. Viral plaque assays were used to determine the viral load at 3, 7 and 14 days following infection. Similar to our previous results, there was approximately two logs less virus detected in the pancreas and one log less virus detected in the heart of NODTGFβ mice infected as compared to infected NOD mice at the peak of CB3 infection (Figure 3.3). Importantly, we found that, following co-treatment with LPS4, there are no significant differences in viral replication in the pancreas, heart or spleen of NODTGFβ mice as compared to NODTGFβ mice infected with CB3 alone (Figure 3.3). We therefore conclude that LPS4 does not abrogate the protection conferred by TGF-β simply by increasing viral replication.
3.2.3 LPS treatment changes the cytokine response of NODTGFβ mice

As TLR4 stimulation, but not TLR2, is able to circumvent protection, we sought to determine the mechanism behind this difference. Upon recognition of their cognate ligand, TLRs induce downstream signaling cascades that eventually lead to the production of several pro-inflammatory cytokines. Accordingly, we determined the inflammatory cytokine levels in the serum of mice following infection with CB3 alone or CB3 combined with either LPS2 or LPS4.

Significant changes in the cytokine profile were observed following CB3/LPS4 treatment of NODTGFβ mice when compared to mice treated with CB3 alone or CB3/LPS2 (Figure 3.4). NODTGFβ mice infected with CB3 and LPS4 produced significantly increased amounts of IL-6, macrophage chemotactic protein (MCP)-1 and TNF-α at 24 hours PI as compared to CB3 and CB3/LPS2 treated mice (Figure 3.4). Interestingly, the level of cytokine production observed in nontransgenic NOD mice was lower than what was observed for NODTGFβ mice (data not shown). This indicates that the presence of TGF-β potentiates the response to various TLR agonists particularly at early time points following infection. These results suggest that the increased production of TLR4 signaled pro-inflammatory cytokines plays an important role in circumventing TGF-β conferred protection.

As the cytokine response of NODTGFβ mice was affected in vivo, we asked whether the continual presence of TGF-β affects the capacity of APCs to produce cytokines in response to TLR stimulation. APCs from NODTGFβ mice and their nontransgenic littermates were sorted by flow cytometry and isolated based on the expression of CD11b and CD11c surface markers. We purified two distinct populations, one containing mainly macrophages (CD11b+CD11c-) and another containing several subsets on dendritic cells (DCs) (all CD11c+ with certain subsets also positive for CD11b). Cells were stimulated in vitro either with CB3 alone or with CB3 and LPS2 or LPS4 for 24 hours. Secretion of IL-6 into the culture media was measured by ELISA. We detected only minimal levels of IL-6 in response to CB3 alone from both cell types and from both mouse strains. We observed that macrophages and dendritic cells could both be induced to produce IL-6 in the presence of LPS. More importantly, we observed that macrophages produced higher levels of IL-6 than
DCs (Figure 3.5). These data suggest that macrophages are the primary producers of IL-6 \textit{in vivo}. Furthermore, we observed that macrophages and DCs from NODTGFβ mice responded equally to \textit{in vitro} stimulation from either LPS2 or LPS4. (Figure 3.5) This was particularly surprising in light of the significant difference observed \textit{in vivo} following the different LPS treatments of NODTGFβ mice. Therefore, while the presence of TGF-β is refractory to TLR2 signaling \textit{in vivo}, this is not observed \textit{in vitro} where TGF-β is not available. Therefore, the TGF-β mediated regulation is transient in nature.

### 3.2.4 Pancreatic expression of TGF-β affects the activation of APCs in response to infection

TGF-β directly affects APCs and has previously been shown to affect antigen presentation specifically by downregulating MHC class II (20). We asked whether the protection conferred by pancreatic expression of TGF-β could be explained by changes in the activation state of APCs following infection. APC populations were analyzed by flow cytometry for surface expression of costimulatory molecules and MHC class II on day 3 PI. We observed that macrophages (CD11b+CD11c-) from NODTGFβ mice failed to upregulate the CD40 following infection with CB3 in contrast to NOD macrophages where CD40 upregulation is clearly observed following infection (Figure 3.6A). Interestingly, NODTGFβ macrophages still upregulate other costimulatory molecules such as CD80 (Figure 3.6A) and CD86 (data not shown) indicating that TGF-β expression specifically alters CD40 expression. This altered expression was not observed on DCs and suggests that DCs from NODTGFβ mice respond to infection by upregulating MHC class II and the costimulatory molecules (Figure 3.6B).

Surprisingly, MHC class II expression was observed to be upregulated to a greater degree on macrophages of NODTGFβ mice compared to nontransgenic NOD mice following CB3 infection (Figure 3.6A). These results indicate that NODTGFβ macrophages retain the ability to present antigen following infection but that they may do so without the upregulation of CD40 potentially inducing tolerance rather than activation of autoimmune T cells. Infection of NODTGFβ mice with CB3 and LPS4 did not significantly alter expression pattern of CD40 on macrophages or dendritic cells suggesting that protection is circumvented.
in a CD40-independent manner (Figure 3.7). Take together these results indicate that the increased production of pro-inflammatory cytokines observed following LPS4 co-treatment is sufficient to overcome protection despite the observed CD40 defect. Additionally, it has been previously demonstrated that IL-6 is a downstream effector of CD40 responsible for abrogating the tolerogenic potential of APCs (21) suggesting a similar mechanism for our model.

3.3 Discussion

LPS treatment combined with CB3 infection is sufficient to overcome protection from autoimmune myocarditis induced by the local expression of TGF-β specifically in the pancreas of NOD mice. Interestingly, all adjuvants are not capable of overcoming protection, as LPS from P. gingivalis a TLR2 agonist was unable to induce disease in combination with CB3. This result is particularly interesting in the context of observations indicating that LPS2 is sufficient to break genetic resistance in C57BL/6 mice infected when co-administered with CB3 (M. Poffenberger and M. Horwitz, unpublished observation). This suggests that the mechanisms involved in circumventing TGF-β mediated protection are distinct from those involved in breaking genetic resistance. This study highlights that the stimulation provided to APCs influences their activation and maturation and determines whether a pathogen response is allowed to progress to autoimmunity.

Here we demonstrate that expression of TGF-β affects the cytokine response of APCs upon stimulation. Specifically, NODTGFβ mice responded vigorously to CB3/LPS4 treatment producing greater levels of TNF-α, MCP-1 and IL-6, than following infection with CB3 alone. Any of these three cytokines may play an important role in overcoming protection and/or in the observed pathology. TNF-α is a downstream effector of LPS and TLR4 that can functionally replace LPS in the induction of chronic myocarditis in genetically resistant mice (13). MCP-1 has recently been demonstrated to play an important role in the pathology of EAM. Kaya and colleagues demonstrated that blocking MCP-1 with monoclonal antibodies or a dominant negative inhibitor of MCP-1 delivered by gene therapy were highly effective at reducing EAM severity (22). IL-6, which demonstrated a significant increase in our study is likely crucial to overcoming the protection conferred by TGF-β. IL-6
is a pluripotent cytokine produced by several cell types and it was recently demonstrated to be an important regulator of suppression by Tregs (23). It was further described that IL-6 production induced by TLR signaling allowed proper immune activation by relieving effector T cells from the suppression by Tregs (23). IL-6 may be directly involved in the pathology of autoimmune heart disease since IL-6 deficient mice on an otherwise susceptible genetic background were protected from EAM (24). Interestingly, although only modest amounts of IL-6 are produced in the susceptible NOD mice following CB3 infection, disease still develops. This strongly suggests that the increased level of IL-6 is likely required to circumvent TGF-β protection and may play less of a direct role in inflammation and pathology in susceptible mice.

It is interesting to note that several groups have ascribed a role for TGF-β in the generation of Tregs. In particular, *in vitro* experiments demonstrated that T cell stimulation in the presence of TGF-β could convert CD4+CD25-FoxP3- effector T cells into CD4+CD25+FoxP3+ Tregs (25). It was also shown that a short pulse of TGF-β protects NOD mice from spontaneous diabetes by increasing the Treg population (26). An increase in Tregs is certainly a potential explanation for the TGF-β-mediated protection and will be a subject of future investigations.

We further demonstrated that the pancreatic expression of TGF-β rendered APCs more sensitive to LPS4 stimulation *in vivo* leading to increased production of pro-inflammatory cytokines. Although TGF-β is generally considered to play an anti-inflammatory role, it has also been shown to potentiate the production of several pro-inflammatory mediators, including IL-6, from monocytes (27-29). Intriguingly, the presence of TGF-β seems to specifically abrogate inflammatory cytokine production in response to *in vivo* TLR2 stimulation. Our results indicate that this inhibition is transient since APCs from NODTGFβ mice respond strongly to *in vitro* stimulation with LPS2. It was recently observed that TGF-β inhibits myeloid differentiation 88 (MyD88) dependent signaling (30). MyD88 is an adaptor molecule involved in signaling of both TLR2 and TLR4 (30). Since TLR2 but not TLR4 signaling is entirely dependent on MyD88, we speculate that the lack of response of NODTGFβ mice to LPS *P. gingivalis in vivo* is due to the inhibition of this adaptor molecule.

We observed that NODTGFβ mice were deficient in their capacity to upregulate the costimulatory molecule CD40 following infection. CD40 is expressed on the surface of
APCs and interacts with CD154 (CD40L) on the surface of T cells during the course of a normal immune response leading to T cell proliferation, cytokine production and further APC maturation (31). Several lines of evidence have implicated CD40-CD154 interactions in the decision between tolerance and activation as well as in the induction of autoimmunity (31). Indeed, it has been demonstrated that blocking CD40 can effectively inhibit T cell responses and lead to the generation of regulatory T cells (32). Conversely, ligation of CD40 on otherwise tolerogenic CD8+ lymphoid DCs was shown to be sufficient to render these DCs immunogenic by decreasing their ability to metabolize tryptophan and induce T cell apoptosis (33). It was later described that this mechanism is mediated by IL-6 (21).

Furthermore, it was demonstrated that the induction of oral tolerance of CD8 T cells can be reversed in the context of CD40 ligation (34). The potential role of CD40 in the development of autoimmunity was further confirmed by studies demonstrating that injection of APCs matured in the presence of LPS and anti-CD40 antibodies and loaded with self-antigen was sufficient to induce autoimmune heart pathology (15). TGF-β has previously been linked to the regulation of the CD40-CD154 pathway since TGF-β deficient mice were shown to express elevated levels of CD154 on all cell types indicating that TGF-β may normally act to lower levels of CD154 (35). We have observed that despite failing to upregulate CD40 following CB3 infection, NODTGFβ derived macrophages still upregulated surface MHC class II expression. Therefore, this suggested that the macrophages from NODTGFβ mice likely confer protection by presenting self-antigen in the absence of costimulation thereby tolerizing rather than activating autoreactive lymphocytes following CB3 infection.

We then observed that LPS4 overcame the protection conferred by TGF-β protection without significantly altering the pattern of CD40 expression on APCs following treatment. This indicated that LPS can bypass requirements for CD40 signaling in order to induce activation of autoreactive T cells. This observation is supported by a previous report demonstrating that LPS treatment was sufficient to induce diabetes in otherwise protected CD40 deficient mice (36). The mechanism through which LPS can bypass CD40 and activate autoimmunity is unclear but our observations strongly suggest that changes in cytokine expression specifically IL-6 likely leads to the release of autoreactive T cells from the control of Tregs as well as the reversal in the ability of DCs to establish tolerance (21, 23).
In conclusion, we report that infection of NODTGFβ mice with CB3 co-administered with LPS4 is sufficient to overcome protection and induce chronic autoimmune heart disease. Further, we demonstrate that cytokine production mediated by a TLR4 specific ligand (LPS from *S. minnesota*) but not a TLR2 specific ligand (LPS from *P. gingivalis*) correlates with induction of disease. Our results are a further indication that an individual’s history of infection can have profound effects on the ability of their immune system to mount a proper response that can efficiently control infection without degenerating into autoimmune pathology.

3.4 Materials and methods

**Mice.** NOD/Ltj mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) and maintained in our rodent breeding colony at the University of British Columbia (Vancouver, British Columbia). NODTGFβ transgenic mice expressing TGF-β under the control of the human insulin promoter were generated in the laboratory of Dr. Nora Sarvetnick (Scripps Research Institute, La Jolla, California) (37) and were bred and maintained in our rodent facility. Nonfasting blood glucose levels were monitored from tail vein bleeds using a standard glucometer with a range of 20 to 600 mg/dl. NOD mice were used as nontransgenic control because the transgenic mice were derived on the NOD background. NOD mice spontaneously develop diabetes by 13-15 weeks in our colony. All mice were tested prior to infection to ensure that none were diabetic. All procedures performed on these mice followed the guidelines of the institutional animal care committee.

**Virus and LPS.** Virus stocks of coxsackievirus group B type 3 (CB3, Nancy Strain) were originally obtained from the laboratory of Dr. Charles Gauntt (University of Texas at San Antonio). Virus stocks of CB3 were prepared on monolayers of HeLa cells using a multiplicity of infection of 0.1 in DMEM. Virus was collected following several freeze-thaw cycles, filtered and stored at -80°C. Viral titers were measured using a standard plaque assay on HeLa cell monolayers. Mice were infected intraperitoneally at 6-10 weeks of age with sublethal doses (less than half LD₅₀) of 400 plaque forming units of CB3. Following infection, viral plaque assays were used to determine viral loads from individual organs that were harvested, aseptically weighed and homogenized in diluent. Data are representative of 2
separate experiments. Where indicated, mice were injected intraperitoneally with 25µg of LPS *S. Minnesota* (Sigma) or 25µg of LPS *P. Gingivalis* (InvivoGen) at the time of infection.

**Immunohistochemical staining.** Organs were harvested from euthanized mice at various time points post-infection and placed in 10% formaldehyde for 24 hours before being processed for paraffin sectioning. Paraffin sections were cut at a thickness of 4 microns for immunohistochemistry. Staining was performed using standard procedures for hematoxylin and eosin as well as Masson’s trichrome (Wax-it, Vancouver, British Columbia) to determine and compare pathology. Serial sections of the heart were graded according to a 4-tier scoring system: grade 1, less than 1% pathology; grade 2, 1-25%; grade 3, 26-50%; grade 4, greater than 50%.

**ELISA.** Porcine cardiac myosin (Sigma) was coated onto 96-well NUNC MaxiSorp™ High Protein-Binding Capacity ELISA plates at a concentration of 0.2 g/well. Excess binding sites were blocked with 2% bovine serum albumin and 1% fetal bovine serum. Serial dilutions of sera (100 µl per well) were added to the coated wells. Antibody against cardiac myosin (Alexis, San Diego, California) was used to standardize the assays. Antibody was detected with a horseradish-peroxidase-conjugated goat antibody specific for mouse IgG Fab (Sigma) or a combination of purified antibody specific for mouse IgG3 (BDbiosciences), biotinylated antibody specific for rat IgG (Sigma) and streptavidin conjugated HRP (Sigma). Alternatively, ELISA plates were coated with whole heart extract prepared as described previously (19). Data are representative of 4 separate experiments.

**Cytokine Bead Array.** Sera was collected from tail vein bleeds at 24 hours post-infection and stored at -80°C until analysis. Cytokine levels were measured using a BD Bead array inflammation kit (BDbiosciences) allowing simultaneous quantitative measurement of IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70 from a single sample. Samples were prepared according to manufacturer’s instructions and analyzed on a BDFacsArray equipped with FCAP software (BDbiosciences). Data are representative of 3 separate experiments.

