MDA5 AND A TYPE 1 INTERFERON SIGNATURE IN THE DEVELOPMENT OF TYPE 1 DIABETES

by

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Abstract

Type 1 diabetes (T1D) is a debilitating disease involving the autoimmune destruction of insulin-producing pancreatic β-cells. The personal and economic burden of this disease is enormous, therefore simpler and more cost effective therapeutic approaches than those currently available must be explored.

In children at risk for T1D, a unique type 1 interferon (IFN-I) transcriptional signature precedes islet autoimmunity. Recent onset of T1D strongly associates with infection by RNA viruses like coxsackievirus that induce IFN-I. Importantly, genetic variants in the T1D risk locus *IFIH1* are linked to protection from T1D and result in reduced expression of the RNA virus sensor melanoma differentiation-associated protein 5 (MDA5), which is also a critical component in establishing the IFN-I signature.

In chapter 2 we describe a novel model where we have translated the reduced MDA5 expression phenotype observed in patients onto the non-obese diabetic (NOD) mouse and established its importance in T1D. We describe the first observations that a reduction in MDA5 in the NOD mouse protects from spontaneous and coxsackievirus B4 (CB4)-induced T1D. We also establish the importance of a specific IFN-I signature in the development of T1D as a result of reduced (not eliminated) MDA5 sensing of CB4 that allows for regulatory T cells (Tregs) at the site of autoimmunity and protects from CB4 induced T1D.

In chapter 3 we show that this unique IFN-I signature is limited to MDA5 and disease pathogenesis is linked to the specific IFN-I response induced by the virus as a strain of CB3 failed to modify the IFN-I signature associated with disease. Further RNA sequencing discussed in Chapter 4 demonstrates unique tissue-specific differential gene profiles associated with a reduction in MDA5 following CB4 infection.

Our results support our hypothesis that there is a direct correlation between the IFN-I signature induced following environmental challenge with the induction of a strong

effector T cell and a matched Treg response. This work demonstrates the essential role of MDA5 signaling in regulating the IFN-I signature, implicates MDA5 in T1D susceptibility and in protection against IFN-I and T1D-inducing agents like CB4 and suggests restricting MDA5 function as a potential T1D therapeutic.

Preface

All of the work presented henceforth was conducted in the Life Sciences Center at the University of British Columbia, Point Grey campus. Animal studies were conducted in the Centre for Disease Modeling at the Life Sciences Center and in the Modified Barrier Facility in the Pharmaceutical Sciences building at the University of British Columbia. RNA sequencing was conducted at the UBC Biomedical Research Centre by Ryan Vander werff. Dr. Marc Horwitz was the supervisory author on this thesis and was involved throughout for all the projects in concept formation and manuscript composition.

A version of Chapter 2 was recently published with the title "Reduced expression of the MDA5 gene IFIH1 prevents autoimmune diabetes" in the journal Diabetes with Pamela J. Lincez, Iryna Shanina and Dr. Horwitz as contributing authors to the manuscript. I contributed to the design and conducted all the experiments and wrote the manuscript, Iryna Shanina conducted experiments and Dr. Horwitz contributed to the design of experiments and co-wrote the manuscript.

For the work discussed in Chapters 3 and 4, I was the lead investigator for all the experiments with advice, guidance and revision of work throughout by Dr. Horwitz. The RNA sequencing experiments and preliminary analysis discussed in Chapter 4 was performed by Ryan Vander werff.

All animal work was performed under strict accordance with the recommendations of the Canadian Council for Animal Care. The protocol was approved by the Animal Care Committee (ACC) (certificate numbers: A08-0415, A08-0622, and A130116) and the Biosafety Committee (certificate number B140086) of the University of British Columbia.

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APC	antigen presenting cell
BBDR	Bio-breeding diabetes resistant rat BCL2 B-cell leukemia/lymphoma 2
CAR	coxsackie and adenovirus receptor
CB3	coxsackievirus B3
CB4	coxsackievirus B4
CBV	coxsackievirus group B
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cell
DCM	dilated cardiomyopathy
DMEM	Dulbecco's modified eagle medium
ds	double stranded
EAE	experimental autoimmune encephalomyelitis
EAM	experimental autoimmune myocarditis
ELISA	enzyme-linked immunoabsorbent assay
EMCV	encephalomyocarditis virus
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
Foxp3	forkhead box p3
GSIS	glucose-stimulated insulin secretion
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV	herpes simplex virus
Idd	insulin-dependent diabetes
ICA	islet cell autoantigen
IFIH1	interferon induced helicase protein 1
IFN	interferon
IFN-I	type 1 interferons
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IRF	interferon regulatory factor 4
ISG	interferon-stimulated genes
ISRE	interferon-stimulated response element
KO	knock out
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
MAVS	mitochondrial antiviral signaling adaptor
MCP-1	monocyte chemoattractant protein 1
MDA-5	melanoma differentiation-associated gene-5
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1

MS	multiple sclerosis
MyD88	myeloid differentiation primary response gene 88
NK	natural killer cell
NO	nitric oxide
NOD	non-obese diabetic
nsSNP	non-synonymous single nucleotide polymorphism
OAS	oliogoadenylate cyclase synthase 1b
PAMP	pathogen associated molecular pattern
PD-L1	programmed death ligand 1
PFU	plaque forming unit
PI	post-infection
PI3K	phosphoinositide 3-kinase
PIV	paramyxovirus
PLN	pancreatic lymph node
PRR	pattern recognition receptor
PTPn22	protein tyrosine phosphatase non-receptor type 22
RANTES	regulated on activation normal T cell expressed and secreted
RIG-I	retinoic-acid-inducible-gene I
RLR	RIG-I-like receptors
RNA	ribonucleic acid
SCID	severe combined immunodeficient
SNP	single nucleotide polymorphism
SEM	standard error on the mean
T1D	type 1 diabetes
Tbet	T-box expressed in T cells
TCR	T-cell receptor
TGF-β	transforming growth factor-β
Th	helper T cell
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
Treg	regulatory T cell
Teff	effector T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
VSV	vesicular stomatitis virus
WT	wild-type

Acknowledgements

When I was a girl I wanted to be a marine biologist. Didn't we all? Well, twenty five or so years later, after thirteen years of school after high school and a journey through biochemistry to biotechnology to microbiology and immunology, from the East coast to the West coast- I have become what I think I really meant I wanted to be- a scientist. There are many people to acknowledge for helping me through the years it took to get from kindergarten to now completing a doctoral degree.

First I'd like to thank my doctoral team, my lab members and give my utmost gratitude to my supervisor Marc Horwitz for his patience, support, advice and faith that I'd find my way as a scientist. To my committee members Pauline Johnson, Ninan Abraham and Ru Tan, thank you for your helpful insights and support over the years, especially for your interest in my science communication endeavors. And thank you Banff alumni for letting me get creative, weird, terrified and courageous about SciComm.

Next, I'd like to acknowledge those more personally acquainted, putting the ladies first, I'd like to give much love to all the amazing women in my life. First and foremost, thank you Mom. You've always told me I was born under a shining star and I thank you for your endless encouragement. Thank you Nana for playing piano with me and for your amazing one-liners. Thank you to the GLB girls and their little girls for all the love. Thank you to my Van buds- RD, AJ, ZG, AO, EL, AC, SG, BP, SL, CD you're all a force to reckon with. EL thanks for teaching me a box slide. AJ as you said, there's nothing but big ideas- let's keep climbing mountains. RD I'm glad we took 14hrs to get home and KM let's go surfing! Thank you Joni, Stevie, Aretha, Etta, Nina, Erykah, Whitney, Mariah, Janet, Lauryn, Chantal, Esthero and Gwen for the sweet sounds.

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Thank you Mac for always being there. You are the best listener I know. Thank you Mat for always making it a competition, for bringing Taessa and Aurelia in to our lives and making me feel so darn SMRT. Thank you Paul for taking me fishing. Thank you JD for making me take Phys/Cal, KP for telling me to always go forwards and never backwards, SG for the studio, vinyl and much aloha, and BH for making life a little bit more exciting. Thank you Van, Bob, Neil, Mick, Michael, Marvin, James, Paul, George, Ringo, John, Jack, Jimmy and Jim for the soundtrack to my life.

Last but not least, thank you to my grandfather Sydney Mathews who has inspired my dreams of scientific discovery and has always motivated me to keep on to the end of the road, whatever that end may be.

Dedication

To my family…

Chapter 1: Introduction

1.1 The pancreas

The findings presented in this work offer a glimpse of the complex process that ensues in type 1 diabetes (T1D). Before we delve into the detail and all the parts of the process, I think it appropriate to first discuss and appreciate the structure and function of the whole part, the pancreas, of which the disease consumes.

Mammals, birds, reptiles and amphibians have a pancreas. Invertebrates do not have a pancreas, though pancreatic-like cells have been identified in the gut and brain. The organization of pancreas tissue is species-specific, where the organ is compact in primates, dogs and hamsters; is compact in part (the tail or splenic section) in rats and mice; and is dispersed across the mesentery in rabbits. The pancreas is a glandular organ with two functional units, the exocrine and endocrine pancreas that develop from a common progenitor cell population [1].

The exocrine pancreas is comprised of acinar, centroacinar and ductal cells and accounts for close to 90% of the organ's cell mass. Together, the cells of the exocrine pancreas serve a digestive function by secretion of digestive enzymes, ions, mucous and water into the duodenum of the gastrointestinal tract. With a loss in exocrine pancreas function, as a result of acute or chronic inflammation in the cases of acute or chronic pancreatitis, or in cases of cystic fibrosis, malabsorption of nutrients and malnutrition progresses, ultimately leading to a condition known as exocrine pancreas insufficiency (EPI). The exocrine pancreas is further divided into peri- and tele-insular regions, with peri-acinar cells lying in close proximity to the islets of Langerhans in the endocrine pancreas. In a rat pancreas, thousands of lobules form a vascular bed within the exocrine pancreas, where hundreds of islets lie within lobules (intralobular) or in tissue lining the secretory ducts (interlobular) creating an islet-acinar portal system. This islet-acinar axis creates an integrated vascular bed where the exocrine pancreas gains access to arterial blood flowing from splenic and superior mesenteric arteries into pancreatic islets. Blood drains from intralobular islets and through islet-acini portal vessels, flowing across the exocrine

acini and into the venous system. With the islet-acinar axis, the exocrine pancreas is also exposed to hormones, antigens and neurotransmitters released from endocrine cells like the hormone insulin produced by islet β cells of the endocrine pancreas. The exposure to insulin, though positive for exocrine homeostasis and function as well as pancreatic growth, has implicated the exocrine pancreas in the autoimmune disease T1D, where depletion in β cell production of insulin, as observed with T1D, has shown to affect exocrine pancreas structure and function [2]. An increase in immune cell infiltrates in the exocrine pancreas of T1D patients has also been observed though the contribution to the development or maintenance of disease is not well understood [3].