**Cell sorting and in vitro cytokine assay.** APC populations were purified based on their surface expression of CD11b and CD11c. Single cell suspensions were generated from spleens of uninfected animals, stained with fluorescently labeled antibodies and sorted by flow cytometry using a FACSaria cell sorter (BDbiosciences). Cells were distributed in a 96 well plate in DMEM at a concentration of 10^5 cells per well (macrophages) and 0.5 X 10^5
cells per well for dendritic cells. Cells were treated with $10^5$ PFU of CB3 virus alone or in combination with 1µg/ml of either LPS from *S.minnesota* or LPS from *P.gingivalis*. Cells were incubated for 24 hours at 37°C. Supernatants were harvested and IL-6 levels analyzed by ELISA using a Ready-SET-Go IL-6 detection kit (eBiosciences). Data are representative of 3 separate experiments.

**Flow cytometry.** Spleens were harvested at appropriate time points post-infection. Single cell suspensions were generated following standard procedures and red blood cells were lysed in a hypotonic buffer. Non-specific antibody binding was prevented by pre-treating cells for 15 minutes at 4°C with Mouse BDFcBlock™ (Clone 2.4G2). Cells were then stained with appropriate antibodies for 30 minutes at 4°C, washed once with FACS buffer (1X PBS containing 2% fetal bovine serum) and stained with fluorescently conjugated streptavidin when biotin-conjugated antibodies were used, cells were washed once more before being fixed in 1% buffered paraformaldehyde. Samples were then analyzed on an LSRII cytometer (BDbiosciences) and using FlowJo (Tree Star Inc.) analysis software. Fluorescently conjugated antibodies directed against CD11b (clone M1/70) and CD11c (clone HL3) were purchased from eBiosciences while biotin conjugated antibodies directed against CD40 (clone 3/23), CD80 (clone 16-10A1), CD86 (clone GL1), MHC class II (I-A^d^, clone AMS-32.1) were purchased from BD Biosciences. Data are representative of 3 separate experiments.

**Statistical Analysis.** The unpaired Student’s t-test and Mann-Whitney U test were used for all plaque assays, serum titer and cytokine analyses. A p value of less the 0.05 was considered significant.
Figure 3.1: CB3/LPS4 treatment of NODTGFβ mice is sufficient to circumvent protection and induce cardiac fibrosis

Representative Masson’s Trichrome-stained cardiac section from NODTGFβ mice (left column, n=6 for each treatment) or their nontransgenic NOD littermates (right column, n=6 for each treatment). Mice were infected with CB3 (top row) with or without co-administration of LPS4 (middle row) or LPS2 (bottom row). Sections were taken 28 days PI. Data are representative of 4 separate experiments. Magnification:400X.
Figure 3.2

(A) Absorbance (490nm) vs Log of Titer

(B) Anti-CM titer (IU/mL)

- CB3
- CB3 + LPS4
- CB3 + LPS2
- Uninfected

- NOD-Total IgG
- NOD TGFβ - Total IgG
- IgG3
Figure 3.2: ELISA analysis of autoantibodies directed to cardiac myosin
Sera obtained from uninfected (open circles, n=8), CB3 infected (blue circles, n=8), CB3/LPS2 infected (red triangles, n=8) or CB3/LPS4 (green squares, n=8) infected NODTGFβ mice was collected at 28 days post infection and analyzed for anti-cardiac myosin antibodies. NODTGFβ mice infected with CB3 produce low levels of cardiac myosin specific antibodies. We observed a significant increase in production of autoantibodies following treatment with CB3/LPS4 (p<0.05) but no significant differences following treatment with CB3/LPS2. Data are representative of 4 separate experiments. B) Antibody endpoint titer analysis for cardiac myosin-specific antibodies generated following infection of mice with CB3 alone or CB3 co-administered with either LPS2 or LPS4. Analysis of the ELISA results of sera obtained at day 28 post-treatment of NOD (black bars, n=6 for each treatment) or NODTGFβ (white bars, n=6 for each treatment) was done to determine the antibody endpoint titers. Antibody endpoint titers were calculated from the sera of an individual mouse as the last positive dilution of antibody within 2 standard deviations of the titer from normal uninfected NOD mouse serum. Black and white bars represent total anti-cardiac myosin IgG titers and yellow bars represent anti-cardiac myosin IgG3 titers. We observed no significant changes in the proportion of cardiac myosin specific IgG3 produced following any of the treatments. Results are representative of 3 separate experiments.
Figure 3.3

A

Log10 pfu/g

NOD + CB3

NOD + CB3/LPS4

NODTGF-β + CB3

NODTGF-β + CB3/LPS4

Pancreas  Heart  Spleen

B

Log10 pfu/g

Pancreas  Heart  Spleen

C

Log10 pfu/g

Pancreas  Heart  Spleen
Figure 3.3: Measurement of viral titers following infection

NODTGFβ mice (blue bars, n=4 at each time point) and their nontransgenic littermate (red bars, n=4 at each time point) were sacrificed (A) 3, (B) 7 or (C) 14 days PI following infection with either CB3 alone (solid bars) or CB3 co-administered with LPS4 (lined bars). Viral load in pancreas, heart and spleen was measured by standard plaque assay. The viral titers are presented as Log10 pfu/gram of tissue and represent the average values of duplicates from at least 4 mice in each group. Despite a reduction in viral load in the pancreas and heart of NODTGFβ mice following infection, we observed no significant differences between NODTGFβ mice infected with CB3 alone or co-administered with LPS4. Data are representative of 2 separate experiments.
Figure 3.4

A. IL-6

B. MCP-1

C. TNF-α
Figure 3.4: *In vivo* cytokine production profile following infection

Cytokine levels in the sera of infected mice were measured by cytometric bead array 24 hours following infection of NODTGFβ mice with either CB3 alone (n=9) or CB3 co-administered with LPS2 (n=9) or LPS4 (n=10)). Infection with CB3/LPS4 yielded significant increases in the production of (A) IL-6, (B) MCP-1 and (C) TNF-α compared to infection with either CB3 alone or CB3/LPS2 (**, P<0.01; ***, P<0.001) Each point represents an individual mouse and the line represents the average value. These data represent the pooled values from 4 separate experiments.
Figure 3.5: NODTGFβ APCs produce IL-6 in response to either LPS2 or LPS4 stimulation *in vitro*

Production of IL-6 by NODTGFβ (white bars) or NOD (black bars) macrophages (M∅) or dendritic cells (DC) in response to *in vitro* stimulation with either CB3 alone or CB3 in combination with LPS2 or LPS4 was investigated by ELISA. Macrophages and dendritic cells were sorted by flow cytometry and separated based on surface expression of CD11b and CD11c. We observed that macrophages (CD11b+CD11c-) from NOD and NODTGFβ mice produce equivalent quantities of IL-6 in response to CB3 combined to either LPS while DCs (CD11c+) produced lesser amounts of IL-6. The data are representative of 3 separate experiments.
Figure 3.6: NODTGFβ macrophages do not upregulate CD40 in response to viral infection

Maturation state of APCs was analyzed by flow cytometry 3 days post-infection with CB3. Shown are representative histograms of CD40, CD80 and MHC class II expression of (A) macrophages (gated on CD11b+CD11c- cells) or (B) DC (CD11c+) from either NODTGFβ mice (blue line, n=4) or nontransgenic NOD controls (red line, n=4). Appropriate isotype controls are illustrated as shaded grey areas. We observed that NODTGFβ mice fail to upregulate CD40 in response to infection (left panel) while maintaining normal upregulation of CD80 (middle panel), CD86 (data not shown) and increased upregulation of MHC class II (right panel). DCs from transgenic and non-transgenic mice upregulated CD40 normally. The data are representative of 3 separate experiments.
Figure 3.7

A

B

CD40-PE

CD40-PE

NODTGFβ + CB3
NODTGFβ + CB3/LPS4
Isotype control
Figure 3.7: LPS4 co-treatment does not alter pattern of expression of CD40 on NODTGFβ antigen presenting cells in response to CB3 infection

CD40 expression was analyzed by flow cytometry 3 days post-infection with CB3 alone (blue line, n=4) or CB3 co-administered with LPS4 (black line, n=3). Shown are representative histograms of CD40 expression of (A) macrophages (gated on CD11b+ CD11c- cells) or (B) DCs (CD11c+) from NODTGFβ mice. Appropriate isotype controls are illustrated as shaded grey areas. LPS4 co-treatment does not significantly alter the pattern of CD40 expression. The data are representative of 3 separate experiments.
Table 3.1: Histology scores of cardiac tissue from NOD and NODTGFβ mice following CB3 infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Histology Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NOD + CB3 (n=6)</td>
<td>17% (1/6)</td>
</tr>
<tr>
<td>NOD + CB3/LPS4 (n=6)</td>
<td>0% (0/6)</td>
</tr>
<tr>
<td>NOD + CB3/LPS2 (n=6)</td>
<td>33% (2/6)</td>
</tr>
<tr>
<td>NODTGFβ+ CB3 (n=6)</td>
<td>100% (6/6)</td>
</tr>
<tr>
<td>NODTGFβ+ CB3/LPS4 (n=8)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td>NODTGFβ+ CB3/LPS2 (n=8)</td>
<td>100% (8/8)</td>
</tr>
</tbody>
</table>

Serial sections of the heart were graded according to a 4-tier scoring system: grade 1, < 1% pathology; grade 2, 1-25%; grade 3, 26-50%; grade 4, >50%
3.5 References


Chapter 4

Regulatory T cells protect from type 1 diabetes following induction by coxsackievirus infection in the context of transforming growth factor-beta

4.1 Introduction

Coxsackieviral infections commonly precede the onset of type 1 diabetes (T1D) in patients (1) and in animal models, coxsackievirus B4 (CB4) infection significantly accelerates diabetes onset (2, 3). In non-obese diabetic (NOD) mice, islet destruction and development of T1D are preceded by a period of non-invasive peri-insulitis strongly suggesting that there is a window of time in which peripheral tolerance is partially maintained (4). Several groups have reported that, in the NOD mouse, regulatory T cells (Tregs) gradually lose their capacity to suppress effector T cell proliferation correlating with the spontaneous onset of T1D (5-7). This loss of functional Tregs results in impaired peripheral tolerance to β cell antigens and represents an important checkpoint in disease progression (8).

Several reports have ascribed a role for transforming growth factor (TGF)-β in the function and/or generation of Tregs in the periphery (6, 9-14). In particular, Chen and colleagues demonstrated that in vitro stimulation of naïve T cells in the presence of TGF-β led to the expression of the Treg specific transcription factor Foxp3 and functional suppression by these Foxp3 expressing cells (9). Further, a short pulse of TGF-β in the islets of NOD mice suppressed spontaneous onset of diabetes through an expansion of Foxp3+ Treg cells within the islets of the pancreas (15). A systemic TGF-β gene therapy approach also demonstrated enhanced survival of transplanted islets cells that correlated with increased Treg numbers in the pancreas (16). Although these reports highlight the potential importance of TGF-β in diabetes, it remains to be determined whether TGF-β can induce protective Tregs in a clinically relevant model of viral-induced diabetes.

In this report, we demonstrate that TGF-β induced Tregs can be activated/generated following viral infection to protect from T1D. NOD mice expressing TGF-β specifically in the β cells of the pancreas (NODTGFβ mice) (17) were infected with CB4 and despite meeting all the criteria for susceptibility to viral-induced diabetes these mice were protected from T1D induction. Protection from T1D was correlated with an increased presence of Tregs in the pancreatic lymph nodes (PLNs) and pancreas. Furthermore, we demonstrated that recombinant TGF-β administered systemically post-infection (PI) could replace transgenic TGF-β and was sufficient to protect NOD mice from CB4-induced diabetes. Our
data indicate that TGF-β induces Tregs to maintain self-tolerance to anti-islet autoimmunity without suppressing the response to the virus.

4.2 Results

4.2.1 CB4 infection in the context of TGF-β protects from type 1 diabetes

As reported previously (2), infection of NOD mice with CB4 resulted in a significant acceleration of diabetes in more than 60% of infected mice as compared to uninfected age-matched controls (Figure 4.1A). This occurs regardless of gender, as viral-induction of T1D does not follow the same gender bias observed for spontaneous disease. NOD mice harboring a transgene driving expression of TGF-β specifically in the pancreas (NODTGFβ) were previously described (17) and they spontaneously develop diabetes, albeit at a reduced rate compared to non-transgenic NOD mice (Figure 4.1B) (17). Furthermore, these mice develop autoreactive T cells with diabetes transfer potential (17). Importantly, these mice present with relatively normal pancreatic organization as opposed to other described models (17, 18). Previous reports have linked the presence of autoreactive T cells and the degree of insulitis with susceptibility to viral-induction of disease (2, 19). Islet inflammation in uninfected NODTGFβ mice was not significantly different from their NOD counterparts with nearly 30% of islets presenting with invasive insulitis at the time of infection (10-12 weeks old) (Figure 4.2A and Table 4.1), indicating the presence of activated β cell specific autoreactive T cells within the pancreas. As such, NODTGFβ mice meet the criteria previously described (2, 19) for susceptibility to CB4-induced T1D. Strikingly, NODTGFβ mice infected with CB4 did not develop T1D unlike their NOD counterparts (Figure 4.2B). Upon infection, no significant change in islet inflammation was observed in NODTGFβ mice whereas by 7 days PI, significant increases in insulitis were observed in NOD mice (Figure 4.2A and Table 4.1). This was particularly marked in NOD mice that were diabetic by day 7 PI as more than 90% of islets in these mice presented with invasive insulitis (n=5). Additionally, the percentage of islets free of insulitis was not significantly decreased in NODTGFβ mice following CB4 infection (Figure 4.2A and Table 4.1) indicating that no new islets were being targeted following infection. This phenotype is reminiscent of both the BDC2.5 TCR transgenic
model and the non-obese resistant (NOR) mouse where Tregs prevent T1D by precluding the progression of islet pathology from peri-insulitis to invasive insulitis (20, 21).

4.2.2 **NODTGFβ mice are polarized to a Th1 response following infection**

Previously, NODTGFβ mice were found to be polarized towards a Th2 phenotype at steady-state (17). Furthermore, it has recently been established that TGF-β acts as a co-factor with IL-6 in the generation of Th17 cells (22-24). As such, we investigated whether changes in T cell polarization were involved in the protection from T1D following infection. Cytokine production from splenic T cells was analyzed *ex vivo* prior to infection and at 7 days PI. Prior to infection, very few T cells were observed to produce cytokines and slightly more T cells from NODTGFβ mice compared to NOD mice were observed to produce IL-4 although this difference was not statistically significant (Figure 4.3). As predicted following a viral infection, T cells preferentially produced Th1 cytokines (IFN-γ and TNF-α) in both NODTGFβ and NOD mice (Figure 4.4). Interestingly, despite previous reports of Th2 polarization prior to infection (17), T cells from NODTGFβ mice did not abundantly produce IL-4 (Figure 4.4) clearly confirming a Th1 response following viral infection. Finally, only a few CD4 T cells were observed to produce IL-17 and, more importantly, there was no significant increase in the percentage of Th17 cells in NODTGFβ mice compared to NOD mice (Figure 4.4). These data clearly indicate that NODTGFβ mice mount a Th1 response similar to NOD mice following infection and that polarization towards a Th2 or Th17 phenotype was not involved in the protection from T1D.