Most of what we understand about the events leading and contributing to the autoimmune process that ensues in the pancreas of T1D patients is from studies in mice and studies that focus primarily on the events in the endocrine pancreas. In rats and mice this second functional unit comprises only about 2% of the pancreas and is where we find the islets of Langerhans. The number of islets across the gastric lobe, duodenal head and tail anatomical structures of the pancreas is strain-specific in mice. The non-obese diabetic (NOD) mouse, the most commonly used mouse model for T1D, has typically 120 islets in the gastric lobe, comparable to the number found in an age-matched CD1 mouse, a mouse model for type 2 diabetes, whereas in the duodenal head and tail, the NOD mouse has 549 and 489 islets compared to 453 and 686 observed in a CD1 mouse[4]. A human pancreas has close to a million islets comprised of α, β, δ, ε, and pancreatic polypeptide (PP) cells[5]. The distribution of cell types in human islets is quite varied with α cells that secrete glucagon representing 35-40%, the somatostatin producing δ cells about 10-15% and the β cells, the hallmark cells of T1D that provide insulin and amylin, representing close to 50% of all cells in human islets[6]. Whether the differences between species, in the distribution of cell types within the islet or the islet architecture are relevant in disease development remains unclear.

The architecture of human islets is distinct from rodents in that all cells in the islet border capillaries as well as form heterologous contacts with β cells towards the center of the

islet [7]. This heterologous contact between β and non-β cells facilitates islet paracrine function. In rodents, β cells primarily form a homologous core, demarcated with a border of non-β cell types. Rodent and human islets also differ in their innervation patterns, where rodent islets are richly innervated from acetylcholine releasing paracrine inputs and human islets are scarcely and indirectly innervated from α cell cholinergic signaling[8]. Hormone secretion and nervous system function are critical for proper endocrine function. In human islets, α cell secreted acetylcholine is critical in priming β cells to release insulin as a response to wavering blood glucose concentrations and maintain endocrine regulated glucose metabolism [8]. The multi-cellular architecture of islets allows for multiple signals (humoral, nervous stimulation, and cell-cell contact communication through connexions, cadherins, ephrins etc.) that regulate proper β-cell function under both basal and glucose-stimulated conditions[9]

1.2 Glucose-stimulated insulin secretion

Insulin is the only hormone secreted by the adult mammal body that can reduce blood glucose concentrations. Most of our knowledge of insulin regulation, its role in glucose metabolism and the detrimental effects of its insufficiency that lead to diabetes mellitus have been extensively studied in rodents, with the aim of translating this knowledge to understand human diabetes [10]. The challenge in the field continues to be defining normal islet biology in contrast to biological phenotypes associated with diabetes. Since Toronto researchers Frederick Banting and Charles Best identified in the 1920s[11] that insulin is released by the pancreas and acts as an anti-diabetic factor, a comprehensive model for glucose-stimulated insulin secretion (GSIS) has been developed to establish insulin regulation in a normoglycemic state. The central dogma of GSIS, pioneered by work from Dean and Matthews in 1968, begins when food intake gradually increases plasma glucose levels and flushes glucose through specific transporter molecules on the plasma membrane of pancreatic β cells[12].

Upon entry into the β cell, glucose is phosphorylated and enters glycolysis to produce pyruvate. The metabolism of pyruvate then generates adenosine triphosphate (ATP), which stimulates ATP-sensitive K^+ channels to close and induces β cell membrane

depolarization. The induction of membrane potential opens voltage-dependent Ca^{2+} channels (VDCC), leading to an influx in Ca^{2+} and stimulation of insulin exocytosis. Release of insulin into the bloodstream, through the islet's intricate vascular architecture, allows the hormone to bind the α-subunit of its tetrameric membrane anchored receptor on cells and act in a receptor-ligand complex[13]. Stimulation of the tyrosine kinase insulin receptor induces a series of intracellular kinase activity leading to phosphoinositide 3 (PI3)-kinase isolation and transport of passively diffused glucose to the mitochondria, where glucose is used to make ATP or stored as glycogen.

Glucose homeostasis is tightly regulated by proper GSIS function as well as by the secretion of a variety of hormones and molecules that exert glucoregulatory functions according to fasting or fed, diabetic and non diabetic states[14]. Cyclic AMP (cAMP) produced as a result of glucose metabolism and incretin receptor activation contributes to β cell responsiveness to glucose. Glucagon released by islet α-cells, acts opposing to insulin, stimulates glucose production from hepatic stores and increases plasma glucose levels[15]. The gut derived incretin hormone glucagon-like peptide-1 (GLP-1) and β cell produced hormone amylin are also critical regulators of glucose homeostasis[16, 17].

Many layers have been added and have expanded the GSIS model over the past four decades-from the biphasic insulin release and time-dependent potentiation mechanisms identified in the late 1970s to the recently described role of 1-deoxysphinganine and its inhibition of GSIS and induction of diabetes[18-21]. Biphasic release of insulin, involving the release of insulin rapidly for a few minutes followed by the sustained insulin release, is a critical process in maintaining glucose homeostasis, endocrine function and is also thought to play an autocrine role in regulation of GSIS[22]. Changes in insulin secretion dynamics have detrimental consequences to glucose tolerance. Inhibition of the first insulin response and a reduction to the second are hallmark features of type 2 diabetes (T2D) mellitus- the most common chronic form of diabetes. As a better physiological understanding of GSIS components unfolds therapeutics targeting GSIS pathway molecules can be developed to prevent GSIS inhibition and the onset of diabetes [23, 24]. Insulin secratagogues targeting ATP-sensitive K^+ channels have been

used in T2D therapy and most recently bispecific antibodies coupling a GSIS targeted monoclonal antibody (mAb) and a therapeutic arm mAb targeting another diabetes-associated factor have successfully prevented T1D in NOD mice[23]. Restoring proper GSIS function and glucose homeostasis can effectively reverse T2D, however with T1D, where the autoimmune destruction of pancreatic beta cells leads to complete loss of insulin secretion, the inhibition of GSIS and subsequent lack of insulin production is unfortunately only one casualty in a very complex disease.

1.3 Autoimmune diabetes

The immune system is an intricate network of components and mechanisms biologically designed to protect their host from danger, whether the danger is a foreign invader or an inappropriate, misguided self-directed attack. Under normal homeostasis circumstances, central tolerance should eliminate T cells in the thymus that react with self-antigens and peripheral tolerance by way of autoantigen specific natural regulatory T cells, should contain self-reactivity in the periphery. Unfortunately, though tightly regulated, immune tolerance can become dysregulated and mount an inappropriate response against itself leading to autoimmunity. The aberrant self-directed immune responses that lead to autoimmunity can destroy tissues and instigate systemic and organ-specific disorders categorized as autoimmune diseases.

Diabetes umbrellas metabolic diseases, divided into two etiopathogenic categories: type 1 and type 2, characterized by abnormal insulin secretion, insulin function or both that lead to chronic hyperglycemia[25]. In the type 1 category, diabetes is an organ-specific chronic autoimmune process where the T cell-mediated destruction of pancreatic β cells results in the complete loss of insulin secretion. Disease onset occurs after a large majority of β cells have been destroyed and presents years in advance of clinical symptoms. The second diabetes category, T2D, encompasses most (90-95%) diabetes cases, results in defective insulin responsiveness and secretion, but not in the autoimmune destruction of pancreatic β cells[26, 27].

1.4 Type 1 diabetes

1.4.1 Clinical presentation

At the time of clinical presentation, more than 90% of T1D patients test positive for one or more autoantibodies. Autoantibodies against glutamic acid decarboxylase (GAD65), islet antigen-2 (IA2, also known as ICA512 and PTPRN), insulin, islet-specific glucose-6-phosphatase catalytic subunit-related protein IGRP (also known as G6PC2), zinc transporter 8 (ZNT8) and islet cell autoantigen (ICA) are among those commonly detected. Relatives of T1D patients can also be determined by autoantibody screening. T1D typically manifests between the ages of 6 and 15 years, however, rates of diagnosis are becoming increasingly common in children younger than 5 years of age. Disease incidence can also peak in later stages of adolescence. At diagnosis, some patients can present with polyuria, polydipsia, weight loss, visual abnormalities, high levels of glycosylated haemoglobin A1c and even severe metabolic abnormalities including ketoacidosis that all suggest hyperglycaemia and insulin deficiency has ensued. Other asymptomatic patients can be diagnosed inadvertently, prior to insulin deficiency, by routine urine and blood tests. Following clinical presentation, some patients experience what has been described as a 'honeymoon' phase of disease, where natural insulin production is restored. This phase does not last in patients progressing to chronic autoimmunity, where eventually patients will depend again on exogenous insulin and dietary restrictions. The proper maintenance of disease, or capacity for endogenous insulin production is evaluated by the level of C peptide, a by-product of proinsulin processing by β-cells, that remains above 0.2nmol/L. Proper monitoring of blood glucose levels by measuring glycosylated haemoglobin A1c levels helps patients avoid chronic eye and renal diseases and acute instances of insulin-induced hypoglycaemia[28].

1.4.2 Disease incidence

On a global scale, T1D accounts for 5-10% of diabetes cases and is most prevalent in Northern European populations and individuals of European descent. The number of patients with T1D worldwide is estimated at about 3 million with about 78,000 children diagnosed every year and incidence greatly varying between countries[29]. Presentation of the disease is more prevalent in boys and men, unlike most other autoimmune disease

with a greater bias in female incidence. Diagnosis peaks in children 5-7 years old and at or close to puberty. Disease is also diagnosed, though less commonly, in adults. Disease incidence and even development of autoimmunity up to years before symptomatic T1D presentation has demonstrated seasonal synchronization. Diagnosis has been more frequent in autumn and winter seasons and births in spring have been more closely tied with increased susceptibility. There is also a degree of variation observed in geographical incidence with adjacent regions like Finland and Estonia recording separate incidence rates and totals. Even the rates of increase in incidence across age groups is variable where in Europe, disease incidence is rising in children of ages younger than 5[30]. All of these observations of incidence variability from seasonal to geographical to age distribution variance strongly suggest that an environmental factor is contributing to and influencing disease pathogenesis. Specific diets, the gut microbiome, hygiene, vitamin D levels, and virus infections have all been implicated as etiologic agents affecting T1D susceptibility and epidemiology.

1.4.3 Disease susceptibility

In a landmark paper in 1986, George Eisenbarth proposed a model of T1D etiology that suggested a trifecta of immunologic, genetic and environmental factors was responsible for the natural development of the disease[31]. This classical model suggests that in a genetically susceptible individual, harboring a certain number of β cells, an encounter with an environmental agent consequentially triggers activated, β cell directed autoreactive T cells to progressively reduce β cell numbers and induce a substantial loss in insulin secretion to a point that eventually leads to the presentation of clinical disease. Over the past three decades, in an effort to better understand the events leading to the onset of islet autoimmunity and to develop a means to prevent disease, researchers have refined and extended this classical model (Figure 1.1) by identifying genes that influence individual susceptibility, describing mechanisms and their components that drive autoimmunity and tolerance dysregulation (Figure 1.2), and identifying environmental factors in new onset cases and, or, associated with disease pathogenesis[28].