4.2.3 **CB4 infection of NODTGFβ mice leads to a significant increase in the number of Tregs in the pancreatic lymph node and pancreas**

It has been demonstrated that stimulation of T cells in the presence of TGF-β can induce the conversion of naïve T cells to a Treg phenotype (9-12). Accordingly, we examined whether increases in Treg presence were responsible for the protection from diabetes observed in NODTGFβ mice. Following CB4 infection, significantly increased levels of CD4⁺ Foxp3⁺ Tregs were found in PLNs (Figure 4.5A-D), but not the spleen (Figure
4.6) of NODTGF\(\beta\) mice as compared to uninfected littermates. Infection of NOD mice also resulted in significant increases in Tregs in the PLNs (11.9\% in uninfected mice, \(n=7\) vs. 16.8\% in infected mice, \(n=10\)) and this is analogous to a prior report in which similar increases were associated to insulitis severity and/or onset of spontaneous T1D (7). This implies that measuring mere increases in the proportion of Tregs in the PLNs may not be directly predictive of Treg mediated protection. A prior study reported that Tregs from BDC2.5 mice did not efficiently suppress activation of diabetogenic T cells in the PLNs and inferred that Treg function may be limited to within the confines of the pancreas (20). In the pancreas, we observed a significantly greater percentage of CD4\(^{+}\) Foxp3\(^{+}\) Tregs in CB4-infected NODTGF\(\beta\) mice as compared to similarly infected NOD mice, uninfected NOD mice and uninfected NODTGF\(\beta\) mice (Figure 4.5E). As expected, no differences in activation were observed between T cells in the PLNs of NODTGF\(\beta\) or NOD mice following infection (Figure 4.7). These data suggest that Tregs may primarily act directly in the pancreas rather than in the draining lymph nodes to suppress diabetogenic T cells and prevent onset of T1D.

4.2.4 Infection of \(\beta\) cells of the pancreas is required for induction of Tregs

It was previously demonstrated that CB4 infection induced T1D via presentation of pancreatic \(\beta\) cells and their self-antigens to the pre-existing population of diabetogenic T cells (25). In order to determine whether infection of \(\beta\) cells was also necessary to activate or generate functional Tregs, NODTGF\(\beta\) mice were infected with a closely related virus, CB3. Both CB3 and CB4 infect the acinar tissue of the pancreas causing considerable pathology and inflammation, however, only CB4 infects pancreatic \(\beta\) cells (19). CB3 infection did not lead to any changes in the proportions of Tregs in the PLNs (Figure 4.5 C-D) suggesting that presentation of \(\beta\) cell antigens is necessary to induce protective Tregs in this model and that mainly \(\beta\) cell antigen-specific Tregs are activated or generated to protect against diabetes.
4.2.5 Functional inactivation of Tregs reestablishes susceptibility of NODTGFβ to type 1 diabetes

To confirm the functional role of these TGF-β-induced Tregs in the protection from diabetes, CB4 infected NODTGFβ mice were treated with an anti-CD25 antibody that has been previously demonstrated to functionally inactivate and/or deplete Tregs (26). Antibody treatment PI reestablished susceptibility of NODTGFβ mice to CB4-induced T1D as disease developed with the same kinetics and incidence to that observed for NOD mice following infection while mock-treated mice remained protected from disease (Figure 4.8A, 1B). These data confirm that T1D can be induced in NODTGFβ mice and they are not simply impervious to the induction of disease following viral infection. Instead, suppression of diabetes is actively induced and maintained. By demonstrating loss of function through antibody-mediated functional inactivation, this experiment showed that TGF-β-induced Tregs are responsible for the protection from diabetes.

4.2.6 Adoptive transfer of Tregs from NODTGFβ mice protect CB4 infected NOD mice from T1D

To further demonstrate the functional role of TGF-β induced Tregs in the protection from T1D, Tregs were purified from the PLNs of NODTGFβ mice at 7 days PI and adoptively transferred to NOD mice 24 hours post-CB4 infection. Following Treg transfer, recipient NOD mice that were adoptively transferred with donor Tregs from infected NODTGFβ mice were protected from diabetes development for over 15 days PI while mock-treated mice still developed accelerated diabetes following infection (Figure 4.8B). However, the observed protection may only be transient as one of the adoptively transferred mice developed diabetes 17 days post-infection (Figure 4.8B). This may infer that a source of TGF-β is required to maintain protection. These data demonstrate gain of function further confirming the role of Tregs in the protection observed in our model.
4.2.7  Tregs maintain protection from T1D in a CTLA-4 dependent manner

The costimulatory molecule CTLA-4 is expressed at high levels on the surface of Tregs and has been demonstrated to play an important role in both the function (27) and the TGF-β mediated conversion of Tregs in vitro (28). To investigate the functional requirement of CTLA-4 in TGF-β induced Treg mediated protection, we treated CB4 infected NODTGFβ mice with a neutralizing antibody directed against CTLA-4 at 24 hours PI. Antibody treated NODTGFβ mice developed diabetes with increased incidence compared to mock-treated NODTGFβ mice (Figure 4.8C). This suggests that Tregs maintain protection from T1D in a CTLA-4 dependent manner.

4.2.8  NODTGFβ mice show reduced upregulation of costimulatory molecule following infection

TGF-β treated antigen presenting cells (APCs) have previously been shown to induce tolerance in a Treg dependent manner (29, 30). Compared to infected NOD mice, flow cytometry analysis revealed that macrophages isolated from the pancreas, PLNs and spleen of infected NODTGFβ mice at day 7 PI have significantly reduced surface expression of the costimulatory molecule CD40 (Figure 4.9A-B). A similar trend is observed with the costimulatory molecules CD80 and CD86 (Figure 4.9 C-D). Their surface expression was significantly reduced on macrophages isolated from the spleen of infected NODTGFβ mice, this reduction was also observed on macrophages isolated from the PLNs although this difference was not statistically significant (Figure 4.9 C,D). Interestingly, this reduced upregulation was not observed on dendritic cells in the spleen (Figure 4.10) or the PLNs (data not shown). It is interesting to note that NOD and NODTGFβ macrophages express similar levels of costimulatory molecules prior to infection (Figure 4.11) and that these molecules are upregulated to the same extent in both mice at day 3 PI (Figure 4.12). Differences in surface expression of costimulatory molecules were not observed until day 7 (Figure 4.9). This time frame coincides with the kinetics of increases in the number of Tregs following infection in the NODTGFβ mice, suggesting that presentation of pancreatic self-antigen by these “semi-mature” macrophages may act in the generation or activation of these
protective Tregs in the NODTGFβ mice. Furthermore, we observed that functional inactivation of Tregs did not reestablish upregulation of costimulatory molecules on macrophages (data not shown) indicating that they are unlikely to be the targets of suppression in our model. Interestingly, viral clearance of both CB3 (31, 32) and CB4 (Figure 4.13A) was not affected in NODTGF-β mice when compared to infected NOD mice indicating that the influence of TGF-β does not negatively affect the protective immune response directed against the virus. This is further supported as delayed clearance typically results in a fatal outcome and no increase in death was observed in the NODTGFβ mice following coxsackievirus infection. These data indicated that changes in costimulatory molecule expression on macrophages are more relevant to the induction of autoimmunity than to the immune response to viral infection.

4.2.9 Systemic TGF-β treatment protects NOD mice from T1D

To assess the potential therapeutic role of TGF-β during viral-induced autoimmunity and to validate the biological relevance of our results in this transgenic model, we asked whether systemic TGF-β treatment would also be sufficient to protect from coxsackievirus-induced T1D. One day following CB4 infection, NOD mice (10-12 weeks old) were treated with a single dose of recombinant TGF-β and monitored for induction of diabetes. We observed a significant reduction of diabetes incidence by day 15 PI (Figure 4.14A). This TGF-β mediated protection correlated with increases in Tregs in both the PLNs (Figure 4.14B) and the spleen following infection (Figure 4.14C) compared to similarly treated mock-infected mice. Protection was transient, however, as disease induction was observed by day 28 PI (data not shown). Mice were only given a single dose of TGF-β and given the short half-life of TGF-β in vivo (33) it would not be expected to persist in the mice. This suggests that a multi-dose regimen would likely extend protection. Similar to what we observed for transgenic expression of TGF-β, systemic treatment with TGF-β did not affect clearance of the viral infection (Figure 4.13B), suggesting that treatment does not reduce the capacity of the host to mount an immune response to the virus. Most notably, this indicates that TGF-β could be administered after exposure to virus and act to modulate disease induction without adverse effects on the host.
4.3 Discussion

Viral infections clearly represent the last step of disease progression in animal models and require a pre-existing population of autoreactive T cells (2, 3). As viruses, such as coxsackievirus, are common human pathogens, this mechanism also likely operates to induce T1D in humans. This suggests that protective approaches identified in mouse models would likely translate into potential therapies. Our data build on previous reports on the protective role of TGF-β in order to demonstrate that the immune system can be manipulated so that infection with a virus normally associated with acceleration of disease, such as CB4, can actively lead to the induction of mechanisms of tolerance and ultimately lead to protection from diabetes. Importantly, our data indicate that changes in the cytokine milieu can lead to protection from diabetes without compromising the capacity of the immune response to control viral infection.

The presence of a pre-existing population of autoreactive T cells has previously been established as the primary criteria for susceptibility to viral-induction of T1D (2, 3). Similar to NOD mice from which they were derived, NODTGFβ mice harbor diabetogenic T cells capable of transferring disease and develop spontaneous diabetes (17). Importantly, the constitutive expression of TGF-β in this model did not result in any profound alterations of pancreatic architecture contrary to a similar model presented in a previous report (18). Since NODTGFβ mice fulfill the criteria for susceptibility to viral-induced disease such as the development of autoreactive T cells and susceptibility to disease following functional inactivation of Tregs our results strongly suggest that NODTGFβ mice are protected by mechanisms that are actively induced following infection in the context of TGF-β rather than simply being impervious to viral-induced T1D. We observed significant increases in the percentage of Tregs in the PLNs and, more importantly, the pancreas of NODTGFβ mice. Interestingly, we do not observe increased presence of Tregs in NODTGFβ mice prior to infection as compared to wild-type counterparts confirming the role of viral infection in the induction of Tregs in our model. These data are in contrast to observations where a pulse of TGF-β induced prior to the end of the priming phase of disease was sufficient to induce Tregs without any further manipulations and mediate protection from spontaneous diabetes (15). We speculate that these discrepancies may arise from differences in the levels or timing...
of TGF-β production. Previous studies have demonstrated that stimulation of naïve T cells in the presence of TGF-β converts these cells into functional Tregs with suppressive capacity (9-12). To our knowledge, this represents the first report of a viral infection in the context of TGF-β actively inducing the generation/activation of Tregs directly in vivo and yielding protection from autoimmunity.

Our data clearly demonstrate that Tregs function to prevent diabetes directly within the pancreas. The activation or generation of Tregs may, however, still occur in the PLNs as adoptive transfer of Tregs purified from the PLNs was sufficient to protect NOD mice from CB4-induced T1D. Similar to the BDC2.5 model, the presence of functional Tregs did not affect the activation of T cells in the PLNs (20); instead Tregs prevented the transition from peri-insulitis to invasive insulitis. Importantly, despite an ongoing autoimmune response at the time of infection, TGF-β induced Tregs prevented new islets from becoming targets thereby allowing for the maintenance of insulin production. We further demonstrated that infection of the islet cells themselves is an important requirement for the generation of Tregs. This strongly implies that self-reactive T cells are converted to Tregs or that self-reactive Tregs are activated in order to prevent disease. As such, the protection from disease following infection in NODTGFβ mice can be explained by two non-exclusive mechanisms. First, protection may be maintained either by a reestablishment or an increase in the suppressive capacity of Tregs. Second, conversion of β cell-reactive T lymphocytes into Tregs may be responsible for decreasing the available pool of activated autoreactive T cells. The antigenic specificity of the generated Tregs may also explain why a small increase in Treg percentage following infection was sufficient to induce protection. In this regard, two separate reports have demonstrated that in vitro expanded antigen specific Tregs have greater T1D suppressive capacity than polyclonal Tregs in NOD mice (34, 35). In another model of T1D, it was demonstrated that adoptive transfer of as little as 2000 Tregs was sufficient to prevent disease further illustrating the potent suppressive capacity of fully functional Tregs (36). This implies that increases in the percentage of antigen specific Tregs in the pancreas observed in NODTGFβ mice following infection should be amply sufficient to completely protect from the induction of T1D.

Several groups have investigated the interaction between Treg and APCs (reviewed in (37)). It has been suggested that immature APCs may lead to the generation of Tregs (29, 30).
and that, in turn, Tregs may maintain tolerance by acting directly on APCs (38). Here, we demonstrate that macrophages from the pancreas, PLNs and spleen of NODTGFβ mice do not mature to the same extent as macrophages from NOD mice in response to infection. Since the timing of this defect corresponds with the increase in Tregs in our model, we speculate that antigen presentation by these “semi-mature” macrophages is responsible for Treg generation. In support of this hypothesis, it was recently demonstrated that monocytes isolated from glatiramer acetate treated mice presented with a similar “semi-mature” phenotype and were capable of inducing expansion of Tregs (39). These type II monocytes were further demonstrated to preferentially secrete immunosuppressive rather than pro-inflammatory cytokines (39). We are currently investigating whether type II monocytes are also involved in the protection observed in our model. Importantly, we saw no differences in viral clearance between NODTGFβ and NOD mice. Viral clearance is likely unaffected since dendritic cells mature normally in response to infection despite the expression of TGF-β.

Overall, these data indicated that macrophages might be more involved in the induction of autoimmunity than in the response to CB4 infection, in our model. This is supported by previous observations in the BDC2.5 model where macrophages engulf islets in response to CB4 infection and are likely responsible for induction of T1D (40). Furthermore, since viral clearance remains unaffected, these data also suggest that the Tregs generated in our model act to suppress only self-reactive lymphocytes and do not affect viral-specific lymphocytes. Taken together, this indicated that the presence of TGF-β at the site of infection has a profound effect on the induction of autoimmunity without affecting the response to pathogen infection suggesting that a TGF-β based therapeutic approach would not run the risk of fatal side effects.

NODTGFβ mice have previously been demonstrated to be polarized towards a Th2 phenotype at steady-state (17). As T1D has been well described as a Th1 driven disease (reviewed in (41)), this change in polarization could well have explained the protection observed in our model. However, following infection, we observed that T cells from NODTGFβ mice responded similarly to NOD mice by producing IFN-γ and TNF-α preferentially over IL-4 demonstrating a strong Th1 response. As such, polarization to a Th2 phenotype is not responsible for the observed protection from T1D in CB4-infected NODTGFβ mice. Further, while it is well established that TGF-β along with other cofactors
such as IL-6 can induce pathogenic Th17 cells (reviewed in (42)), a very limited number of Th17 cells were observed post-infection and no increases in Th17 cells were observed in the NODTGFβ mouse. Despite the continuous presence of TGF-β in the transgenic mice and the induction of IL-6 typically associated with viral infection no differences were observed between infected NOD and NODTGFβ mice. It has been reported that IFNγ can inhibit development of Th17 cells (43) and this likely explains their absence following infection. Taken together, these results strongly indicate that suppression of T1D was not the result of polarization of T helper cells towards a Th2 phenotype.

Finally, our data support a therapeutic role for TGF-β, as systemic treatment was sufficient to significantly reduce T1D incidence by day 15 PI. Protection was transient, indicating that treatments maintaining more prolonged exposure to TGF-β will be necessary to achieve long-term protection from diabetes. More specifically, TGF-β may need to be present continuously throughout the course of viral infection to ensure that the cytokine is present at the time of self-antigen presentation. To this effect, it was recently determined that continuous TGF-β exposure is necessary to maintain Foxp3 expression and suppressive capacity of Tregs converted in vitro (44). Alternatively, treatments using TGF-β agonists or delivery methods that maintain longer expression of TGF-β may help maintain tolerance, although these approaches would need to be fully tested to ensure that the side-effects of treatment do not outweigh the benefits. Besides validating our studies in the transgenic mouse, these data signify an important short-term therapeutic role for this cytokine against viral-induced autoimmunity without striking side-effects in terms of the anti-viral response.