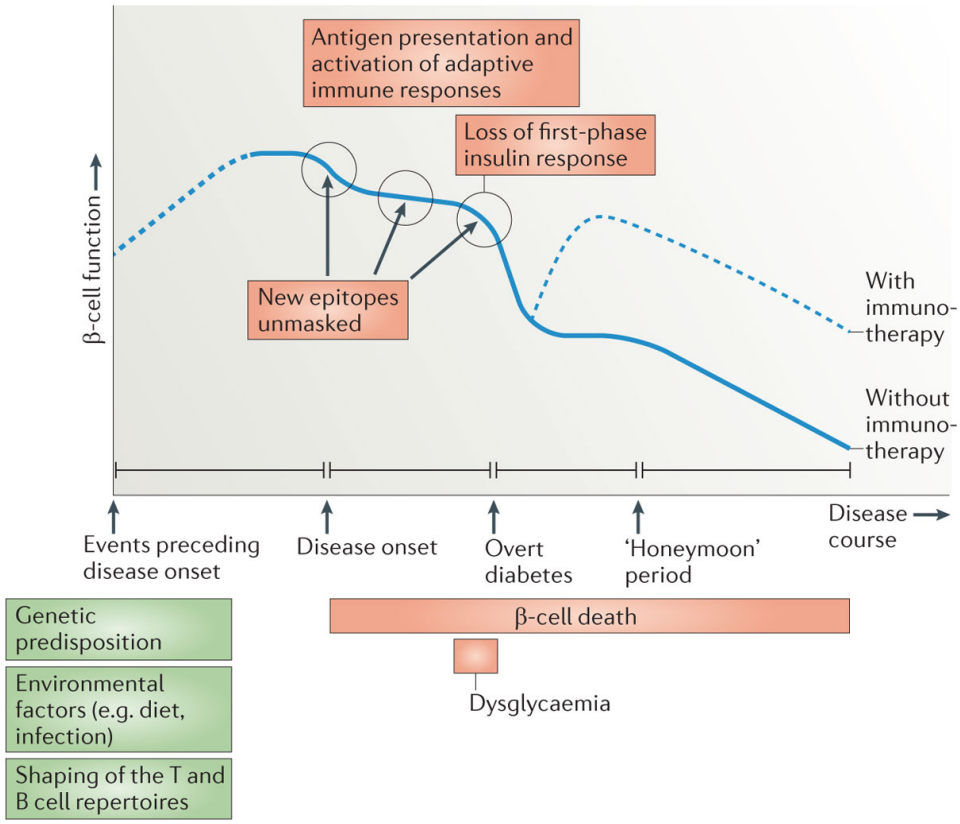


Figure 1.1 The natural progression of T1D. Over the past three decades, in an effort to better understand the events leading to the onset of islet autoimmunity and to develop a means to prevent disease, researchers have refined and extended this classical model by identifying genes that influence individual susceptibility, describing mechanisms and their components that drive autoimmunity and tolerance dysregulation, and identifying environmental factors in new onset cases and, or, associated with disease pathogenesis. Reprinted by permission from Nature Publishing Group: Nature Reviews Immunology, Kevan C. Herold, Dario A. A. Vignali, Anne Cooke & Jeffrey A. Bluestone. Type 1 diabetes: translating mechanistic observations into effective clinical outcomes © 2013

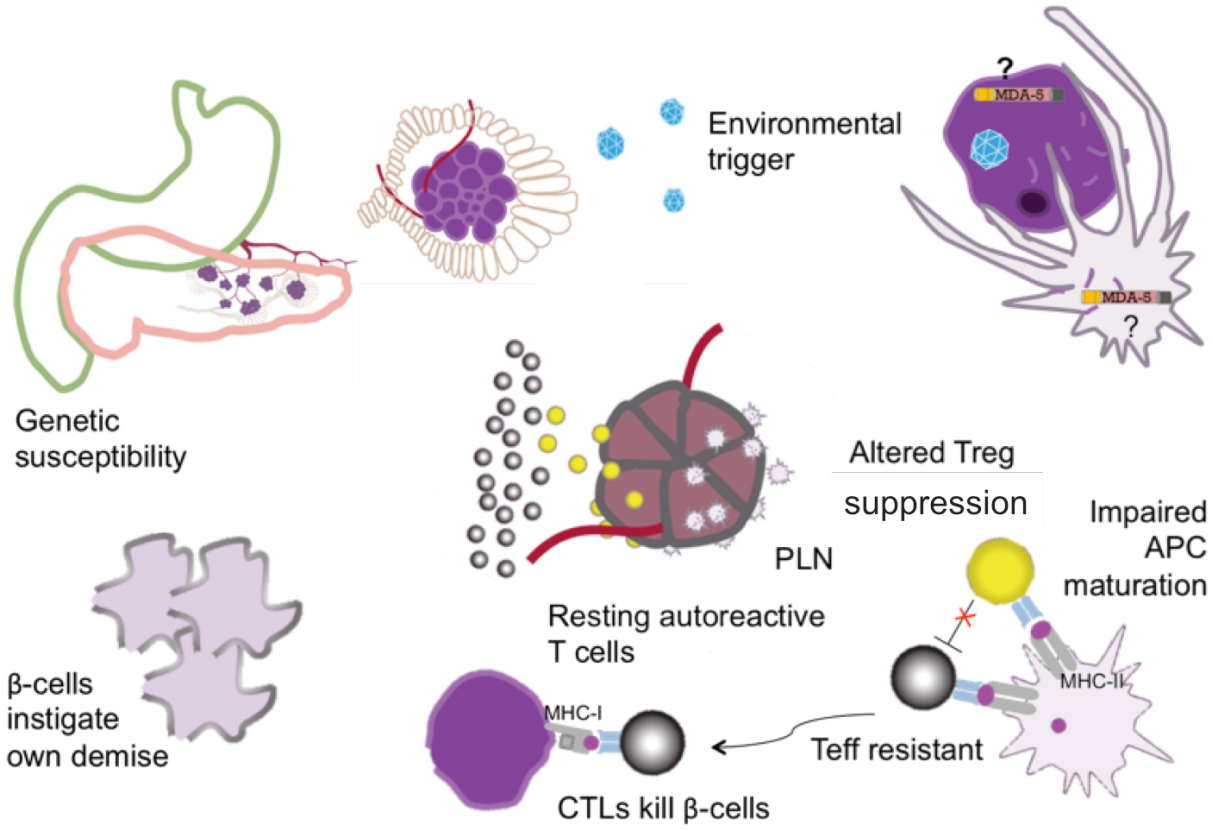


Figure 1.2 Factors suggested to contribute to the destruction of β-cells in T1D. Genetic risk and environmental agents like enterovirus infections, together with potentially altered type 1 interferon (IFN-I) signalling from virus receptors like MDA5 and other immunological factors such as impaired antigen presenting cell (APC) maturation, defects in regulatory T cell (Treg) function and/or improved effector T cell (Teff) function and the existence of resting autoreactive memory T cells in the pancreas and pancreatic lymph node (PLN) and potential inherent defects within β-cells are all suggested contributors to the development of type 1 diabetes.

1.4.3.1 Immunological factors

It is clear from animal and human studies that the loss in β-cell mass leading to insulitis and hyperglycemia and ultimately autoimmune disease in T1D is at foremost, an immune-mediated process. Components from both innate and adaptive immune systems contribute to the immunopathogenesis of T1D (Figure 1.3).

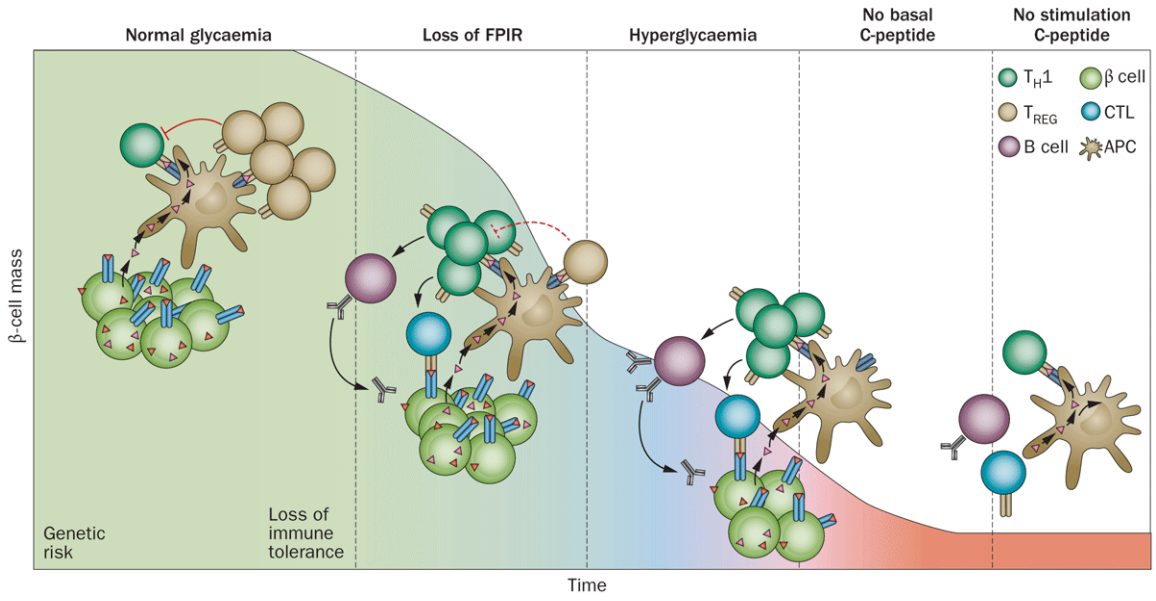


Figure 1.3 Components from both innate and adaptive immune systems that contribute to the immunopathogenesis of T1D. Under immune homeostasis, after birth, regulatory T cells (Tregs) keep islet autoreactive effector T cells (Teffs) from activation. With genetic risk or a combination thereof with as yet defined environmental and/or immunological factors, islet-autoreactivity tolerance can be disrupted where Tregs no longer retain Teffs from activation, B cells produce autoantibodies and autoreactive Teffs proliferate. In the first-phase insulin response (FPIR), the β-cell mass insufficiently regulates increasing blood glucose levels though Tregs still suppress islet-autoreactivity. With time however, and the actions of undefined immunoregulatory and possibly inherent factors, Treg suppression collapses and autoreactivity dominates. Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T cell; FPIR, first-phase insulin response; TH1, T helper 1; TREG, regulatory T cell; T1DM, type 1 diabetes mellitus. Reprinted with permission from the Nature Publishing Group: Nature Reviews Endocrinology. Roep, B. O. & Tree, T. I. M. (2014) Immune modulation in humans: implications for type 1 diabetes mellitus. © 2014

After birth, islet autoreactive effector T cells (Teffs) are restrained from activation by regulatory T cells (Tregs). With genetic risk or a combination thereof with as yet defined environmental and/or immunological factors, this islet-autoreactivity tolerance can be disrupted. Loss of immune tolerance where islet autoreactive T cells are let loose from Treg suppression, allows the activation of B cells to produce autoantibodies and proliferation of Teffs. In what has been described as a first-phase insulin response (FPIR), the β-cell mass looses the ability to sufficiently regulate increasing blood glucose

levels and Tregs are still able to suppress islet-autoreactivity. With time and the actions of undefined immunoregulatory and possibly inherent factors, Treg suppression collapses and autoreactivity dominates. It is at this stage that clinical hyperglycemia manifests and the β-cell mass inadvertently deteriorates and ultimately suffers a loss in glucose-stimulated functions such as endogenous insulin production that can be measured by the level of C peptide -a by-product of proinsulin processing [32]**.**

The dysregulation of tolerance in T1D has been well described in the NOD mouse and BB/W rat models of T1D[33-35]. Disease onset in these models follows the immune diversion from two key regulatory events or 'checkpoints'. Dysregulation in islet antigen recognition, mediated either developmentally or with islet cell damage, is suggested as the first checkpoint and as the second event- the progression from non-destructive to destructive insulitis[28]. Together, these events ultimately lead to T cell-mediated destruction of β cells and autoimmunity. The progression of destructive insulitis likely culminates from several immunological events that favour the function and production of autoreactive effector T cells. Improved effector rather than regulatory T cell function, a loss in negative signalling, a rise in pro-inflammatory factors and the revealing of new β cell antigens may act to perpetuate and advance the autoimmune process in T1D.