In conclusion, we provide evidence that cytokines like TGF-β can be used to manipulate the immune response to infection in order to maintain tolerance to self-antigens while still allowing for proper control of infections. By changing the cytokine milieu in the pancreas, coxsackievirus infection results in the induction of suppression as opposed to activation of autoimmunity without concomitant loss of the anti-viral response. Our data build on previous reports on the role of TGF-β and, in a clinically relevant model of viral-induced autoimmunity, clearly demonstrates that Tregs can be generated/activated following viral infection in the context of TGF-β and protect from T1D. Taken together, our results further attest to a potential role for TGF-β in therapies directed at preventing viral-induced autoimmune diseases.
4.4 Materials and methods

Mice. NOD/ShiLtJ mice were obtained from The Jackson Laboratory (Bar Harbor, USA). NODTGFβ transgenic mice expressing TGF-β under the control of the human insulin promoter were generated in the laboratory of Dr. N. Sarvetnick (The Scripps Research Institute, La Jolla, USA) (17). All mice were bred and maintained in our rodent facility and tested for diabetes prior to infection. All procedures performed followed the guidelines of the institutional animal care committee.

Virus. Stocks of CB4 Edwards strain 2 were prepared as described previously (32, 45). 10-12 week old mice were infected intraperitoneally with sublethal doses (less than half LD₅₀) of 100 PFU.

Flow cytometry. Single cell suspensions were stained for the appropriate markers and analyzed by flow cytometry. Fluorescently conjugated antibodies directed against CD11b (clone M1/70), CD11c (clone HL3), CD4 (clone L3T4), CD25 (clone PC61 or 7D4) and foxp3 (clone FJK-16s) were purchased from eBioscience (San Diego, USA) while biotin conjugated antibodies directed against CD40 (clone 3/23), CD80 (clone 16-10A1) and CD86 (clone GL1) were purchased from BD Biosciences (Missisauga, Canada).

Immunohistochemical staining. Tissue sections were prepared as previously described (32). Staining was performed using standard procedures for hematoxylin and eosin (iCapture center, Vancouver, British Columbia). Serial sections of the pancreas were graded for islet pathology based on a three-tier scale.

Isolation of pancreatic infiltrating cells. Pancreata were isolated from infected NOD and NODTGFβ mice and mechanically disrupted. Single cell suspensions were treated for 10 minutes at 37°C in a PBS solution containing 1mg/ml of collagenase. Recovered cells were stained for flow cytometry.

Regulatory T cell functional inactivation: CB4 infected NODTGFβ mice received intravenous injection of 450µg anti-CD25 antibody (clone PC61) at day 3 and day 6 PI. Alternatively, mice were injected intraperitoneally with a single dose of 100µg of purified anti-CTLA-4 (clone UC10-4B9, eBioscience, San Diego, USA) at 24 hours PI.

Regulatory T cells adoptive transfer: PLNs were harvested from CB4 infected NODTGFβ mice at day 7 PI. Tregs were purified using a Robosep automated cell separator (Stem cell
CD4+ CD25+ T cells were sequentially purified using modified CD4 and CD25 enrichment kits (Stem cell technologies, Vancouver, Canada). 1 X 10^5 purified Tregs were adoptively transferred intraperitoneally into NOD mice at 24 hours PI.

**Intracellular cytokine staining:** Splenocytes were restimulated with PMA (500ng/ml) and ionomycin (10ng/ml) in the presence of Golgi Plug (BD Biosciences, Missisauga, Canada) in IMDM containing 10%FBS. Cells were stained for surface markers, fixed, permeabilized, stained for cytokines and analyzed by flow cytometry. Fluorescently conjugated antibodies to CD4 (clone L3T4), CD8 (clone 53-6.7), IL-17 (clone TC11-18H10.1) and TNFα (clone MP6-XT22) were obtained from eBiosciences (San Diego, USA). Fluorescently conjugated antibodies to IL-4 (clone 11B11) and IFNγ (clone XMG1.2) were obtained from BD Biosciences (Missisauga, Canada).

**Systemic TGF-β treatment.** NOD mice were injected intraperitoneally with 100ng of recombinant human TGF-β1 (Sigma-Aldrich, Oakville, Canada) 24 hours PI with CB4.

**Statistical Analysis.** The unpaired Student’s t-test (flow cytometry analysis) and the Mann-Whitney U test (diabetes incidence curves and insulitis index) were used. A P value of less than 0.05 was considered significant. Stars denote significance.
**Figure 4.1: CB4 infection accelerates diabetes induction in NOD mice**

A) Diabetes incidence of 10 weeks old NOD mice infected with CB4 (filled squares) or mock-infected with DMEM (open circles). B) Spontaneous incidence of diabetes of uninfected NOD (filled squares) or NODTGFβ mice (open diamonds).
Figure 4.2: CB4 infection of TGF-β expressing NOD mice does not induce T1D

A) Histological analysis of pancreata from NOD and NODTGFβ mice 7 days PI with CB4 or mock-infection with DMEM. Consecutive pancreatic sections were stained with H&E and scored for islet pathology. Data are presented as percentages and were obtained from a minimum of 140 scored islets representing at least 8 mice per group. B) Diabetes incidence of NOD (filled squares) and NODTGFβ (open diamonds) mice following infection with CB4. Stars denote significant change in overall phenotype and change in mice presenting with insulitis.
Figure 4.3: Cytokine production in NOD and NODTGFβ mice prior to infection

Cytokine production from A) CD4 T cells and B) CD8 T cells from the spleen of NOD (black bars) and NODTGFβ (white bars) mice was measured \textit{ex vivo} by intracellular flow cytometry following restimulation with PMA and ionomycin. Data are presented as mean ± s.e.m. and are representative of 4 mice per group from 2 separate experiments.
Figure 4.4: T cells from NODTGFβ mice are polarized to a Th1 phenotype following CB4 infection

Cytokine production from A) CD4 T cells and B) CD8 T cells from the spleen of NOD (black bars) and NODTGFβ (white bars) mice was measured *ex vivo* by intracellular flow cytometry following restimulation with PMA and ionomycin. Data are presented as mean ± s.e.m. and are representative of 4 mice per group from 2 separate experiments.
Figure 4.5: CB4 infection of NODTGFβ mice leads to increases in Tregs in the pancreatic lymph nodes and in the pancreas

Representative histograms of Foxp3 expression by CD4+ T cells in the PLNs of NODTGFβ mice 7 days following A) mock-infection with DMEM or infection with B) CB4 or C) CB3. Numbers shown on the histograms represent percentage of Foxp3 positive cells. Isotype controls are represented by shaded grey areas. D) Average percentage of Foxp3+ CD4+ T cells in the PLNs of NODTGFβ mice following mock-infection with DMEM (black bars, n=10) or infection with CB4 (white bars, n=13) or CB3 (grey bars, n=12) E) Average percentage of CD4+ cells expressing Foxp3 in the pancreas of NOD (black bars, uninfected: n=4, infected: n=14) or NODTGFβ mice (white bars, uninfected: n=4, infected: n=7). Data are presented as mean ± s.e.m. from at least 2 separate experiments. Stars denote significance.
Figure 4.6: CB4 infection does not significantly increase Tregs in the spleen of NODTGFβ mice

Representative histograms of Foxp3 expression by CD4⁺ T cells in the spleen of NODTGFβ mice 7 days following A) mock-infection with DMEM or infection with B) CB4 or C) CB3. Numbers shown on the histograms represent percentage of Foxp3 positive cells. Isotype controls are represented by shaded grey areas. D) Average percentage of Foxp3⁺ CD4⁺ T cells in the spleen of NODTGFβ mice following mock-infection with DMEM (black bars, n=10) or infection with CB4 (white bars, n=13) or CB3 (grey bars, n=12). Data are presented as mean value ± s.e.m. and are representative of at least 3 separate experiments. Stars denote significance.
Figure 4.7: Pancreatic expression of TGF-β does not affect T cell activation in the PLN following CB4 infection

Representative histograms of the surface expression of CD69 (A, B) and CD62L (C, D) on CD4+ (A, C) or CD8+ (B, D) T cells from the PLN of either NOD (black lines) or NODTGFβ (grey lines) mice. Data are representative of at least 3 separate experiments.
Figure 4.8

A

B

C

Percent Diabetic (>300 mg/dL)

Time Post-infection (weeks)

- NOD TGFβ + CB4 + anti-CD25 (n=10)
- NOD TGFβ + CB4 (n=7)

- NOD + CB4 (n=17)
- NOD + CB4 + NOD TGFβ Tregs (n=8)

- NODTGFβ + CB4 + anti-CTLA-4 (n=9)
- NODTGFβ + CB4 (n=12)
Figure 4.8: TGF-β induced Tregs protect from T1D diabetes in a CTLA-4 dependent manner
A) Diabetes incidence of CB4 infected NODTGFβ mice treated with anti-CD25 (filled diamonds) antibodies or mock-treated with DMEM (open diamonds). B) Diabetes incidence of CB4 infected NOD mice adoptively transferred with NODTGFβ Tregs (open diamonds) or mock-treated (filled squares) C) Diabetes incidence of CB4 infected NODTGFβ mice treated with anti CTLA-4 antibodies (filled diamonds) or mock-treated with DMEM (open diamonds).
Figure 4.9: Pancreatic expression of TGF-β reduces upregulation of costimulatory molecules on macrophages following CB4 infection

A) Representative histograms of CD40 expression on macrophages (CD11b⁺ CD11c⁻) from NOD (black line) or NODTGFβ (grey line) mice 7 days PI with CB4. B) Average mean fluorescence intensity of B) CD40, C) CD80 and D) CD86 expression on macrophages (CD11b⁺ CD11c⁻) from NOD (black bars) or NODTGFβ (white bars) mice. Data from spleen, PLNs and pancreas are presented as mean ± s.e.m. and are representative of at least 4 mice per group from at least 2 separate experiments. Stars denote significance.
Figure 4.10: Pancreatic expression of TGF-β does not affect upregulation of costimulatory molecules on dendritic cells following CB4 infection

Representative histogram of A) CD40, B) CD80 and C) CD86 on dendritic cells (CD11c+) from NOD (black line) or NODTGFβ (grey line) mice 7 days PI with CB4. Average mean fluorescence intensity of D) CD40, E) CD80 and F) CD86 on dendritic cells (CD11c+) from NOD (black bars) or NODTGFβ (white bars) mice. Data are presented as mean ± s.e.m. and are representative of at least 5 mice per group from at least 2 separate experiments.
Figure 4.11: Costimulatory molecule expression on antigen presenting cells from NODTGFβ mice is similar to NOD mice prior to infection

Average MFI of costimulatory molecule expression on macrophages (CD11b+CD11c-, A, B, C) or dendritic cells (CD11c+, D, E, F) from NOD (black bars) or NODTGFβ (white bars) mice at day 3 PI. Data are presented as mean value ± s.e.m. and represent at least 2 separate experiments and 5 mice per group.
Figure 4.12: Antigen presenting cells from NODTGFβ mice respond normally to viral infection at early time points

Average MFI of costimulatory molecule expression on macrophages (CD11b+CD11c-, A, B, C) or dendritic cells (CD11c+, D, E, F) from NOD (black bars) or NODTGFβ (white bars) mice at day 3 PI. Data are presented as mean value ± s.e.m. and represent at least 2 separate experiments and 5 mice per group.
Figure 4.13: Clearance of viral infection is not affected in the presence of TGF-β

Viral load in pancreas of A) NOD mice (black bars, n=5) and NODTGFβ mice (white bars, n=6) or B) NOD mice (black bars, n=5) and NOD mice treated systemically with 100ng of recombinant TGF-β (white bar, n=5) were measured post-CB4 infection. Data are presented as log 10 plaque forming units per gram of tissue and represent the average from duplicate values obtained from each mouse in the group. Data are representative of at least 2 separate experiments. Any samples not yielding any plaque forming units were assigned a value of 2 log 10 pfu/g representing the limit of detection of the assay.
**Figure 4.14:** Systemic TGF-β treatment transiently protects from diabetes in a Treg dependent manner

A) Diabetes incidence of CB4 infected NOD mice treated 24 hours PI with 100ng of recombinant human TGF-β (open diamonds) or mock-treated with DMEM (filled squares). Average percentage of Foxp3+ CD4+ T cells in the B) PLN or C) spleen in TGF-β treated NOD mice 7 days following infection with CB4 (white bars, n= 9) or mock-infection with DMEM (black bars, n=6). Data are presented as mean value ± s.e.m. and are representative of at least 2 separate experiments. Stars denote significance.
Table 4.1

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Table 4.1: Histological analysis of pancreatic tissue of NOD and NODTGFB mice 7 days following infection with CB4 or mock-infection with DMEM
4.5 References


Chapter 5

Type 1 diabetes induced by coxsackievirus is regulated by CD11b+CD11c- antigen presenting cells. Richer MJ, Lavallée DJ, Shanina I and Horwitz MS

5 A version of this chapter will be submitted: Type 1 diabetes induced by coxsackievirus is regulated by CD11b+CD11c- antigen presenting cells. Richer MJ, Lavallée DJ, Shanina I and Horwitz MS
5.1 Introduction

Type 1 diabetes (T1D) is a T-cell mediated autoimmune disease that results from the destruction of the insulin producing beta cells of the pancreas. Susceptibility to T1D is dictated by a complex interplay between genetic determinants and environmental influences (1, 2). Among these environmental factors, viral infections have long been associated with the development of T1D (3). Particularly, coxsackieviral infections have been described as a common precursor to T1D in patients (3) and the diabetogenic properties of coxsackievirus B4 (CB4) infections have been well documented in mouse models (4-6).

In both humans and non-obese diabetic (NOD) mice, T1D development is progressive and there is evidence that partial peripheral tolerance to islet antigens is maintained for a certain period of time (7). Several reports have demonstrated an important role for regulatory T cells (Tregs) in the maintenance of this peripheral tolerance towards pancreatic self-antigens (8-10). Furthermore, in both mice and humans a progressive loss in the suppressive capacity of Tregs correlates with disease development (11-13). Recent data suggest that the loss of suppressive capacity is not due to Treg intrinsic defects but rather to a decreased capacity of antigen presenting cells (APCs) to maintain Treg function (14). Anderson and colleagues have demonstrated that in NOD mice, CD11b+CD11c- APCs are responsible for the decrease in Treg suppressive function and mapped this defect to a region encoding the gene for IL-2 (14). Importantly, APCs from T1D patients have been demonstrated to be similarly defective in their capacity to maintain Treg function (15) and present with an inflammatory phenotype that may favor the development of potentially pathogenic IL-17-producing T cell clones (Th17) (16). Taken together, this suggests that therapies aimed at modifying the APC response may allow for the reestablishment of peripheral tolerance and allow for long-term protection from T1D. To this effect, it has previously been demonstrated that treatment with glatiramer acetate prevents the induction of experimental autoimmune encephalomyelitis (EAE) in mice by inducing “semi-mature” type II monocytes (M2) that in turn induce protective Tregs (17).

We previously demonstrated that CB4 infection in the context of TGF-β induced Tregs and protection from T1D rather than disease acceleration (18). This correlated with the presence of “semi-mature” CD11b+CD11c- APCs in the pancreatic lymph nodes (PLNs) and
pancreas of infected mice (18). Interestingly, the capacity of the host to clear viral infection remained unaffected. This strongly suggested that APCs could be manipulated in order to prevent autoimmunity without affecting the capacity of the host to respond to infection. In this report, we demonstrate that CD11b+CD11c- APCs are central to the induction of T1D following CB4 infection. Furthermore, we demonstrate that CB4 infection in the context of localized TGF-β expression yields “semi-mature” CD11b+CD11c- APCs with reduced inflammatory properties. In turn, due to the reduced expression of the costimulatory molecule CD40, these APCs are responsible for the induction of Tregs in the PLNs and pancreas of NOD mice. Our results suggest that the development of therapeutics aimed at modulating APC function may represent a powerful tool for reestablishing peripheral tolerance to beta cell antigen and providing long-term protection from T1D.