It is well accepted that autoreactive T cells and autoantibodies as a part of adaptive immune responses ultimately contribute to and mark islet destruction and autoimmunity in T1D. What remains unclear are the immune system components and events that contribute to aberrant adaptive responses and a loss in tolerance to β cell antigens. One of the first events described to precede islet infiltration by autoreactive T cells is the hyperexpression of major histocompatibility complex (MHC) class I. Tissue cells such as those in the islets, only express MHC I, therefore presentation of self-antigens by tissue cells as in T1D is restricted to $CD8^+$ T cells. As islet cell destruction ensues with T cell infiltration and islet cell contents are acquired directly or passively as tissue cells are engulfed by antigen presenting cells (APC), antigenic peptides can be expressed and presented in the pancreatic lymph node to $CD4^+$ T cells by way of MHC II presentation in addition to cross presentation to $CD8^+$ T cells. During the early stages of disease

onset, in addition to MHC I hyperexpression, cytokines, such as type 1 interferons (IFN-I) that are a part of anti-viral responses, and the chemokine CXCL10 that recruits immune cells expressing CXCR3 as a part of innate immune responses are also present in the islets prior to autoreactive lymphocyte infiltration[36-38].

The repertoire of immune system components and their respective mechanisms responsible for the onset of autoimmunity continues to expand and diversify. For autoimmune diabetes, the immunological factors responsible for the break in tolerance to self-proteins is still not well defined. The detection of autoantibodies in the serum of T1D patients provides an initial indication of an ongoing autoimmune response though it is still unclear if a specific self antigen or multiple self antigens or combinations thereof are responsible in propagating islet directed autoreactive T cells[39]. In 85-90% of T1D patients, the progression of autoimmunity can be measured by the serological presence of one or more islet cell, insulin, glutamate decarboxylase (GAD), zinc transporter 8 (ZNT8), tyrosine phosphatase IA-2 and IA-2β autoantibodies well before clinical symptoms emerge.

Preclinical models and human genome studies have suggested insulin (or its prohormone precursor proinsulin) as the instigating autoantigen of disease and the islet-specific IGRP and chromogranin A autoantigens as supportive drivers of disease progression[39-42]. Epithelial cell presentation of tissue-specific proteins by way of the transcription factor AIRE in the thymus, assists central tolerance to peripheral and tissue-specific proteins like the islet protein insulin[43-45]. In mice lacking the insulin gene *Ins2*, expressed in both the thymus and islets, a breakdown in central tolerance is suggested to accelerate autoimmune diabetes and in patients carrying mutations in the *INS* promoter of the insulin-dependent diabetes mellitus 2 (IDDM2) risk gene, thymic *INS* expression is reduced[46]. Moreover, in NOD mice, it appears that acceleration versus protection from T1D in respect to the *Ins1* and *Ins2* genes comes down to their individual B9-B23 gene sequences[47, 48]. In both NOD mice and T1D patients, during diabetes development, autoreactive T cells that recognize an epitope within the S(B9)-G(B23) residues of proinsulin have been detected. In patients carrying the T1D susceptible HLA DR4

haplotype, other immunodominant peptides in proinsulin and within the insulin A-chain (A1-A15) sequence have been reported[49]. Further, insulin B-chain peptides have been shown to stimulate IFN-γ production from peripheral blood mononuclear cells (PBMCs) isolated from recent onset and diabetic patients[50]. Defects in the presentation of tissue-specific antigens in the thymus that lead to the escape of autoreactive T cells in to the periphery and ultimately the onset of autoimmunity, represents one of the many potential immunological mechanisms suggested in the etiology of T1D[28].

Intrinsic defects in β cell development, or signaling or endolysosomal function have also hinted that the β cell may be responsible for its own demise in T1D. The identification of almost 60% of T1D susceptibility genes expressed in the pancreatic β cell and evidence from several preclinical functional and human pathology studies have also supported intrinsic abnormalities in the β-cell leading to T1D [51]. Furthermore, cellular stress induced by viral infections, endoplasmic reticulum dysfunction or vitamin D deficiency or combinations thereof, can induce abnormal immunogenicity of β cells, and propagate "danger" signaling ultimately progressing disease towards T1D[52-54].

1.4.3.2 Genetic factors

With a simple change or single nucleotide polymorphism (SNP) in the sequence of a gene, variant alleles can form, creating a gateway or protecting from disease susceptibility. For T1D, immunological dysfunction is strongly influenced by inherent polygenic effects. Susceptibility based on heritability or familial association has established that siblings of T1D patients can acquire almost 6% risk of developing the disease, which is a risk 15-fold greater than for individuals with non-diabetic siblings. Almost half of the genetic risk of acquiring T1D is attributed to specific variants in the human leukocyte antigen (HLA) locus that encodes major histocompatibility complex (MHC) I and MHC II molecules. In clinical studies HLA screening helps identify individuals at risk for T1D and is used as a marker for inclusion in experimental studies and clinical trials[55].

Specific haplotypes are strongest in European populations, whereas other HLA risk alleles are more prominent in Japanese and Korean populations[28, 56-58]. Protective HLA alleles such as the HLA-DQB1*0602 have also been identified. Due to their functions in antigen presentation, MHC molecules are instrumental in discrimination of self and non-self and the establishment of tolerance, as such, variants in the HLA locus greatly contribute to the risk for dysfunctional immune tolerance ultimately leading to T1D. Expression of MHC I on β cells has been implicated in the development of T1D, where studies examining autopsy samples of recent-onset patients, *in vitro* culture experiments with pancreatic islets cells, and in animal models, hyperexpression of MHC I with correlative IFN-I signalling and IFN-α production have been observed[37, 59, 60].

In NOD mice, insulin-dependent diabetes (Idd) rick loci (Idd1-Idd26) have been associated with T1D development[61, 62]. Variants in the NOD Idd1 and Idd16 genes that map to the MHC I and II molecules respectively, seem to confer susceptibility to T1D through loss of function[63, 64]. Another MHC-associated locus outside the Idd1 and Idd16 genes has been identified whilst the rest of Idd loci have been deemed as non-MHC linked. MHC I alleles confer susceptibility independently of MHC II risk alleles, with MHC I B*5701, B*3906 and A*0201 associated with the greatest T1D risk. However, the highest risk for T1D has been relayed to specific MHC II genotypes as MHC II molecules are predominantly expressed on immune cells[65-67].

Apart from the HLA risk genes, more than 50 non-HLA susceptibility loci have been associated with T1D [28]. Prior to the era of genome-wide association studies (GWAS), variants in the *Ins* (resulting in preproinsulin expression), *CTLA-4* (co-stimulatory molecule expressed on T cells), *PTPN22* (encoding lymphoid protein tyrosine phosphatase (LYP)), and *IL2RA/CD25* (expressing the α-chain of the IL-2 receptor complex-also referred to as CD25)) genes have been reported with strong correlation to T1D risk[55, 58, 66]. The identification of single nucleotide polymorphisms (SNPs) and variant alleles underlying the complexity in T1D risk has been greatly aided by the improvement of high throughout sequencing methodologies and the introduction of large-scale GWAS.

Except for the strong correlation of the MHC, the majority of T1D risk loci identified by GWAS though moderately frequent, have relatively low risk and carry polymorphisms also seen with other autoimmune diseases. This implies perhaps a commonality in autoimmune pathogenesis in the function of these risk loci[55, 68, 69]. Additionally, most of the T1D risk loci are expressed in cells of innate and adaptive immune responses as well as the pancreatic islets (Figure 1.4) and their expression is linked to possible disease-relevant pathways (Figure 1.5). In line with this, more than 50% of T1D risk genes encode cell-surface receptors and intracellular signaling components[51, 70].

In 2006, from one of the first GWAS, the interferon induced with helicase C domain 1 (*IFIH1)* gene, encoding the RNA sensor melanoma differentiation-associated gene 5 (MDA5), was introduced as another significantly correlative T1D risk locus. A non-synonymous SNP (nsSNP) (rs1990760) in the IFIH1 gene resulting in the Thr^{946} allele, was shown to increase disease risk whereas the wild-type Ala^{946} allele was found to be associated with protection from T1D. The GWAS study revealed that the Thr^{946} allele increased risk by 16% in 65% of the population sampled and that individuals homozygous for the Thr^{946} allele that represented 42% of the group sampled, had 35% increased risk of T1D compared to individuals expressing the Ala^{946} allele[71]. From a subsequent deep sequencing study of T1D patients and healthy controls, four rare variants in the *IFIH1* gene (Figure 1.6) with higher allele frequency were identified in controls rather than T1D subjects. These variants resulting in altered splicing, truncation of the MDA5 protein, or nonsynonymous amino acid changes in MDA5 have demonstrated to either reduce or completely disable *IFIH1* function [71-73]. Moreover, it has been demonstrated that chromosomes harbouring the Thr^{946} allele produce more *IFIH1* transcripts [74]. Observations from these studies and from other subsequent functional studies including a recent mutagenesis study in mice, have demonstrated that the variants Gly821Ser (chemically induced in mice), Ala946Thr, and at positions 327 and 826 in the *IFIH1* gene are gain-of-function mutations that induce changes in MDA5 conformation compatible for constitutive activation, IFN-I signaling and that ultimately lead to autoimmunity[74-78].