5.2 Results

5.2.1 CD11b+CD11c- APCs from CB4 infected NOD mice are sufficient to accelerate T1D in uninfected NOD mice

Previous reports have detailed an important role for APCs in the induction of T1D following CB4 infection in T-cell receptor transgenic BDC2.5 mice (19, 20). Additionally, we have recently demonstrated that changes in the maturation status of CD11b+CD11c- APCs (a population containing a large proportion of monocytes and macrophages) are correlated with protection from T1D in TGF-β expressing NOD mice (NODTGFβ) (18). As such, we asked whether CD11b+ CD11c- APCs were involved in the acceleration of T1D typically observed in CB4 infected NOD mice. Uninfected 10-12 week old NOD mice were adoptively transferred with CD11b+CD11c- APCs or CD11c+ (dendritic cells, DCs) APCs purified from CB4 infected NOD mice at day 7 post-infection (PI). Adoptive transfer of CD11b+CD11c- APCs resulted in accelerated disease kinetics and increased T1D incidence compared to mock-transferred NOD mice (Figure 5.1). Conversely, adoptive transfer of DCs did not accelerate disease induction although disease incidence was slightly higher than in mock-transferred mice by day 28 (25% for CD11c+ transferred, n=8 vs 10% for mock-
transferred, n=10) (data not shown). These results demonstrate that CD11b+CD11c- APCs play a central role in the acceleration of T1D following CB4 infection in NOD mice.

5.2.2 CB4 infection in the context of TGF-β yields CD11b+CD11c- APCs with reduced inflammatory properties

We previously demonstrated that CB4 infection in the context of TGF-β induces “semi-mature” CD11b+CD11c- APCs with lower surface expression of costimulatory molecules at day 7 PI compared to similarly infected wild-type (WT) NOD mice (18). Here, we asked whether infection in the presence of TGF-β also affected other inflammatory properties of these APCs. CD11b+CD11c- and CD11c+ cells from NOD and NODTGFβ mice were sorted by flow cytometry at day 7 following CB4 infection and cultured directly ex vivo for 24 hours. Cytokine secretion in the culture supernatant was measured by bead array. Production of the inflammatory cytokines TNF-α (Figure 5.2A) and IL-6 (Figure 5.2B) from CD11b+CD11c- APCs purified from CB4 infected mice NODTGFβ mice was significantly reduced compared to the same population purified from CB4 infected NOD mice. No significant production of IL-12p70 or IFN-γ was observed (data not shown). Furthermore, while we observed no significant production of the anti-inflammatory cytokine IL-10 (data not shown), we observed a trend for increased TGF-β production from CD11b+CD11c- APCs purified from infected NODTGFβ mice although this difference did not reach statistical significance (Figure 5.3). This suggests that the primary effect of TGF-β in this model is to reduce the inflammatory properties of APCs rather than increase their anti-inflammatory properties. Interestingly, infection in the context of TGF-β did not affect the capacity of DCs to produce inflammatory cytokines (Figure 5.4). Taken together, these data demonstrate that CB4 infection in the context of TGF-β yields a population of CD11b+CD11c- APCs with reduced inflammatory properties.

Reduced production of inflammatory cytokines has been previously associated with alternatively activated or type II monocytes/macrophages (M2) (17). As such, we asked whether infection in the context of TGF-β resulted in the polarization of monocytes/macrophages towards an M2 phenotype. To address this question, we measured surface expression of the macrophage scavenger receptor (MSR-A, CD204) and of the
macrophage mannose receptor (MMR, CD206) two markers associated with M2 monocytes/macrophages (21) and of the negative costimulatory molecule programmed death ligand-1 (PD-L1, B7-H1) a molecule associated with the maintenance of peripheral tolerance (22). No differences were observed in the percentage of CD11b+CD11c- APCs expressing CD204, CD206 or CD204 and CD206 between NOD and NODTGFβ mice prior to infection (Figure 5.5A, Figure 5.6) or at day 7 PI (Figure 5.5A, B). Similarly, we observed no differences in PD-L1 expression between CD11b+CD11c- APCs from NOD or NODTGFβ mice at day 7 following CB4 infection (Figure 5.7). This suggests monocytes/macrophages with a typical M2 phenotype are not likely generated following CB4 infection in the context of TGF-β.

5.2.3 CD11b+CD11c- APCs from CB4 infected NODTGFβ mice are sufficient to increase Treg levels in uninfected NOD mice

T1D protection in mice expressing TGF-β is mediated by an increased Treg presence in the pancreas and pancreatic lymph nodes (PLNs) of infected animals correlating with the induction of a “semi-mature” population of CD11b+CD11c- APCs (18). As “semi-mature” APCs have been previously associated with the induction of Treg populations (17), we asked whether CD11b+CD11c- APCs were responsible for the increase of protective Tregs observed in NODTGFβ mice following CB4 infection. CD11b+CD11c- APCs were sorted by flow cytometry from NOD or NODTGFβ mice at day 7 PI with CB4 and adoptively transferred to uninfected 10-12 week old NOD mice. We observed a significant increase in Treg levels in the PLNs (Figure 5.8A) and pancreas (Figure 5.8B) of NOD mice that had been adoptively transferred with CD11b+CD11c- APCs from NODTGFβ mice compared to mock-transferred NOD recipients. Conversely, transfer of the same population purified from infected NOD mice was not sufficient to significantly increase Treg levels in either the PLNs (Figure 5.8A) or the pancreas (Figure 5.8B). These data build on our previous observations (18) and further strengthens the hypothesis that CD11b+CD11c- APCs are central to the induction of Tregs and protection from T1D following CB4 infection in the context of TGF-β. Importantly, transfer of CD11b+CD11c- APCs from NODTGFβ mice infected with CB3, a closely related virus strain that unlike CB4 does not infect pancreatic beta cells, was not
sufficient to induce increases in Treg levels (Figure 5.9). This suggests that infection of pancreatic beta cells and subsequent presentation of self-antigen by “semi-mature” CD11b+CD11c- APCs is a critical requirement for the induction of Tregs.

5.2.4 Reduced expression of CD40 on CD11b+CD11c- APCs is responsible for Treg induction following CB4 infection

CD11b+CD11c- APCs from NODTGFβ mice have previously been demonstrated to express lower surface levels of costimulatory molecules following CB4 infection (18). As such, we asked whether this “semi-mature” phenotype was linked to their capacity to induce Tregs. To determine the importance of CD80 and CD86 signals, we crossed NOD mice deficient for CD28 with NODTGFβ mice (NODTGFβCD28KO) to generate mice expressing TGF-β in the pancreas but lacking the capacity to translate signals from either CD80 or CD86. NODTGFβCD28KO mice were infected with CB4 and Treg levels were measured at day 7 PI. As opposed to what was previously observed in NODTGFβ mice (18), infection of NODTGFβ mice deficient for CD28 did not result in increased Treg levels in the PLNs (Figure 5.10) suggesting that CD28 signals provided by either CD80 or CD86 are required for the induction of Tregs following infection in the context of TGF-β. To address the role of CD40, NOD mice deficient for CD40 (NODCD40KO) were infected with CB4. At day 7 PI, we observed a significant increase in Treg levels in the PLNs (Figure 5.11A) and pancreas (Figure 5.11B) of infected NODCD40KO mice compared to mock-infected mice suggesting an important role for reduced surface expression of CD40 in conferring CD11b+CD11c- APCs with the capacity to induce Tregs. In order to confirm the contribution of CD11b+CD11c- APCs in increasing the Treg levels in NODCD40KO mice following CB4 infection, CD11b+CD11c- APCs were sorted from CB4 infected NODCD40KO mice and adoptively transferred to uninfected 10-12 week old NOD mice. Following adoptive transfer, we observed significantly increased levels of Tregs in the PLNs compared to mock-transferred mice (Figure 5.11C). Taken together, these data suggest that the protection from T1D conferred by pancreatic expression of TGF-β results from the reduction of CD40 expression on CD11b+CD11c- APCs, and this is sufficient to preferentially increase Tregs rather than self-reactive effector T cells.
5.2.5 Treg increases induced by CB4 infection in the context of TGF-β do not result from conversion of naïve T cells to a regulatory phenotype

To better understand the mechanism of protection from T1D, experiments were designed to determine how Tregs levels are increased following CB4 infection in the context of TGF-β. It has been well documented that T cell stimulation in the presence of TGF-β can convert conventional naïve T cells to a Treg phenotype by inducing Foxp3 expression (23, 24). As such, we asked whether Treg increases observed in NODTGFβ mice following CB4 infection resulted from the conversion of non-regulatory T cells. NODThy1.1 mice were crossed with previously described NOD mice expressing a Foxp3GFP reporter (25, 26). Conventional T cells were sorted by flow cytometry as CD4+GFP- cells and adoptively transferred in 10-12 week old NOD or NODTGFβ recipients (expressing the Thy1.2 allele allowing for the differentiation of donor cells). Mice were challenged with CB4 at 24 hours post-transfer and analyzed by flow cytometry at day 7 PI. CD4 T cells of donor origin (Thy1.1+) were readily identifiable in the spleen, PLNs and pancreas of both NOD and NODTGFβ mice (Figure 5.12 and data not shown). However, none of the recovered donor CD4 T cells were positive for GFP in either NOD or NODTGFβ recipients indicating that infection in the context of TGF-β does not induce Foxp3 expression in CD4 T cells (Figure 5.12). These data suggest that conversion does not significantly contribute to the increased levels of Tregs observed in NODTGFβ mice.

As conversion does not significantly contribute to enhance Treg presence following CB4 infection in the context of TGF-β, experiments were designed to address other potential mechanisms. First, we asked if increases in Tregs were due to an outgrowth of a particular Treg clone. Vβ repertoire analysis of Tregs from the PLNs of CB4-infected NOD or NODTGFβ mice revealed no significant changes in Vβ chain usage among these Tregs (Figure 5.13). This suggests that increases in Tregs are not due to an outgrowth of one specific Treg clone but a rather a more generalized increase. We next determined whether infection in the context of TGF-β yielded Tregs that were more resistant to apoptosis. Expression of the anti-apoptotic effector BCL2 was analyzed by intracellular flow cytometry. No significant difference in the level of BCL2 expression (data not shown) or in the
percentage of Tregs expressing BCL2 (Figure 5.14A) was observed between NODTGFβ mice and NOD mice at day 7 PI with CB4. This strongly suggests that Treg increases are not due to a heightened resistance to apoptosis. Finally, we asked whether increased levels of Tregs were due to changes in the capacity of Tregs to proliferate. Expression of the cell division marker Ki67 was analyzed by intracellular flow cytometry. Following infection, we observed a significant increase in Ki67+ Tregs from both NOD and NODTGFβ mice compared to their mock-infected littermates (Figure 5.14B). Further, we observed increases in Tregs expressing Ki67 in CB4 infected NODTGFβ mice compared to similarly infected NOD mice (Figure 5.14B). Although this difference did not reach statistical significance, the number of mice in which greater than 1/3 of Tregs were actively dividing (Ki67+) was higher among NODTGFβ mice (12 of 17 mice, 70.5%) than NOD mice (6 of 16 mice, 37.5%). These results suggest that enhanced Tregs proliferation is one of the contributing factors leading to the increase in Tregs in the pancreas and PLNs following infection.

5.3 Discussion

It is becoming increasingly clear that defects in the maintenance of peripheral tolerance are linked to the development of T1D. Specifically, a decline in the capacity of Tregs to suppress autoreactive T cells has been associated with increased destruction of pancreatic beta cells and progression to T1D in both humans and mice (27). Recent evidence has demonstrated that this decline in suppressive capacity is not due to Treg intrinsic defects but rather may be due to changes at the level of APCs (14, 15). Here, we demonstrate that CD11b+CD11c- APCs play a central role in the acceleration of T1D induced by coxsackievirus B4 infection. Furthermore, we demonstrate that infection in the context of TGF-β reduces the inflammatory properties of this APC population leading to the induction of Tregs and protection from T1D.

Anderson and colleagues have recently demonstrated a link between the CD11b+CD11c- subset of APCs and the declining function of Tregs in NOD mice (14). Together with our data demonstrating that CD11b+CD11c- APCs but not CD11c+ DCs are sufficient to accelerate T1D following CB4 infection, this suggests that CD11b+CD11c- APCs play a major role in the progression of both spontaneous and viral-induced T1D. As
such, our findings suggest that therapies aimed at targeting the inflammatory properties of CD11b+CD11c- APCs may represent a long-term solution to reestablishing peripheral tolerance to pancreatic antigens. In this regard, it has been demonstrated that GA treatment protects from autoimmunity in a mouse model of multiple sclerosis (MS) by acting at the levels of APCs. Specifically, drug treatment reduced the maturation status and inflammatory properties of CD11b+ monocytes and increased their capacity to induce Tregs (17). The similarities in phenotypes observed in the MS model and in NOD mice infected with CB4 in the context of TGF-β including changes in the maturation status and reduced inflammatory cytokine production suggest that glatiramer acetate treatment may represent an interesting therapeutic avenue for the treatment of T1D. Interestingly, it was recently demonstrated that glatiramer acetate treatment of NOD mice increased Treg levels and partially protects from T1D acceleration induced by cyclophosphamide treatment (28). Taken together, these data strongly suggest that CD11b+CD11c-APCs represent a key target for therapies aiming to reestablish tolerance and protect from T1D.

The capacity of CD11b+CD11c- APCs to increase Tregs following infection in the context of TGF-β is correlated with three important features. First, we previously demonstrated that CD11b+CD11c- APCs present with a “semi-mature” phenotype characterized by reduced surface expression of costimulatory molecules (18). Here, we further demonstrate that reduced expression of CD40 is particularly important to heighten the capacity of CD11b+CD11c-APCs from NOD mice to increase Treg levels. This strongly suggests that while CD40 signals are likely involved in the acceleration of T1D by CD11b+CD11c-APCs following CB4 infection, reduction or absence of these signals tips the balance towards Treg induction and reestablishment of tolerance. This has been supported by data from other animal models in which blocking the CD40/CD154 interaction through genetic ablation of either molecule or blocking antibody treatment has been associated with decreased alloimmune responses and increased transplant tolerance (29). Not surprisingly, our data also demonstrated that CD28 signals are required for the induction of Treg following CB4 infection in the context of TGF-β. It is well established that both thymic development and peripheral homeostasis of Tregs are critically dependent on CD28 signals (30, 31). As such, therapies aimed at reducing CD40 expression on CD11b+CD11c- may prove particularly effective at reestablishing Treg function and peripheral tolerance. Second,
CD11b+CD11c- APCs generated following CB4 infection produce lower levels of the inflammatory cytokines TNF-α and IL-6. This may have several effects on disease progression. In particular, T1D acceleration following CB4 infection has been hypothesized to progress through a bystander activation mechanism that relies upon presentation of previously sequestered antigens in an inflammatory milieu (4). As such, reduced inflammation likely curtails the activation and/or expansion of autoreactive T cells. IL-6 has previously been associated with blocking Treg function (32) and preventing the expression of the Treg specific transcription factor Foxp3 in order to favor the generation of Th17 clones (33). As such, decreased IL-6 production may be linked to the capacity of CD11b+CD11c- APCs from NODTGFβmice to increase Treg levels. Third, infection of pancreatic beta cells appears to be a critical requirement for the induction of Tregs by CD11b+CD11c- APCs. This strongly suggests that the presentation of pancreatic self-antigens by semi-mature APCs is an important requirement for the generation of protective Tregs in this model. Taken together, our data demonstrate that infection in the context of TGF-β contributes to protection from T1D by decreasing the capacity of CD11b+CD11c- APCs to activate autoreactive responses and increasing their immunoregulatory properties.

The generation of protective Tregs following infection in the context of TGF-β may represent an extension of a natural process that allows maintenance of tolerance to self-antigens, following viral infections. Infections with LCMV have long been described to protect NOD mice from the development of T1D (34). Filippi and colleagues have recently demonstrated that this protection is associated with increases in several immunoregulatory mechanisms that are likely activated to prevent immunopathology following the resolution of an antiviral response. In particular, they demonstrated that the resolution of an acute LCMV infection is associated with increased TGF-β production and that increased levels of Tregs can protect from the induction of T1D (35). This suggests that any mechanism that serves to increase the production of TGF-β may be sufficient to reestablish long-term tolerance and protect from T1D.

Finally our data demonstrate that the heightened Treg levels observed following infection in the context of TGF-β are not due to conversion of naïve T cells to a regulatory phenotype or an increase in Treg survival. Instead, we observed a higher percentage of actively dividing Tregs suggesting that increased proliferation may, in part, be responsible
for the higher Treg levels observed following CB4 infection in the context of TGF-β. In particular, this was supported by our data demonstrating a large increase in the percentage of NODTGFβ mice in which greater than 1/3 of Tregs were observed to be actively dividing compared to infected NOD mice. Interestingly, the percentage of NOD mice in which greater than 1/3 of Tregs were dividing correlated with the percentage of WT NOD mice that are typically observed to be protected from the development of T1D following CB4 infection (approximately 30 to 35%) (5, 18). As such, it is possible to hypothesize that under certain circumstances, CB4 infection in the absence of constitutive TGF-β expression is sufficient to induce Treg proliferation and protect rather than accelerate disease. This mechanism is likely still dependent on TGF-β as was described in NOD mice following LCMV infection (35) and will warrant further investigation. Other mechanisms may also contribute to the induction of Tregs following CB4 infection in the context of TGF-β. Particularly, recent data have demonstrated that Foxp3 expression is unstable in NOD mice and that Tregs can convert to a highly inflammatory phenotype and can directly contribute to T1D pathogenesis (36). It has yet to be determined whether CB4 infection in the context of TGF-β may function to stabilize Foxp3 expression and this will warrant further investigation.

In conclusion, our results demonstrate that CB4 infection in the context of TGF-β confers immunoregulatory properties to CD11b+CD11c- APCs, a population that appears to play a critical role in the progression of both spontaneous and viral-induced T1D. Our results provide clear evidence that reducing the inflammatory properties of this APC subset is sufficient to switch the effects of CB4 infection from a diabetogenic to a protective role. Taken together with evidence that the decline in Treg function associated with disease progression is not due to Treg intrinsic defects (14), this argues for the targeting of this APC subset as a powerful therapeutic tool for the long-term reestablishment of peripheral tolerance to beta cell antigens.

5.4 Materials and methods

**Mice.** NOD/ShiLtJ mice were obtained from The Jackson Laboratory (Bar Harbor, USA). NODTGFβ transgenic mice expressing TGF-β under the control of the human insulin promoter were generated in the laboratory of Dr. N. Sarvetnick (University of Nebraska
NODCD40KO and NODThy1.1 and NODFoxp3GFP mice were obtained from the JDRF Center on Immunological Tolerance at Harvard Medical School. NODCD28KO mice were obtained from Jackson laboratories (Bar Harbor, USA) and crossed with NODTGFβ mice to generate NODTGFβCD28KO mice. All mice were bred and maintained in our rodent facility and tested for diabetes prior to infection. Non-fasting blood glucose levels were measured with a standard glucometer and mice with two consecutive readings of >300mg/dl were considered diabetic. All procedures performed followed the guidelines of the institutional animal care committee.

**Virus.** Stocks of CB4 Edwards strain 2 and CB3 Nancy strain were originally obtained from the laboratory of Dr. Charles Gauntt (University of Texas at San Antonio) and were prepared as described previously (38, 39). 10-12 week old mice were infected intraperitoneally with sublethal (less than half LD<sub>50</sub>) doses of 400 plaque forming units (PFU).

**Flow cytometry.** Single cell suspensions were stained for the appropriate markers and analyzed by flow cytometry on an LSRII cell analyzer (BD Biosciences, Missisauga, Canada). Fluorescently conjugated antibodies directed against CD11b (clone M1/70), CD11c (clone N418), PD-L1 (clone M1H5), CD4 (clone L3T4), CD25 (clone PC61), BCL2 (clone 10C4) and Foxp3 (clone FJK-16s) were purchased from eBiosciences (San Diego, USA). Fluorescently conjugated antibodies directed against Ki67 (clone B56) were purchased from BD Biosciences (Missisauga, Canada). Fluorescently conjugated antibodies directed against CD204 (clone 2F8) and biotin-conjugated antibodies directed against CD206 (clone MR5D3) were purchased from AbD Serotec (NC, USA). Data were analyzed with FlowJo software (Tree Star).

**APC adoptive transfer.** Single cell suspensions were generated from spleens of CB4 infected NOD or NODTGFβ mice at day 7 following CB4 infection. Cells were stained with fluorescently conjugated antibodies directed against CD11b and CD11c and sorted on a FACSARia flow cytometer (BD Bioscience, Missisauga, Canada). 2 x 10<sup>5</sup> purified CD11b+CD11c- or the corresponding percentage of CD11c+ cells were adoptively transferred intraperitoneally into uninfected NOD mice and spleens, pancreata and pancreatic lymph nodes were harvested at day 7 post-transfer for Treg analysis or followed for T1D incidence.
**Ex vivo cytokine analysis.** CD11b+ CD11c- cells and CD11c+ cells were sorted by flow cytometry as described above and 1X10^5 cells were cultured for 24 hours at 37°C in IMDM containing 10% fetal bovine serum. Supernatants were harvested and cytokine levels were measured using a BD CBA inflammation kit (BD Bioscience, Mississauga, Canada) or a Mouse TGF-beta1 ELISA Ready-SET-Go! kit from eBioscience (San Diego, USA) Samples were prepared according to manufacturer's instructions and bead array samples were analyzed on a BD FacsArray equipped with FCAP software (BDBiosciences). Cytokine levels were normalized and are presented as a percentage of cytokine production compared to WT NOD mice.

**Isolation of pancreatic infiltrating cells.** Pancreata were isolated from infected NOD and NODTGFβ mice and mechanically disrupted. Single cell suspensions were treated for 10 minutes at 37°C in a PBS solution containing 1mg/ml of collagenase. Recovered cells were stained for flow cytometry.

**Treg conversion assay.** Single cell suspension from NODFopx3GFPThy1.1 mice were stained with fluorescently conjugated antibodies directed against CD4 and sorted by flow cytometry. 2 X 10^6 CD4+GFP- (non-Tregs) cells were adoptively transferred intraperitoneally into NOD mice or NODTGFβ mice 24 hours prior to infection with 400 pfu of CB4. At day 7 post-infection, conversion to a Treg phenotype was analyzed by measuring GFP (Foxp3) expression in CD4+Thy1.1+ cells from the spleen, pancreas and pancreatic lymph nodes.

**Statistical analysis.** Statistical analysis was performed with Prism GraphPad software. The unpaired Student’s t-test (flow cytometry analysis) was used for statistical analysis. A P value of less than 0.05 was considered significant. Stars denote significance.
Figure 5.1: Adoptive transfer of CD11b+CD11c- APCs from CB4-infected NOD mice is sufficient to accelerate T1D onset

Diabetes incidence of 10-12 week old uninfected NOD mice adoptively transferred with CD11b+CD11c- APCs FACS sorted from CB4-infected NOD mice at day 7 PI (black line) or mock-transferred (grey line).
Figure 5.2: CD11b+CD11c- APCs produce lower levels of inflammatory cytokines following CB4 infection in the context of TGF-β

Ex vivo production of A) TNF-α or B) IL-6 from CD11b+CD11c- APCs FACS sorted from CB4 infected NOD (black bar) or NODTGFβ (white bar) mice. Cytokine levels were measured from culture supernatants following 24 hours of incubation. Data represent mean ± s.e.m of normalized cytokine levels from pooled mice in 6 separate experiments. Stars denote significance.
Figure 5.3: CD11b+CD11c- APCs produce higher levels of TGF-β following CB4 infection in the context of TGF-β

*Ex vivo* production TGF-β from CD11b+CD11c- APCs FACS sorted from CB4 infected NOD (black bar) or NODTGFβ (white bar) mice. Cytokine levels were measured from culture supernatants following 24 hours of incubation. Data represent mean + s.e.m of normalized cytokine levels from pooled mice in 3 separate experiments.
Figure 5.4: CB4 infection in the context of TGF-β does not affect the capacity of DCs to produce cytokines

Ex vivo production of A) TNF-α or B) IL-6 from CD11c+ DCs FACS sorted from CB4 infected NOD (black bar) or NODTGFβ (white bar) mice. Data represent mean + s.e.m of normalized cytokine levels from pooled mice in 3 separate experiments.
Figure 5.5: CB4 infection in the context of TGF-β does not increase expression of type II monocytes/macrophage markers on CD11b+CD11c-APCs

A) Representative flow cytometry plots of CD204 and CD206 expression on the surface of CD11b+CD11c-APCs from NOD (top panels) or NODTGFβ (bottom panels) mice infected with CB4 (right panels) or mock-infected with DMEM (left panels) B) Percentage of CD11b+CD11c-APCs from NOD (black bars) or NODTGFβ (white bars) mice positive for surface expression of CD204, CD206 or CD204 and CD206 at day 7 post-infection with CB4. Data represent mean ± s.e.m from 3 separate experiments.
Figure 5.6: CD11b+CD11c- APCs from NODTGFβ mice do not express increased levels of type II monocyte/macrophage surface markers

Percentage of CD11b+CD11c- APCs from uninfected NOD (black bars) or NODTGFβ (white bars) mice positive for surface expression of CD204, CD206 or CD204 and CD206. Data represent mean + s.e.m from 3 separate experiments.
Figure 5.7: CD11b+CD11c- APCs from NODTGFβ mice do not express increased levels of PD-L1 compared NOD mice following CB4 infection

A) Representative histogram of surface PD-L1 expression on CD11b+CD11c- APCs from NOD (black line) or NODTGFβ (grey line) mice at day 7 following CB4 infection. B) Average mean fluorescence intensity (MFI) of PD-L1 expression on the surface of CD11b+CD11c- APCs from NOD (black bar) or NODTGFβ (white bars) mice at day 7 following CB4 infection. Data represent mean + s.e.m. from 2 separate experiments.
Figure 5.8: Adoptive transfer of CD11b+CD11c- APCs from CB4 infected NODTGFβ mice is sufficient to increase Treg levels in uninfected NOD mice

Average percentage of CD4+ T cells expressing Foxp3 in A) the PLNs or B) the pancreas of 10-12 week uninfected NOD recipients mock transferred with DMEM (grey bars) or adoptively transferred with CD11b+CD11c- APCs FACS sorted from CB4 infected NODTGFβ (white bars) or NOD mice (black bars). Data represent mean + s.e.m. from at least 4 separate experiments. Stars denote significance.
Figure 5.9: Adoptive transfer of CD11b+CD11c- APCs from CB3-infected NODTGFβ mice is not sufficient to increase Treg levels in uninfected NOD mice

Average percentage of CD4+ T cells expressing Foxp3 in the PLNs of 10-12 week uninfected NOD recipients mock transferred with DMEM (grey bars) or adoptively transferred with CD11b+CD11c- APCs FACS sorted from CB3 infected NODTGFβ (white bars) mice (black bars). Data represent mean + s.e.m. from 3 separate experiments.
Figure 5.10: CB4 infection in the context of TGF-β is not sufficient to increase Treg levels in the absence of CD28 signaling

Average percentage of CD4+ T cells expressing Foxp3 in the PLNs of NODTGFβCD28KO mice mock-infected with DMEM (white bar) or infected with CB4 (black bar). Data represent mean + s.e.m. from 3 separate experiments.
Figure 5.11: Absence of CD40 on CD11b+CD11c- APCs is sufficient to increase Treg levels following CB4 infection

Average percentage of CD4 T cells expressing Foxp3 in the A) PLNs or B) pancreas of NODCD40KO mice mock-infected with DMEM (white bars) or infected with CB4 (black bars). C) Average percentage of CD4+ T cells expressing Foxp3 in the PLNs of uninfected 10-12 week old NOD mice adoptively transferred with CD11b+CD11c- FACS sorted from CB4 infected NODCD40KO (black bar) or mock-transferred with DMEM (white bar). Data represent mean + s.e.m from at least 3 separate experiments. Stars denote significance.
Figure 5.12: CB4 infection in the context of TGF-β does not induce conversion of naïve T cells to adaptive Tregs

Representative flow cytometry plots of CD4 and GFP (Foxp3) expression of donor (Thy1.1+) cells recovered from the spleen (left panels) or PLNs (right panels) of NOD (top panels) or NODTGF-β (bottom panels) recipient mice at day 7 following infection with CB4.
Figure 5.13: CB4 infection in the context of TGF-β does not significantly alter the Treg repertoire

Percentage of CD4+Foxp3+ cells from the PLNs of NOD (black bars) or NODTGFβ (white bars) mice expressing a specific Vβ chain at day 7 following CB4 infection.
Figure 5.14: Heightened Treg levels induced following infection in the context of TGF-β is partially due to an increase in proliferation

A) Percentage of CD4+Foxp3+ T cells expressing the anti-apoptotic effector molecule BCL2 in the PLNs of NOD (black bars) or NODTGFβ (white bars) mice infected with CB4 or mock-infected with DMEM. B) Percentage of CD4+Foxp3+ T cells expressing the cellular proliferation marker Ki67 in the PLNs of NOD (black bars) or NODTGFβ (white bars) mice infected with CB4 or mock-infected with DMEM. Data represent mean ± s.e.m. from at least 4 separate experiments.
5.5 References

rather than quantitative age-dependent changes in pathogenic T-cells. *Diabetes* 54:1415-1422, 2005


Chapter 6

Conclusions and future directions
6.1 Discussion

The principal objective of this thesis was to gain a better understanding of the link between innate signals on APCs and the induction of autoimmune diseases following coxsackievirus infection. In particular, experiments were designed to test the hypothesis that the functional consequences of innate recognition of viruses by a subset of APCs could ultimately lead to the induction of autoimmunity. By extension, we also hypothesized that modulation of this APC subset may represent a therapeutic avenue to ensure the maintenance of tolerance following viral infection rather than the induction of autoimmunity. Taken together, the data presented in this thesis highlight a critical role for CD11b+CD11c- APCs (a subset containing a large proportion of monocytes and macrophages) in both protection of the host from the consequences of viral infection as well as the development of autoimmunity following infection.

My results have indicated that CD11b+CD11c- APCs play a critical role in the early immune response to CB4 infection in a TLR3-dependent manner (1). TLR3 deficiency results in greatly enhanced viral replication and cardiac damage leading to a dramatic increase in mortality following sub-lethal challenges with CB4 (1). Interestingly, I demonstrated that transfer of CD11b+CD11c- cells from WT mice was sufficient to reduce cardiac damage and protect TLR3 deficient mice following CB4 infection (1). Furthermore, I demonstrated that TLR3 deficiency only affected the production of a subset of inflammatory cytokines and chemokines and did not affect cellular activation of either innate or adaptive effectors (1). Conversely, while MyD88-dependent signals were not necessary for survival, my results indicated that signaling through the MyD88 pathways was required for the activation of several innate and adaptive cell types (1). Importantly, these results suggest that TLR3 signals do not function to bridge the innate and adaptive immune responses. Rather, it seems that recognition of coxsackievirus infection by TLR3 serves primarily to activate innate mechanisms of protection, likely in macrophages and monocytes. In turn, these protective mechanisms ensure that cardiac viral loads do not reach lethal levels before the adaptive immune response is fully activated and able to mediate viral clearance. My results suggest that this may be in part due to the reduction in chemokines that attract macrophages and monocytes to the heart as well as to a reduction of TNF-α. A decrease in TNF-α
production may be of particular significance as this likely results in lower nitric oxide production, a molecule previously described to be critical for cardiac protection following coxsackievirus infection (2). Together with a previous publication (3), my results demonstrated that TLR3 signaling may be particularly critical for host protection following infection with cardiotropic viruses. It is also intriguing to note that host protection from RNA viruses is not always dependent on the same TLR signaling pathways even when they are members of the same virus family. For example, TLR3 has been demonstrated to play only a minor role in the immune response to TMEV, another picornavirus (4). As such, requirements for specific PRRs in the host response to infection may be more closely linked to the tissue tropism of a virus rather than simple virus family commonalities. This suggests that the wide variety of PRRs capable of recognizing viruses is not simply part of a redundant system but rather may serve to facilitate the development of an immune response tailored to a specific virus.