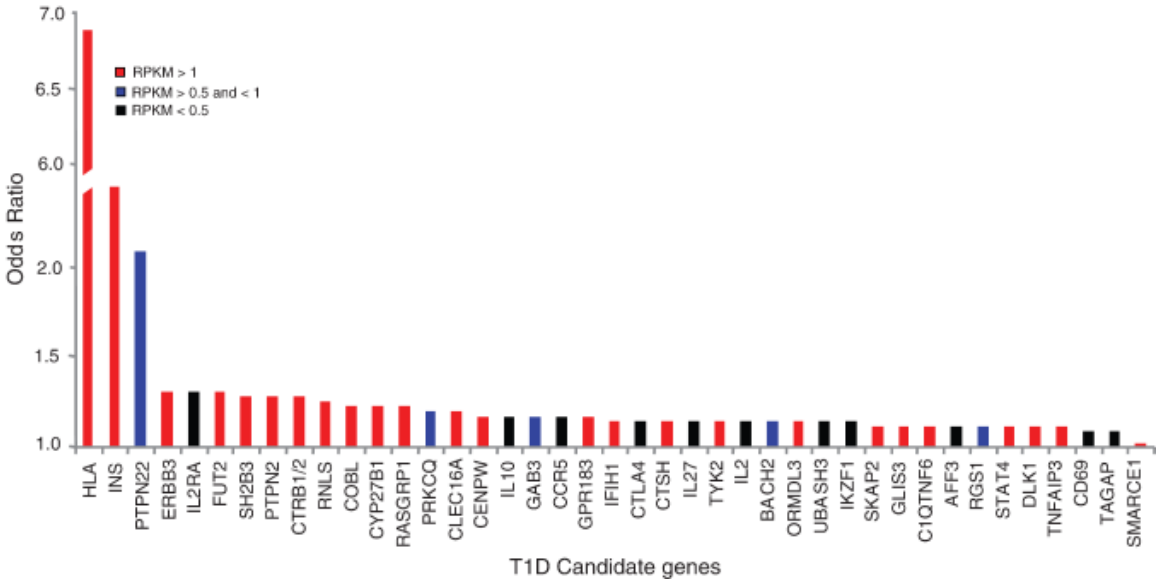


Figure 1.4 T1D risk loci expressed human pancreatic islets. Most of the genes associated with T1D risk are expressed in cells of innate and adaptive immune responses as well as the pancreatic islets and their expression is linked to possible disease-relevant pathways. Reprinted with permission from John Wiley and Sons: Diabetes, Obesity and Metabolism, I. Santin and D. L. Eizirik. Candidate genes for type 1 diabetes modulate pancreatic islet inflammation and β-cell apoptosis. © 2014

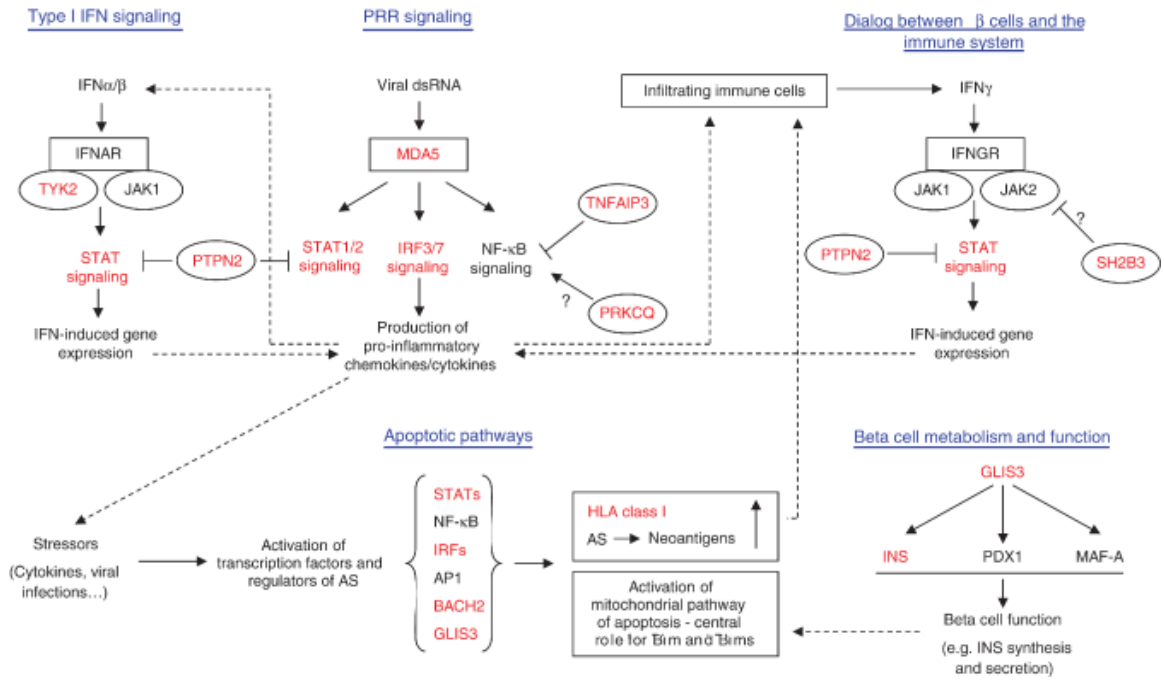


Figure 1.5 Involvement of T1D risk loci in potential disease-relevant pathways. The majority of T1D risk loci are expressed by β-cells and their expression is linked to possible disease-relevant pathways. In line with this, more than 50% of T1D risk genes encode cell-surface receptors and intracellular signaling components including the RNA sensor MDA5 that has been implicated in T1D pathogenesis with the identification of protective variants in the *IFIH1* gene. Reprinted with permission from John Wiley and Sons: Diabetes, Obesity and Metabolism, I. Santin and D. L. Eizirik. Candidate genes for type 1 diabetes modulate pancreatic islet inflammation and β-cell apoptosis. © 2014

The gain-of-function effect with Ala946Thr and other subsequent variants in *IFIH1* leading to increased IFN-I production is also associated with other autoimmune and inflammatory diseases including Crohn's disease, ulcerative colitis, systemic lupus erythematosus (SLE), immunoglobulin A deficiency (IgAD), and most recently, Aicardi-Goutieres syndrome (AGS)[75, 79, 80]. Although IFN-I signaling is essential in antiviral immunity, the alleged IFN-I mediated protection, under circumstances of particular polymorphisms as demonstrated with the *IFIH1* gene, can unfortunately, inappropriately sustain and constitutively induce IFN-I responses that ultimately lead to autoimmune disease. The discovery of variants in *IFIH1* that confer protection from T1D and subsequent gain-of-function studies have demonstrated the significance of a threshold of expression of a particular gene in conferring protection from autoimmune disease and have most importantly, implicated anti-viral responses in the development of T1D. In line

with this, we have recently demonstrated that a reduction in *ifih1* gene expression in NOD mice alters IFN-I signalling and protects from coxsackievirus B4-induced T1D[81].

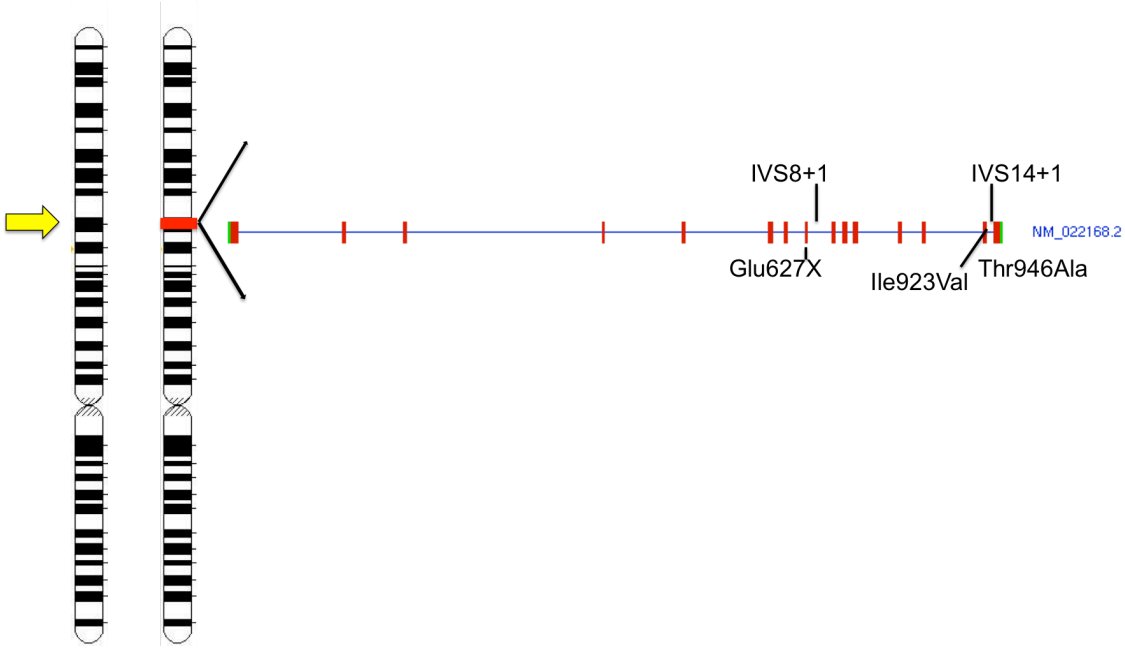


Figure 1.6 Rare variants identified in the *IFIH1* gene in chromosome 2 of patients protect from T1D. Patients carrying the protective variants are heterozygous. Functional experiments have demonstrated the gain-of-function mutations or risk alleles that result in hyperproduction of type 1 interferons and induce autoimmune pathologies.

While genetic predisposition strongly influences risk for T1D, it does not solely represent the etiology of the disease. It is generally thought that the collection of susceptibility alleles tied to T1D risk can act independently or as a collective in conjunction with other non-genetic factors to influence the fate of disease onset[82-85].

1.4.3.3 Environmental factors

1.4.3.3.1 Enteroviruses and type 1 diabetes

Accumulating evidence of a changing demography with rising disease incidence rates in childhood diabetes, especially in children less than 5 years of age, and low concordance around 50% for monozygotic twins, has suggested that other factors aside from genetics

are involved in the development of T1D. Beyond genetics, changes in diet and the environment have been implicated in T1D etiology. Viral infections and how they may trigger and/or exacerbate autoimmune diabetes has long been hypothesized to influence an individual's T1D susceptibility. Many human and animal studies have proposed that viruses including enteroviruses, herpesviruses, rubella virus, mumps virus, cytomegalovirus, rotaviruses, and even endogenous retroviruses are infectious risk factors in T1D, capable of inducing cellular immune responses that can lead to the damage of β cells (Figure 1.7)[86-91]. It is unclear whether viral infections initiate T1D, or accelerate an already established autoimmune process or whether they are opportunistic incidents as a result of increased susceptibility in a T1D host. The most clinically relevant and convincing association of viral infections with T1D comes from studies on enteroviruses, specifically coxsackieviruses[86, 92-95].

In studies examining a possible role for prenatal and perinatal enterovirus exposure in T1D susceptibility, a fivefold increase in risk was found for boys born to mothers positive for enterovirus-specific IgM antibodies[96]. A definitive association of enterovirus infections during pregnancy and the development of T1D in offspring is still uncertain, though many studies have reported a higher incidence of enterovirus-specific IgM antibodies in mothers whose offspring subsequently develop T1D. These studies suggest that in a subgroup of susceptible offspring, disease initiation is likely by enterovirus infection and has occurred *in utero* [96, 97]. Enterovirus infections preceding islet cell autoantibody seroconversion has also been observed in patients, further implicating enteroviral infection in the initiation of T1D. Nevertheless, there has also been accumulating evidence from clinical and animal studies pointing to enteroviral infections as a precipitate rather than instigator of disease pathogenesis.

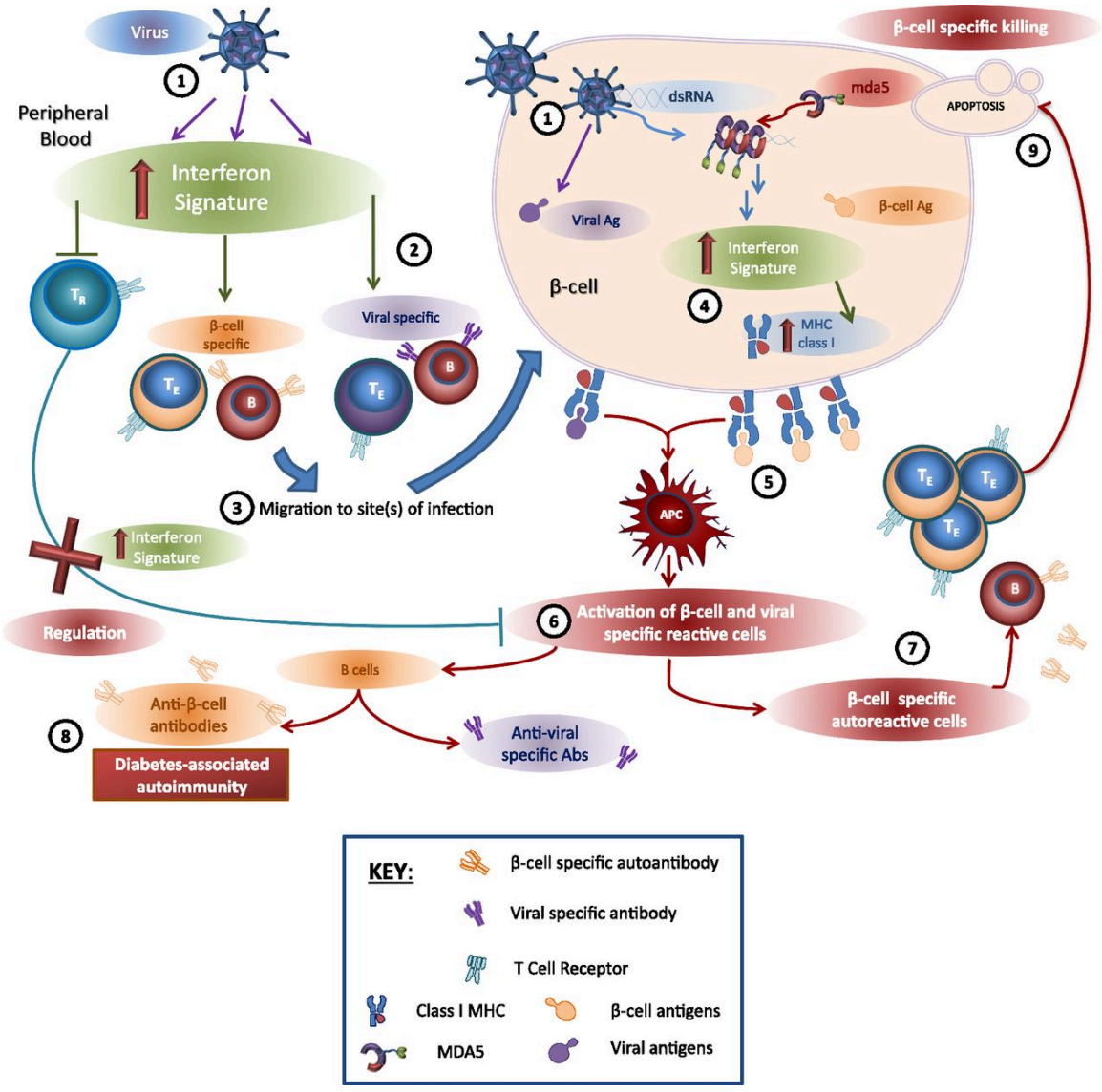


Figure 1.7 Various cellular mechanisms proposed to induce β-cell autoimmunity following viral infection. Viral infections and how they may trigger and/or exacerbate autoimmune diabetes has long been hypothesized to influence an individual's T1D susceptibility. Many human and animal studies have proposed that viruses including enteroviruses, herpesviruses, rubella virus, mumps virus, cytomegalovirus, rotaviruses, and even endogenous retroviruses are infectious risk factors in T1D, capable of inducing cellular immune responses that can lead to the damage of β cells. Reprinted with permission from American Diabetes Association: Diabetes, Sarah J. Richardson, Marc S. Horwitz, Is Type 1 Diabetes "Going Viral"? © 2014, American Diabetes Association.

Enteroviral particles and the enterovirus capsid protein VP1 have been detected post mordem in pancreatic β cells of T1D patients and the VP1 protein has even been detected

post mordem in pancreas tissue from a child positive for islet-cell autoantibodies, preceding autoimmunity[98]. *In situ* hybridization has also identified enterovirus RNA in the pancreas of deceased T1D patients and not in healthy individuals[99]. And a recent meta-analysis of molecular and immunological studies of enteroviral infection has determined a striking correlation of enteroviral infection with T1D and autoimmunity.

1.4.3.3.2 Coxsackievirus B

Poliovirus, echovirus, enteroviruses 67-71 and Coxsackieviruses A and B are enteroviruses that are a part of the Picornaviridae family. Enteroviruses are common human pathogens, transmitted fecal-orally that generally replicate first in the intestinal tract and induce usually clinically inapparent or mildly symptomatic illness. In some cases, enterovirus infection can spread to other organs and cause severe illness specific to the enterovirus type[100].

Severe infections with coxsackievirus A can lead to enteric diseases and severe diseases in the heart, pancreas, and central nervous system can result from infections with coxsackievirus B. There are 23 different serotypes of coxsackievirus A and six serotypes of coxsackievirus B[101]. The six coxsackievirus B serotypes can induce a variety of diseases including the autoimmune diseases myocarditis and type 1 diabetes[86]. All coxsackievirus B serotypes are capable of triggering systemic disease in infants that can devastatingly lead to death [102]. Coxsackievirus B3 has been most commonly associated with myocarditis. CB3 has been linked to approximately 30% of new dilated cardiomyopathy cases per anum though data establishing a direct link between CB3 pathogenesis and the onset of myocarditis in patients has remained difficult[103].

Coxsackieviruses as other enteroviruses have a 7.4-kb single- stranded positive-sense RNA genome containing a VPg (3B) protein at the 5' end. The 7- methyl guanosine-like cap influences replication and translation following virus entry. To gain entry in to a cell, coxsackieviruses interact with both coxsackievirus and adenovirus receptor (CAR) and decay accelerating factor (DAF)- both located in the host cell membrane. Once the virus enters the cytosol and uncoats, its positive-sense genome is released in the cytosol for

translation and later, transcription. As a polyprotein comprised of the virus proteins VP4, VP3, VP2, VP, 2A, 2B, 2C, 3A, 3B, 3C, and 3D emerges from translation at the rough endoplasmic reticulum (ER), it is cleaved into its respective structural and functional proteins. The virally encoded 3Dpol is a RNA-dependent RNA polymerase that transcribes viral positive-sense RNA in to negative-sense RNA strands that serve as intermediates for the transcription of multiple positive-sense RNA strands needed for new progeny virions. After synthesis, the newly generated positive-RNA strands are packaged in new virus particles formed by the newly generated structural and functional virus proteins. The new progeny viruses are then released via plasma membrane by a mechanism likely mediated by viral protein 2B[102]. Uncovering the structural and replicative characteristics of coxsackieviruses and their interactions with host cellular structures and immune mechanisms has greatly contributed to understanding the role these viruses play in autoimmune diseases, most importantly in the etiology of T1D.

1.4.3.3.3 Coxsackievirus B and type 1 diabetes

Of the viruses implicated in the development or pathogenesis of T1D, coxsackievirus B has been the most strongly associated in T1D etiology. High levels of coxsackievirus B RNA and corresponding coxsackievirus-specific antibodies are more frequently detected at the time of clinical T1D onset compared to healthy controls [104-115]. Interestingly, compared to healthy individuals, corresponding increases in IFN-α with increased coxsackievirus B RNA have been measured in the blood of T1D patients at various stages of the disease. Increased levels of IFN-α measured by immunohistochemistry and mRNA expression has also been detected in the pancreatic β-cells of T1D patients, suggestive of a virus infection in the pancreas[60, 116]. Moreover, enterovirus, specifically coxsackievirus B4 (CB4), has been isolated from the pancreas of T1D patients[99, 117] and successful passaging of isolated CB4 in murine pancreatic islets and further studies using this mouse-adapted CB4 strain in NOD mouse models has demonstrated the virus' diabetogenic properties and ability to induce diabetes-like disease[118-120].

In murine models of coxsackievirus B, evidence of viral replication is observed in the heart, pancreas, liver, spleen and brain. Systemic delivery of the virus causes viremia

within 24 hours and persists in target organs for up to 3 to 5 days with viral titres peaking in the pancreas at day 3 post-infection (PI) and the heart by day 5 PI. With the likely intervention of neutralizing antibodies, virus titres are reduced and are generally undetectable by 15 days PI. Despite viral clearance, viral RNA can still be detected in certain organs after weeks and months. Viral pathogenesis and severity of disease leading to chronic disease such as autoimmunity is dependent on host susceptibility and the host-pathogen combination[121].

In the case of CB4 infection in the diabetes susceptible NOD mouse, CB4 infection leads to the induction of T1D. Some studies have previously suggested that autoimmunity from coxsackievirus B infection may result from molecular mimicry, with mimicry between a 2C non-structural coxsackievirus B epitope and the islet derived antigen glutamic acid decarboxylase. Later, studies using diabetes susceptible mice, discounted molecular mimicry as a sufficient induction of autoimmunity and demonstrated that it is likely Coxsackievirus B destruction of the exocrine pancreas and subsequent release of islet cell antigens, sequestered by antigen presenting cells, that prime autoreactive memory T cells and re-invigorate an ongoing autoimmune process[118]. Studies with the NOD BDC2.5 T-cell receptor (TCR) transgenic model that does not spontaneously develop autoimmune diabetes, and wild-type (WT) NOD model have shown that CB4 infects, but does not directly kill pancreatic β cells and instead activates a pre-existing population of autoreactive T cells to elicit autoimmunity[118, 120]. Resident antigen presenting cells (APCs) engulf infected β-cells, present sequestered islet and viral antigens to resting autoreactive T cells and induce a population of T cells directed both at the virus and specific to the pancreatic islets. The β-cell directed T cells then destroy the islets and accelerate insulin loss and T1D disease pathology[118]. Therefore, CB4-mediated T1D in susceptible hosts such as the NOD model, is not directed by molecular mimicry, but rather dependent on the local presence of autoreactive, diabetogenic T cells. The pancreatropic virus encephalmyocarditis virus-D (EMCV-D) has also been used to induce diabetes however this virus directly targets β cells and induces diabetes by virus-directed β cell death [122]. Unlike EMCV-D, CB4 infects, and without directly killing pancreatic β cells induces diabetes [118, 120].