Interestingly, my results have demonstrated that this same subset of CD11b+CD11c-APCs is also involved in the pathogenesis of both autoimmune myocarditis (5) and T1D (6) (Richer et al., manuscript in preparation). In particular, I observed that the adoptive transfer of CD11b+CD11c-APCs from CB4 infected NOD mice is sufficient to accelerate T1D in uninfected recipient mice (Richer et al., manuscript in preparation). Together with my findings that CD11c+DCs are not sufficient to accelerate disease, this strongly suggests that CD11b+CD11c-APCs play a central role in activating autoreactive T cells and accelerating the ongoing autoimmune destruction of beta cells. Alternatively, this population of APCs may directly contribute to the destruction of beta cells. To this effect, macrophages were demonstrated to be sufficient to destroy beta cells in a transgenic mouse model even in the absence of T cells (7). These mechanisms are not mutually exclusive and CD11b+CD11c-APCs may contribute to the pathogenesis of CB4-induced T1D using a combination of both mechanisms. I further observed that protection from coxsackievirus-induced T1D (6) and autoimmune myocarditis (5) in NOD mice expressing TGF-β within the pancreatic islets is correlated with the induction of “semi-mature” CD11b+CD11c-APCs. This strengthens our hypothesis that this APC subset is involved in the pathogenesis of both of these autoimmune diseases following CB4 infection. Results from our group (8) and others (9) have demonstrated that an increase in macrophages and monocytes infiltrating cardiac tissue is
associated with heightened pathology in models of autoimmune myocarditis. Similarly, monocytes and macrophages are among the first cell types to invade islets in humans (10) and mice (11, 12) and depletion of macrophages is sufficient to delay or prevent onset of T1D in mouse models (13, 14). Furthermore, several reports have demonstrated increased inflammatory properties for these cells in T1D patients (15-17). Taken together, this suggests that CD11b+CD11c- APCs are critical for early recognition and protection from virus infection, in a TLR3-dependent manner. However, in susceptible individuals these cells appear to be improperly regulated leading to an increase in inflammatory mediator production, activation of autoreactive T cells and the development of autoimmunity post-infection.

The importance of CD11b+CD11c- APCs to the pathogenesis of coxsackieviral-induced autoimmune diseases suggests that therapies aimed at regulating their function may serve to protect from the induction of autoimmunity post-infection. Here, I demonstrated that infection in the context of pancreatic expression of TGF-β induces “semi-mature” CD11b+CD11c- APCs (6) (Richer et al., manuscript in preparation). In the T1D model, these “semi-mature” APCs present with reduced inflammatory properties and an increased capacity to increase the levels of protective Tregs in both the pancreas and the PLNs of NOD mice (6) (Richer et al., manuscript in preparation). I have further demonstrated that these Tregs are ultimately responsible for protection of NODTGFβ mice following CB4 infection and that ablation of these cells reestablishes susceptibility to CB4-induced T1D (6). The increased capacity of CD11b+CD11c- APCs to induce Tregs is of particular interest. T1D progression in NOD mice is associated with a decline in the capacity of Tregs to suppress autoreactive T cells (18). It was recently demonstrated that, in both human and mice, this does not represent a Treg intrinsic defect but rather a defect in the capacity of APCs to maintain Treg function (19, 20). This suggests that therapies aimed at supplementing T1D patients directly with Tregs may be doomed to fail as APCs would be similarly unable to maintain their suppressive capacity over time. Conversely, therapies allowing for the correction of the APC defect may serve as a long-term tool to reestablish peripheral tolerance. My results demonstrate that even a single dose of TGF-β at the time of viral infection is sufficient to increase Treg levels and transiently protect from T1D (6). This suggests that even short-term therapies aimed at inducing immunoregulatory APCs may prove protective. Of further
interest, the generation of “semi-mature” APCs was also associated with protection from CB3-induced autoimmune myocarditis (5). As CB3 was not observed to induce Tregs following infection in the context of TGF-β (6) this suggests that modulating APC function may also have direct protective effects that are independent of the generation of Tregs.

Any therapeutic approach targeted at CD11b+CD11c- APCs would need to consider the importance of this same subset of APCs in early protection of the host following coxsackievirus infection (1). Importantly, infection in the context of TGF-β did not affect the capacity of the host to respond to infection (6). My results demonstrated that despite a reduction of the inflammatory properties of CD11b+CD11c- APCs the antiviral properties of these cells were unaffected as NODTGFβ mice did not present with increased mortality or harbor increased viral loads following CB4 infection (6). This suggests that the functions of CD11b+CD11c- APCs that are required for protection of the host following coxsackievirus infection do not completely overlap with the functions that contribute to the development of autoimmunity. This is supported by our data that while protection of the host following CB4 infection is independent of the maturation status of this subset (1), the autoimmune promoting properties of CD11b+CD11c- APCs seem to be strongly linked to costimulatory molecule expression, particularly CD40 (Richer et al., manuscript in preparation). Gaining a better understanding of the signals required for the activation of innate function and maturation of this APC subset may provide insight into the development of therapies aimed at preventing viral-induced autoimmune diseases.

In addition to reestablishing long-term peripheral tolerance, therapies aimed at reducing the inflammatory properties of monocytes and macrophages may have additional benefits for T1D patients. Coronary heart disease is one of the major health complications associated with the diabetic state (21). Furthermore, it has been suggested that the increased inflammatory status of monocytes and macrophages in T1D patients may contribute to the increased risk of heart disease (15). This has been particularly linked to increased expression of IL-6 which, in turn, leads to increased production of C-reactive protein, a risk factor for atherosclerosis (15). Interestingly, we observed that CB4 infection in the context of TGF-β yields CD11b+CD11c- APCs producing lower levels of IL-6 (Richer et al., manuscript in preparation). As such, therapies aimed at reducing the inflammatory properties of monocytes
and macrophages may not only serve to reestablish peripheral tolerance but may also reduce some of the secondary health complications associated with T1D.

In summary, the work presented here illustrates a critical role for CD11b+CD11c- APCs in both the immune response to viral infection and the development of autoimmunity. Taken together with reports that glatiramer acetate, a drug commonly used to treat MS patients, protects from EAE in mouse models by reducing the inflammatory properties of this APC subset (22), my work strongly suggests that targeting CD11b+CD11c- APCs represents an interesting therapeutic avenue for treatment of autoimmune diseases. Furthermore, this work highlights the critical importance of immune regulation mechanisms and the delicate balance between protection of the host from an invading pathogen and the development of immune mediated pathology.

6.2 Future directions

6.2.1 Pattern recognition receptors and coxsackievirus-induced type 1 diabetes

Accumulating evidence suggests that recognition of viruses by PRRs and their functional consequences contribute to the development of autoimmune diseases. Although a role for TLRs has been suggested in the pathogenesis of spontaneous T1D (23-25), very little is currently known about the contribution of PRRs to the development of T1D following CB4 infection. Detailing the role of the TLR3 and MDA-5 signaling pathways may be of particular interest. I demonstrated that, as opposed to what is observed for a several viruses, the TLR3 signaling pathway is of critical importance for early immune responses to infection with CB4 (1). Furthermore, TLR3 ligands have been shown to synergize with KRV infections and increase the incidence of T1D in BBDR rats (26). This suggests that TLR3-mediated recognition of CB4 infection may contribute to the pathogenesis of T1D following viral infection. Preliminary results have indicated that NOD mice deficient for TLR3 may not be susceptible to CB4-induced T1D acceleration. Analysis of these data has been inherently complicated by the dramatic increase in mortality observed in TLR3 deficient mice following CB4 infection (1). As such, experiments will need to be repeated with a much greater number of mice in order to make proper conclusions. If TLR3 deficiency is observed to confer
protection from T1D, experiments aimed at understanding the mechanism of protection should be pursued. In particular, the role of CD11b+CD11c- APCs should be addressed. I have demonstrated that TLR3 signals on this APC subset are critical for survival (1). Therefore, it will be of interest to determine whether TLR3 signals on this APC subset are also required for the acceleration of T1D. These questions can be addressed using an adoptive transfer approach as detailed in Chapter 2 and Chapter 5. If TLR3 signals on CD11b+CD11c- APCs are required for the acceleration of T1D following CB4 infection, adoptive transfer of WT CD11b+CD11c- APCs into TLR3 deficient NOD recipient should reestablish the susceptibility of NODTLR3KO mice to CB4-induced T1D. Conversely, adoptive transfer of CD11b+CD11c- APCs from infected NODTLR3KO mice should not be sufficient to accelerate T1D in uninfected NOD recipients, as opposed to what I described for CD11b+CD11c- APCs from infected WT NOD donors (Richer et al., manuscript in preparation). These results would confirm the importance of TLR3 signals for the diabetogenic potential of this APC subset. These experiments should provide some valuable information linking TLR signaling, APCs and the acceleration of T1D following viral infection.

Recent genome-wide association studies have associated another innate detector of viral infection, MDA-5 (also known as interferon induced with helicase C domain 1 (IFIH1)) with the susceptibility to T1D (27, 28). Specifically, one common non-synonymous single nucleotide polymorphism (nsSNP) and several rarer polymorphisms in the gene coding for MDA-5 have been ascribed a protective role for T1D (27, 28). MDA-5 is a cytoplasmic sensor of dsRNA that has been associated with the detection of picornaviruses, including CB3 (29, 30). The association of polymorphisms resulting in reduced MDA-5 function (31) with a reduced risk of T1D development strongly suggests that detection of coxsackievirus infection by MDA-5 plays a significant role in viral induction of T1D. One way to address these questions is to generate NOD mice deficient for MDA-5 as well as knock-in NOD mice in which the WT MDA-5 coding alleles are replaced by mutated alleles expressing the common nsSNPs. The generation of a biologically relevant knock-in mouse model is possible as the residues identified in humans are conserved in mice. The recent description of NOD mouse derived germline-competent embryonic stem cells will further facilitate the generation of these knock-in mice by removing the need to generate this model on a different
genetic background followed by several rounds of backcrossing (32). Following generation of these mouse models, T1D incidence following CB4 infection can easily be measured. If, as hypothesized, NOD mice deficient for MDA-5 or expressing mutated alleles of MDA-5 are protected from CB4-induced T1D, experiments aimed at measuring the effects of MDA-5 on APC, autoreactive effector T cell and Treg activation will be pursued. The generation of these mouse models on the NOD genetic background will allow for the analysis of the contribution of MDA-5 signaling to the induction of T1D following CB4 infection and is likely to offer significant insight into the mechanism of viral-induction of T1D.

The relative contribution of other TLR and TLR signaling adaptors to the induction of T1D following CB4 infection will also be of importance. In particular, it will be interesting to study the role of the TLR2 signaling pathway and the central TLR signaling adaptor MyD88 as they have both been ascribed a role in the development of spontaneous T1D in NOD mice (23, 24). TLR2 has been proposed to detect secondary necrotic cells leading to the activation of autoreactive T cells and TLR2 deficient NOD mice develop T1D with delayed kinetics and reduced onset compared to WT NOD mice (23). TLR2 signaling has further been suggested to transiently suppress Treg function and this may further contribute to the pathogenesis of T1D following detection of secondary necrotic cells (33). MyD88 deficient NOD mice are completely protected from T1D through a mechanism involving changes in the natural flora leading a reduction in the development of beta cell specific autoreactive T cells (24). Preliminary results have suggested that TLR2 or MyD88 deficiency is sufficient to protect NOD mice from the acceleration of T1D following CB4 infection. One possibility is that NODTLR2KO mice and NODMyD88KO mice have reduced numbers of autoreactive T cells and, as such, do not meet the primary criteria for susceptibility to CB4-induced T1D. This question can be easily addressed by measuring the severity of pancreatic infiltration compared to WT NOD mice, as a reduction in pancreatic infiltration may be an indication of a decrease in the number of autoreactive T cells. Alternatively, the relative diabetogenic potential of T cells from TLR2 or MyD88 deficient can also be addressed using an adoptive transfer approach. If protection cannot simply be explained by a lack of autoreactive T cells, it will be interesting to determine the role of TLR2 and/or MyD88 in the pathogenesis of viral-induced T1D. In particular, the signals leading to the activation of the TLR2 signaling pathway following viral infection will need to be clarified. TLR2 is
commonly associated with the detection of bacterial products although evidence suggests that TLR2 can also recognize certain viral (34) and self-derived ligands (35). As such, it is possible to hypothesize that infection of pancreatic islets leads to the increase of certain self-ligands that, in turn, activate the TLR2 signaling pathway and contributes to the pathogenesis of T1D. Alternatively, TLR2 may be directly involved in recognizing viral products. One way to address these questions is to replace viral infection with treatments with a low-dose of a pancreatic toxin, streptozotocin (STZ). In the BDC2.5 model, low-dose STZ treatment induces T1D through an APC dependent mechanism that is very similar to the mechanism of T1D induction following CB4 infection (36). This may allow determining whether TLR2 contributes to the pathogenesis of viral-induced T1D directly by recognizing viral infection or indirectly by recognizing changes in self-antigen expression following infection. A proteomics approach comparing pancreatic beta cells from uninfected, STZ-treated and CB4-infected mice may ultimately allow for the identification of some of the danger signals associated with progression to T1D. Taken together, these future studies would allow for a better understanding of the innate signals linking viral infections with the induction of autoimmune diseases such as T1D.

6.2.2 Cytokines and coxsackievirus-induced type 1 diabetes

One important functional consequence of innate recognition of viruses is the production of inflammatory cytokines. In particular, I have observed that protection from both T1D and autoimmune myocarditis in TGF-β expressing NOD mice is associated with changes in the production of IL-6 (5) (Richer et al., manuscript in preparation). Recent data from our laboratory have demonstrated that as opposed to what has been previously suggested in other models, deficiency of IL-6 does not protect from the induction of autoimmune myocarditis following CB3 infection (8). Instead, IL-6 plays a regulatory role by dampening early immune responses to viral infection (8). The role of IL-6 in viral-induced T1D remains to be elucidated. We have recently backcrossed IL-6 deficient mice to the NOD genetic background. Preliminary data indicate that IL-6 deficient NOD mice still develop spontaneous T1D making these mice a suitable model to study the role of this cytokine in viral-induced T1D. To address the importance of IL-6 in the progression of CB4-
induced T1D, NODIL6KO mice can be infected with CB4 and incidence of T1D and pancreatic pathology can be measured. One possibility is that IL-6 production will be important for the immune response to CB4 infection and infection will result in increased mortality. If this is the case, low-dose STZ treatment may serve as an alternative to viral infection to study the role of IL-6 in induced T1D. If IL-6 deficiency results in protection from CB4-induced T1D, analysis of the effects of this deficiency on the APC, autoreactive effector T cell and Treg populations will need to be addressed. I have recently demonstrated that CD11b+CD11c- APCs play a central role in the acceleration of T1D following CB4 infection (Richer et al., manuscript in preparation). I have further established that the protection observed following CB4 infection in the context of TGF-β correlates with the induction of “semi-mature” CD11b+CD11c- APCs producing lower levels of inflammatory cytokines including IL-6 (6) (Richer et al., manuscript in preparation). As such, it will be interesting to address the importance of IL-6 production for the capacity of CD11b+CD11c-APCs to accelerate T1D following CB4 infection using an adoptive transfer approach as detailed in Chapter 5. If the production of IL-6 by CD11b+CD11c- APCs is necessary for the acceleration of T1D then transfer of CD11b+CD11c- APCs from CB4 infected NODIL6KO mice should not be sufficient to accelerate T1D in uninfected NOD recipients. Taken together, the data generated from these experiment should provide insight into the role of inflammatory cytokines in the induction of T1D following CB4 infection.