The mechanisms driving the induction of autoimmunity following CB4 infection are not as well understood in humans. Some studies have identified subsets of anti-CB4 antibodies from recent onset patients with the capacity to induce β-cell apoptosis, suggesting molecular mimicry as a mechanism driving disease [123, 124]. Other studies with CB4 infection of human pancreatic islet cells have demonstrated nondestructive islet inflammation and impairment of function rather than cell death, suggestive that the virus precipitates, as seen in the NOD model, an already established autoimmune process and leaves the mechanism of CB4-mediated T1D in humans still unresolved. Additionally, despite epidemiological, clinical and animal studies that strongly link CB4 to T1D, in patients CB4 may only be responsible for a subset of new onset cases as other viruses such as the enterovirus coxsackievirus B 1 (CB1) and rotaviruses have more recently been implicated [87]. Nevertheless, considerable research substantiates enteroviral infections as T1D etiological agents and it is rather the causality of enterovirus infection in T1D pathogenesis and the repercussions of immune responses to enteroviral infections preceding and leading to autoimmunity and clinical manifestations that is still a matter of debate[125].

1.5 RNA sensing

Innate immunity is critical for primary detection of pathogens, such as viruses, and for initiating the first wave of defenses [126, 127]. A thorough understanding of virus detection by the innate immune system is critical not only for understanding innate immune mechanisms, but is essential to anticipate viral pathogenesis.

The functional consequences of this early detection of viral infection results in the establishment of an anti-viral response and contributes to the eventual activation of a virus-specific adaptive immune response aimed at clearing viral infection[128]. Pathogen recognition receptors (PRRs) are germ-line encoded cellular sensors that activate in the presence of pathogen associated molecular patterns (PAMPs). Double stranded RNA that is produced during replication of certain viruses is not a common intermediate in uninfected cells and as such is recognized as a PAMP by sensors from two families of PRRs- the Toll-like receptors (TLRs) and the retinoic-acid-inducible-gene I (RIG-I)-like

(RLRs). Intracellular TLRs found within endosomes include TLR3, TLR7, TLR8 and TLR9 that sense double stranded RNA (dsRNA), single stranded RNA (ssRNA), ssRNA in humans only and unmethylated CpG motifs of DNA respectively. The RLRs located intracellularly in the cytoplasm, that detect dsRNA are retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) [129, 130]. Signaling from TLR7 and TLR9 in plasmacytoid DCs (pDCs) induces IFN-I by the MyD88-IRF7 pathway. pDC signalling of IFN-I has been investigated as a potential early source of IFN-I in anti-viral immunity and has been studied for a potential role in the development of diabetes[131].

Detection of viral dsRNA by PRRs is critical to induce an IFN-I and anti-viral response to control viral infection. RIG-I and MDA5 are a part of the family of DExD/H box RNA helicases ubiquitously expressed in the cytoplasm of most cell types. Both proteins detect dsRNAs, though RIG-I specializes in the detection of negative-sense ssRNA viruses like influenza virus and Sendai virus while MDA5 detects positive-strand RNA viruses predominantly from the Picornavirus family mainly encephalomyocarditis virus (EMCV) and coxsackievirus B4, which are both β-cell tropic viruses (Figure 1.8). MDA5 and RIG-I can both recognize ssRNA from Flaviviruses dengue virus and West Nile virus as well as mouse hepatitis virus and other paramyxoviruses, yet both RNA sensors differ in their choice of ligand. RIG-I activates with short dsRNAs and 5'-triphosphate-containing RNAs, setting apart host cytoplasmic from foreign viral RNAs. Whereas, MDA5 prefers long dsRNA such as the artificial RNA polyinosinic:polycytidylic (poly I:C) [132] and dsRNA generated from viral replication within an infected cell[133, 134].

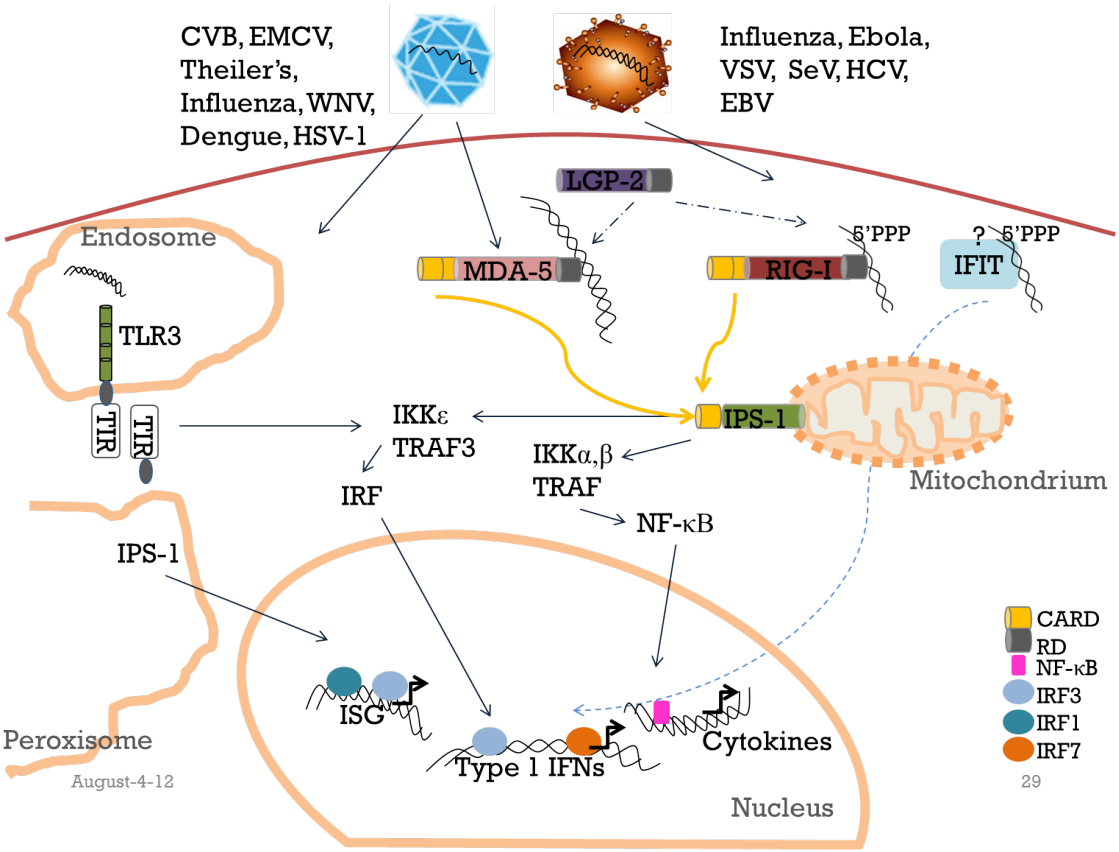


Figure 1.8 Detection of viral double stranded RNA (dsRNA) by PRRs is critical to induce a type 1 interferon (IFN-I) and anti-viral response to control viral infection. RNA viruses signal anti-viral IFN-I responses through specific RNA sensor pathways. Double stranded RNA (dsRNA) produced during viral replication is detected depending on its length and affinity for the dsRNA sensors MDA5, RIG-I and/or TLR3. Activation of MDA5 signals the recruitment of IPS-1 also known as MAVS, leading to the activation of signalling molecules and nuclear translocation of transcription factors to induce the production of type 1 interferons.

Upon recognition of its dsRNA ligand and proper assembly of a filament structure around the dsRNA stem [135], MDA5 signalling activity begins with the phosphorylation and dephosphorylation of its caspase recruitment domains (CARDs) [136]. Once activated, MDA5 CARD domains interact with the mitochondrion-anchored adaptor protein MAVS (also known as IPS-1), which recruits TRAF3, activating NF-κB-binding kinase 1 and IKKε leading to the activation and translocation of IFN regulatory factor-3 (IRF-3) and IRF-7 to the nucleus and the transcription of IFN-I [132, 135, 137]. MAVS recruitment of FADD and receptor-interacting protein-1 can also induce NF-κB activation and the production of inflammatory cytokines.

The RNA mimetic poly i:c has been used as a substitute to viral infections to study MDA5 and TLR3 signaling and subsequent immunomodulatory effects on innate and adaptive responses[131]. Poly i:c can stimulate TLR3 and MDA5 signaling in hematopoietic and stromal cells respectively, causing an increase in IFN-I production that leads to the stimulation of dendritic cells (DCs) and priming and activation of pathogenic $CD4^+$ T cells[138, 139]. IFN-I responses induced through TLR3 and MDA5 activation by poly i:c can also stimulate antigen-specific $CD8^+$ T cell immunity and NK cell activity[140, 141]. Poly i:c and viral infection studies have also substantiated a role for another intracellular dsRNA sensor, LGP2, that does not possess signaling domains, but assists MDA5 affinity for RNA leading to enhanced MDA5-mediated antiviral signaling[142, 143].

Viruses, in particular the paramyxoviruses, find specific means to inhibit host immune responses such as IFN-I responses. In fact, 13 different paramyxoviruses have been demonstrated to use their V protein, encoded by the viral P/V/C gene, to target and inhibit the dsRNA sensor MDA5[144]. The cellular interaction between the viral V protein and MDA5 results in the inhibition of MDA5 and subsequent IFN induction. Interestingly, V proteins specifically target the MDA5 helicase domain, interfering with dsRNA binding, MDA5 activation and subsequent oligomerization of MDA5 helical domains[145]. The V protein from parainfluenza virus 5 (PIV5; previously known as SV5) has been well characterized in MDA5 inhibition studies.

1.6 Type 1 interferon signaling

Type 1 interferons (IFN-I) are a family of interferon cytokines that respond to and interfere with viral infection. Since the role of IFN-I cytokines in anti-viral immune defenses was first described 50 years ago, extensive research has characterized the dominant and broad expression of IFN-I family members IFN-α and β in humans and mice[75, 146-149]. There are thirteen IFN-α isotypes, expressed by several genes, and one IFN-β isotype expressed by a single gene that along with the other IFN-I subtypes, signal through the ubiquitously expressed transmembrane IFN-α receptor (IFNAR). The

IFNAR is heterodimeric with IFNAR1 (low-affinity) and IFNAR2 (high-affinity) representative subunits. IFN-I and the only type 2 interferon, IFN-γ, are functionally similar as modulators of gene expression as a part of innate and adaptive immunity. Where the two IFN types differ is by their receptor and activation of separate STAT pathways. Binding of IFN-I to the membrane bound IFNAR activates Janus kinases Tyk2 and Jak1 and recruits STAT1 to form a heterodimer with STAT2. The STAT1-STAT2 heterodimer crosses into the nucleus where it forms a heterotrimeric complex with the transcription factor IFN regulatory factor (IRF) 9 (p48). The newly formed IFN-stimulated gene factor 3 (ISGF3) heterotrimeric complex then activates transcription of IFN-inducible genes by binding IFN-stimulated response elements (ISREs). IFNAR signaling can also induce the formation of STAT1 homodimers (gamma-activated factor, GAFs) that bind type 2 IFN promoters and activate transcription of IFN-γ–induced genes[75]. Though critical in both IFN-I mediated heterotrimeric (ISGF3) and IFN-γ induced homodimeric complex formation, STAT1 distinguishes IFN-I and IFN-γ mediated immune responses, where STAT1 has been found to be indispensible in DNA cooperativity and gene repression for IFN-γ-induced responses and not IFN-I antiviral immunity[150].