Another inflammatory cytokine, IL-17, has recently been determined to play a pathogenic role in autoimmune disorders, including MS and autoimmune myocarditis (37, 38). Recent evidence has suggested that IL-17 may also be involved in the pathogenesis of T1D although this remains controversial (39-42). As such, it would be interesting to address the role of this cytokine in the development of T1D following CB4 infection. Preliminary results have suggested that IL-17 blockade using a soluble IL-17 receptor molecule (IL-17RA-Fc) protects from the acceleration of T1D following CB4 infection. Further experiments will need to address the role of IL-17 in the pathology of viral-induced T1D. In particular, it will be interesting to determine the source of IL-17 following CB4 infection. Although IL-17 production by Th17 T cell clones is now well established, recent evidence has suggested that innate cell types, including γδ T cells and NK T cells, also contribute to IL-17 production (43, 44). As acceleration of T1D following CB4 infection occurs rapidly (6,
it is possible to hypothesize that production of IL-17 from an innate cell type contributes to the pathogenesis of T1D. The effects of IL-17 production on autoreactive effector T cell activation and Treg function will also need to be addressed. Interestingly, IL-17 production has recently been demonstrated to be important for the establishment of a Th1 response following infection with an intracellular bacterial pathogen (46). As such, it will also be critical to determine the effects of IL-17 blockade on the immune response to CB4 infection. The data from these experiments will allow the determination of what role, if any, IL-17 plays in the immune response to coxsackievirus infection and the development of T1D.

6.2.3 APC subsets and coxsackievirus-induced type 1 diabetes

APCs have previously been demonstrated to be important for the induction of T1D following CB4 infection in the BDC2.5 mouse (47) and here I demonstrated that a particular subset of APCs is central to disease acceleration in the NOD mouse (Richer et al., manuscript in preparation). Further experiments will need to be performed to determine the mechanism of diabetes acceleration by CD11b+CD11c-APCs. Experiments can be designed to address whether this population truly acts as APCs and present antigens leading to the activation of autoreactive T cells. This can first be addressed by adoptive transfer of CD11b+CD11c-APCs sorted by FACS from NOD mice deficient for MHC class I or MHC class II and therefore unable to present antigen to CD8 or CD4 T cells respectively. Loss of diabetogenic potential would indicate a requirement for antigen presentation in order to accelerate T1D and may also provide insight into the relative contribute of CD4 and CD8 T cells in viral-induced T1D. The capacity of CD11b+CD11c-APCs from infected NOD mice to induce T cell proliferation and/or activation of autoreactive T cells can be further addressed using dual adoptive transfer experiments. Uninfected NOD mice would be adoptively transferred with CFSE labeled beta-cell antigen specific CD4 or CD8 T cells from previously described T-cell receptor transgenic mice (BDC2.5 and NOD8.3 mice) and CD11b+CD11c-APCs from infected or uninfected NOD mice. The capacity of this APC subset to induce proliferation and/or activation of T cells can then be measured by flow cytometry by analyzing CFSE dilution and upregulation of surface markers associated with T cell activation on the adoptively transferred T cells recovered from the pancreas or PLNs of
recipient mice. CD11b+CD11c- APCs include a majority of monocytes and macrophages but they may also include other cell types. Therefore, it would be interesting to further characterize the specific cell type involved in disease acceleration. To address these questions, APCs can be purified from CB4 infected NOD mice on the basis of other surface markers, including F4/80 and CD14 to differentiate macrophages and monocytes, and the diabetogenic potential of each subset can be measured following adoptive transfer to uninfected NOD recipients. These experiments would help identify the specific subset of APCs involved in acceleration of T1D following CB4 infection and provide insight into the mechanisms of this acceleration.

Although my data indicate a central role for CD11b+CD11c- APCs in the acceleration of T1D following CB4 infection, other subsets of APCs may also be involved. Of particular interest, marginal zone B (MZB) cells have been suggested to be involved in the progression of T1D by acting as APCs and activating autoreactive T cells in the PLNs (48). As such, it would be interesting to determine the role of this innate subset of B cells in the pathology of coxsackievirus-induced T1D. To address these questions, MZB cells can be sorted by FACS from CB4-infected NOD mice based on their surface expression of the B cell marker CD19 and their relative surface expression of CD21 and CD23 which allows for separation of this B cell subset from other B cells. Adoptive transfer to uninfected NOD mice as detailed in Chapter 5 can then be used to assess the diabetogenic potential of MZB cells. If MZB cells are demonstrated to play an important role in the pathology of CB4-induced T1D, studies should be extended to measure the effects of MZB cells on the activation of autoreactive effector T cells and the suppressive capacity of Tregs. The data gathered from these experiments would extend our knowledge of the mechanisms of viral-induced T1D and may validate MZB cells as a potential therapeutic target for the prevention of both spontaneous and viral-induced T1D.

6.2.4 Modulation of APCs and the prevention of coxsackievirus-induced type 1 diabetes

I have demonstrated that protection from T1D following CB4 infection in the context of localized expression of TGF-β correlates with the induction of “semi-mature”
CD11b+CD11c- APCs that, in turn, are responsible for increased levels of protective Tregs (6) (Richer et al., manuscript in preparation). This strongly suggests that CD11b+CD11c- APCs represent an important therapeutic target for the long-term reestablishment of tolerance and protection from T1D development. TGF-β has a broad-range of effects on several cell types suggesting that systemic therapy with this cytokine may not be practical, as it runs the risk of severe side effects. As such, it would be important to identify alternative approaches to manipulate APC function. Treatment with glatiramer acetate, a drug commonly used for MS patients, is sufficient to protect from the induction of EAE in mouse models (22).

Interestingly, glatiramer acetate-mediated protection occurs through the generation of “semi-mature” monocytes/macrophages that induce protective Tregs (22). Taken together with observations that glatiramer acetate treatment can induce Tregs in NOD mice (49), it would be interesting to determine whether this drug can prevent the induction of viral-induced T1D. To address these questions, mice can be treated with glatiramer acetate during the acute phase of CB4 infection and the incidence of T1D, the maturation and inflammatory status of CD11b+CD11c- APCs as well as the presence of Tregs in the pancreas and PLNs of treated mice can all be measured as described in Chapters 4 and 5. Alternatively, I have demonstrated that reduction or absence of CD40 expression on the surface of CD11b+CD11c- APCs enhances their capacity to induce Tregs (Richer et al., manuscript in preparation). Blockade of CD40-CD154 interactions using specific antibodies has been successful in enhancing transplant tolerance (50). Therefore, it will be interesting to determine whether blockade of CD40 signals is sufficient to induce protection from CB4-induced T1D. To address these questions, mice can be treated with a blocking antibody directed at CD154 (clone MR-1) during the course of CB4 infection and the incidence of T1D, the maturation and inflammatory status of CD11b+CD11c- APCs as well as the presence of Tregs in the pancreas and PLNs of treated mice can all be measured as described in Chapters 4 and 5. Taken together, these experiments would strengthen the hypothesis that CD11b+CD11c- APCs represent a prime therapeutic target and may provide some information leading to the development of therapies aimed at reestablishing tolerance to pancreatic antigens and protection from T1D.
6.2.5 Tregs and the prevention of coxsackievirus-induced type 1 diabetes

I have demonstrated that Tregs play a central role in the protection of NODTGFβ mice following CB4 infection (6). Specifically, I observed increased Treg levels in both the pancreas and PLNs of infected NODTGFβ mice (6). Several questions remain to be addressed regarding the specificity and function of these Tregs. First, it will be important to address whether the Tregs are activated in an antigen-specific manner. Results from my experiments have suggested that one of the requirements for the increase in protective Tregs is the capacity of the virus to infect pancreatic beta cells (6). This suggests that presentation of self-antigens may be required for the induction of Tregs. This is supported by data demonstrating that Tregs do not possess suppressive capacity in the absence of activation with their cognate antigen (51). In order to address this question, we could generate TCR transgenic NOD mice specific for a known antigen that is not expressed in the pancreas. For example, NOD mice expressing the DO11.10 Vβ chain have been previously described (52). These mice express T cells specific for an ovalbumin peptide presented in the context of the I-A^d MHC class II molecule. These TCR transgenic mice can be crossed with NODTGFβ mice to generate NOD mice expressing TGF-β in the pancreas in which the majority of T cells are specific for a non-beta cell antigen (ovalbumin). If our hypothesis that increased Treg levels in NODTGFβ mice requires recognition of beta cell self-antigens is correct, then CB4 infection would not be sufficient to increase Treg levels in DO11.10 TCR transgenic NODTGFβ mice. To further strengthen these observations, we could also generate NODTGFβ mice transgenic for CD4 T cell clones specific for pancreatic beta cell antigens such as the BDC2.5 or BDC6.9 transgenic mice. Provided that their cognate antigen is presented following CB4 infection (which is likely for the BDC2.5 clone as CB4 infection is known to induce T1D in these transgenic mice (53)) then infection of BDC2.5TGFβ or BDC6.9TGFβ mice should results in greatly increased levels of Tregs in the pancreas and PLNs of these mice due to their specificity for self-antigens. Taken together, these results would demonstrate whether recognition of cognate self-antigen by Tregs is a requirement for their induction following CB4 infection in the context of TGF-β. Second, we will need to determine whether Tregs generated following CB4 infection in the context of TGF-β suppress in an antigen-specific manner. It has been suggested that, following activation with
their cognate antigen, Tregs can suppress effector T cells with a broad antigenic specificity (51). However, my results indicate that despite increases in Tregs that can protect from the induction of T1D, the anti-viral immune response remains unaffected suggesting a level of antigenic specificity for the suppressive action of Tregs (6). These questions can be addressed with in vitro suppression assays using T cells from various NOD TCR transgenic mouse strains specific for beta or non-beta cell antigens as a source of effector T cells. If Tregs generated following CB4 infection in the context of TGF-β are specific for self-antigen then only T cells from beta cell specific and not from ovalbumin specific TCR transgenic mice should be suppressed in vitro. These results would indicate whether Tregs generated following infection in the context of TGF-β are broadly suppressive or only suppressive towards autoreactive effector T cells. Taken together, these experiments would provide insight into the mechanism of Treg activation and suppression in this model.

Progression to T1D has been associated with declining Treg function (18) and decreased stability of Foxp3 expression (54). As such, it would be interesting to determine whether CB4 infection of NODTGFβ mice simply results in increased numbers of Tregs or whether it also affects Treg function. We can address these questions in vitro with suppression assays. We can also confirm these results in vivo using an adoptive transfer approach and measuring the relative protective capacity of varying numbers of Tregs from age-matched CB4 infected NOD and NODTGFβ mice. A recent study has demonstrated that Foxp3 expression is unstable on the NOD genetic background and that loss of Foxp3 expression leads to the generation of highly inflammatory self-reactive cells that could contribute to T1D progression (54). As such, it would be interesting to determine whether CB4 infection in the context of TGF-β leads to increased stability of Foxp3 infection. In order to address these questions, reporter NOD mice allowing for the identification of ex-tregs (Foxp3-GFP-Cre X R26-YFP mice) would be crossed with NODTGFβ mice and infected with CB4. Ex-Tregs are readily identifiable by flow cytometry allowing for a relative measurement of Foxp3 stability. These experiments would address whether infection in the context of TGF-β protects from T1D simply by increasing Treg numbers or whether changes in Treg function and stability also play a role in protection.
In conclusion, the work presented here identifies an important subset of APCs that is critical for the immune response to virus while also directly contributing to the development of autoimmunity. We further confirmed our hypothesis that it possible to manipulate this particular subset of APCs in order to prevent the development of autoimmunity while maintaining the capacity of the host to respond to the invading pathogen. Together with the data I have presented in this thesis, the future work proposed here would significantly extend our understanding of the mechanisms that ultimately lead to the development of autoimmunity following coxsackievirus infection. In particular, we would gain a better understanding of the innate signals leading to the deregulation of APC populations and the induction of autoimmunity. Understanding the mechanisms of disease development will provide important insight and potentially reveal new therapeutic targets. This would ultimately allow for the development of targeted therapies aimed at reestablishing peripheral tolerance and preventing the development of autoimmune diseases.
Figure 6.1: Schematic representation of protection from T1D induced following CB4 infection in the context of TGF-β

Similarly to the requirements for acceleration of T1D, induction of protection following CB4 infection in the context of TGF-β appears to be dependent on the capacity of the virus to infect beta cell. Following infection in the presence of TGF-β, CD11b+CD11c-APCs present with a “semi-mature” phenotype and reduced inflammatory properties. These APCs are in turn sufficient to increase levels of Tregs in the pancreas and PLNs leading to protection from T1D. Modified from Autoimmunity Reviews, 8(7), Richer MJ and Horwitz MS, Coxsackievirus infection as an environmental factor in the etiology of type 1 diabetes, 611-5, © 2009, with permission from Elsevier.
6.4 References


43. Lockhart E, Green AM, Flynn JL: IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 177:4662-4669, 2006


The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: B06-0199

INVESTIGATOR OR COURSE DIRECTOR: Marc S. Horwitz

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: Virus mediated autoimmune disease

APPROVAL DATE: August 24, 2009  START DATE: October 26, 2006

APPROVED CONTAINMENT LEVEL: 2

FUNDING TITLE: Signaling through T-like receptors influences the regulation of coxackieviral mediated autoimmune myocarditis
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

FUNDING TITLE: Signaling through toll-like receptors influences regulatory T cells and consequently the development of type 1 diabetes
FUNDING AGENCY: Canadian Diabetes Association

FUNDING TITLE: Chromosomal determinants in the induction of autoimmune myocarditis
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

FUNDING TITLE: Viral-mediated autoimmune disease
FUNDING AGENCY: UBC Start Up Funds

FUNDING TITLE: Chromosomal determinants in the induction of autoimmune myocarditis
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

FUNDING TITLE: Bacterial Expression of Anti-HIV Antibodies for Microbicide Use
FUNDING AGENCY: Beth Israel Deaconess Medical Centre

FUNDING TITLE: Viral-mediated autoimmune disease
FUNDING AGENCY: UBC Dean of Science

FUNDING TITLE: Laboratory of Viral Immunopathology
FUNDING AGENCY: Canada Foundation for Innovation
UNFUNDED TITLE: N/A

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ANIMAL CARE CERTIFICATE

Application Number: A08-0622
Investigator or Course Director: Marc S. Horwitz
Department: Microbiology & Immunology
Animals:

Mice 1700

Start Date: July 1, 2008
Approval Date: September 27, 2009

Funding Sources:
Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Chromosomal determinants in the induction of autoimmune myocarditis

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Signaling through T-like receptors influences the regulation of coxsackieviral mediated autoimmune m...

Funding Agency: Canadian Diabetes Association
Funding Title: Induction of regulatory T cells during viral-mediated type 1 diabetes

Funding Agency: UBC Dean of Science
Funding Title: New Faculty Start Up Grant
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Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

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# ANIMAL CARE CERTIFICATE

## BREEDING PROGRAMS

**Application Number:** A08-0415  
**Investigator or Course Director:** Marc S. Horwitz  
**Department:** Microbiology & Immunology  
**Animals:**

| Mice NOD 300  |
| Mice NODIL4 30 |
| Mice NODBecillko 30 |
| Mice dNTBRII 30 |
| Mice BDC2.5 50 |
| Mice CSS mice 500 |
| Mice MBPNP 30 |
| Mice NODGMCSF 30 |
| Mice IL6KO 200 |
| Mice NODTGFb 200 |
| Mice NODTLRkos 300 |
| Mice NODCD28ko 50 |
| Mice NODCD40ko 30 |

**Approval Date:** August 5, 2009  
**Funding Sources:**  
**Funding Agency:** Canadian Diabetes Association
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