IFN-α subtypes and IFN-β are produced following sensing of pathogens by several germ-line encoded PRRs. Transcription of IFN-β differs from that of IFN-α in that the *Ifnb* gene promoter holds additional binding sites other than the IRF binding sites, including sites for NF-κB and AP-1 binding[151, 152]. As such, IFN-β rather than IFN-α can be produced under different scenarios. With lipopolysaccharide (LPS) stimulation of TLR4 signaling, IRF3 and NF-κB become activated and IFN-β is the only IFN-I produced. IFN-β is also exclusively produced following stimulation by macrophage colony-stimulating factor and by the cytokine RANKL via AP-1 activation[153]. Both IFN-α and IFN-β can signal through IFNAR, however IFN-β can bind to IFNAR1 independently of IFNAR2 and induce distinctive immunological outcomes[154]. Though they have a relatively small (less than 30%) sequence similarity between them, distinct IFNAR binding affinity and have demonstrated different functions, IFN-α subtypes and IFN-β, are together,

essential in anti-viral responses and have more recently been implicated in the pathogenesis of autoimmunity[75, 155, 156].

1.7 Type 1 interferons and autoimmunity

Sensing of microbial products by various cellular PRRs induces the production of IFN-Is from hematopoietic and non hematopoietic lineage cells. The release of IFN-Is feeds an autocrine loop that supports the expression of ISGs and as a part of innate and adaptive immunity, defends the host from an invading pathogen, most particularly from viruses [75]. The stimulation of ISGs and production of IFN-I partially controls pathogen spread by restricting specific cellular processes required for the pathogen's replication. Activation of innate immune cells including APCs and natural killer (NK) cells by IFN-I and the induction of other inflammatory cytokines and chemokines helps create a specific adaptive immune response to clear the pathogen[157, 158]. As a pathogen is cleared and a 'danger' signal emitted by sensing PRRs should subside and IFN-I levels and their respective immunity arms should as well recede. PRR signaling and the subsequent production of IFN-I are critical for early induction of anti-pathogen innate responses. They are also influential on the recruitment of antigen-specific T and B cells as a part of the developing adaptive immune response and as such can have a potential role in supporting exacerbated immune responses that can lead to autoimmunity[75, 157, 158].

The protective role of IFN-I in antiviral immunity during acute viral infections is well described though the immunological consequences of these early IFN-I responses are less understood.

IFN-I can also be induced with nonviral infections (i.e., bacteria, protozoa, fungi, and helminths), and have either protective or damaging effects [159, 160]. Even without a pathogenic insult IFN-I is constitutively secreted at low levels. In the 1980s, after observing antiviral IFN-like activity in uninfected tissues, Velio Bocci suggested that constitutive IFN-I signaling occurred as a result of low level PRR sensing of pathogens in the mucosa and debris from cellular damage and cellular turnover. Animal models lacking the IFNAR or *Ifnβ* gene and neutralizing IFN-I antibodies have since been

developed and have furthered our understanding of the presence and significance of constitutive albeit low secretion of IFN-I as a means to uphold immune homeostasis and evade onset of autoimmunity, cancer and impairment of antiviral responses[161].

What is less understood are the immunomodulatory properties of a persistent and chronic increase in IFN-I and its subsequent ISG expression that together are described as an "IFN-I signature". An IFN-I signature is observed in many autoimmune diseases including systemic lupus erythematosus (SLE), Sjogren's syndrome, rheumatoid arthritis, multiple sclerosis (MS), and T1D. GWAS studies have also characterized an "IFN-I signature" in autoimmune diseases in identifying disease-associated allelic variants in IFN pathway genes and in ISRE sequences [78, 156, 162-164].

From SLE, T1D and other autoimmunity models we know that with the immunologic aberrations observed in the events leading to autoimmunity, persistent stimulation of PRRs and 'danger' signaling can result and lead to heightened and chronic IFN-I production. Damage-associated molecular patterns (DAMPs) that are released as disease ensues, autoantibody production and dysregulated antigen presenting cell (APC) stimulation help disseminate chronic IFN-I production that in turn, can promote effector T and B cell function and ultimately propagate disease and autoimmunity[75].

IFN-Is have both accelerant and suppressive effects in autoimmune diseases[165]. Genetic studies have identified an allelic variant linked to autoimmunity in humans and animals in the tyrosine-protein phosphatase non-receptor type 22 (PTPN22) gene that leads to reduced IFN-I production. A protective role for IFN-Is has also been observed in animal models of arthritis, inflammatory bowel disease and MS. Protection from these autoimmune diseases by IFN-I is likely mediated by their suppressive functions in inhibiting the production of inflammatory cytokines and the activation and proliferation of pathogenic lymphocytes including Th17 cell responses. In MS models, chronic IFN-I can stimulate the production of the immunosuppressive cytokine interleukin (IL)-10 from monocytes, further suppressing innate and adaptive immunity and impeding disease pathogenesis. IFN-β has been used therapeutically for MS patients though it has not

shown prolonged success in patients with relapsing remitting MS (RRMS). Interestingly in these RRMS patients, considerable activation of the IFN-I pathway or an 'IFN-I signature' is observed, suggesting a pathogenic rather than protective role for IFN-I. Several human and animal studies of other autoimmune diseases have supported pathogenic rather than protective effects from IFN-I in autoimmunity. These studies suggest that IFN-I promotes APC activation and presentation of antigens, primes lymphocyte responses and perpetuates disease. Significantly increased STAT1 expression on myeloid cells has been observed in SLE and other autoimmune disease models, indicative of IFNAR engagement and IFN-I pathway activation and allusive to possible IFN-I-mediated effects on cell activation and cell engagement with inflammatory mediators in autoimmunity.

In a recent mouse mutagenesis study, the mutation Ser821 in the autoimmune disease-associated IFN-induced helicase gene 1 (IFIH1) caused constitutive IFN-I production and autoimmune disease with an SLE-phenotype. Localized and systemic increases in the inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and the IFN-I cytokine IFN-β were observed as were $CD4^+$ and $CD8^+$ effector T cells and other inflammatory mediators concomitant with the autoimmune phenotype[78, 166]. High levels of IFN-I have also been detected in patients with autoimmune diseases including MS, SLE and T1D. Interestingly, unique IFN-I signatures are also detected in patients at risk of autoimmune disease before autoimmunity has been established. Further, the expression of key regulators of IFN-I responses varies in different stages of autoimmune disease, suggesting that IFN-I responses fluctuate over the course of disease and likely activate or suppress specific immune responses accordingly, influencing disease pathogenesis.

1.8 Type 1 interferons and type 1 diabetes

Viral infections and antiviral responses have been strongly implicated in the etiology and pathogenesis of many autoimmune diseases including T1D. The contribution of IFN-I production from antiviral responses or the IFN-I signature produced at a given stage of disease in the context of autoimmunity has remained difficult to determine though

mounting clinical and animal research points to a significant involvement of IFN-I in the development of T1D[78, 155, 156, 167, 168]. The precise mechanism of IFN-I participation in T1D remains unclear. What is known is that the cells in the pancreas that predominantly produce IFN-I are the islet β-cells. In children at risk for T1D, an IFN-I transcriptional signature precedes islet autoimmunity[169]. A recent genome-wide transcriptomics study identified dominance in activity of genes involved in innate immunity preceding seroconversion and the detection of autoantibodies in type 1 diabetic children. Specifically, a unique IFN-I signature was identified with interferon response factors (IRFs) as modulators of the interferon-related transcriptional changes[170]. Recent onset of T1D is strongly associated with infection by RNA viruses such as enteroviruses including coxsackievirus, implicating the IFN I signature[91, 171, 172]. Analysis of T1D-associated genes expressed in human pancreatic islets demonstrates a higher number of genes in antiviral response and IFN-I signaling pathways. Greater amounts of IFN-α mRNA and protein are found in T1D patient pancreata compared to non-diabetic patients[60, 116]. IFN-I and downstream genes in the IFN-I signaling pathway are significantly prominent in pancreatic islets as is a unique IFN-I signature in the peripheral blood of T1D patients[116, 173]. The use of IFN-α as a treatment for tumors or viral hepatitis is affiliated with higher T1D incidence. In diabetes-resistant C57/BL6 mice, over-expression of IFN-α induces autoimmune diabetes. Diabetes susceptible NOD mice that lack IFN regulatory factor-1, a critical transcription factor for inducing IFN-β expression, are protected from developing insulitis and T1D. Upregulation of genes induced by IFN-α, IFN-α levels, and increased numbers of IFN-α producing dendritic cells are found in the pancreatic lymph nodes (PLNs) of prediabetic NOD mice[174].

Although, T1D is a complex, multifactorial disease for which the molecular mechanisms guiding the early events leading to autoimmunity are still unclear, accumulating evidence from clinical and animal studies are substantiating a role for endogenous IFN-I in the initiation and likely propagation of autoimmune diabetes. These studies suggest that therapeutic strategies aimed at targeting IFN-I signaling or factors influencing IFN-I responses can prevent or reverse the autoimmune diabetes process.

1.9 Pathogen recognition receptors and type 1 diabetes

Microbial pattern recognition is a central mechanism of innate immunity and occurs by way of PRRs in many cells of the immune system including macrophages and dendritic cells. Other cell types outside the immune system such as pancreatic β-cells also express PRRs that sense not only molecular patterns from pathogens such as viruses, but can also recognize and respond to host tissue, cellular or genetic components released as a result of cell damage and death. The dsRNA sensors TLR3, MDA5 and RIG-I are all expressed in pancreatic β-cells and other PRRs including NOD1 and the nuclear receptor IFI16 have also been detected within human pancreatic islets[70, 175].

In β-cells, PRR recognition of PAMPs activates the JAK/STAT, MAPK and NK-κB signalling pathways[176-178]. The JAK-STAT and chemokine signalling pathways are the central pathways stimulated by RNA viruses including coxsackievirus B [179-181]. Interestingly, the same pathways are regulated in β-cells by T1D candidate genes. Many T1D risk loci influence antiviral responses and consequential IFN-I signalling within β-cells, stimulating the JAK-STAT pathway and activating chemokine and apoptotic responses. Chemokine production from the β-cells recruits inflammatory mediators and together with apoptotic signalling, likely promotes the development of β-cell-directed autoimmunity (Figure 1.9) [181].

1.10 Antigen presenting cells and virus-induced type 1 diabetes

As a result of PRR signaling, antigen presenting cells (APC)s including macrophages and DCs become activated. This matures the cells and induces antiviral responses and consequently also activates adaptive immune responses. As such, APCs likely have a central role in the development of T1D and following infection. Several studies have emphasized an important role of APCs in T1D and in viral-mediated T1D[182].

In coxsackievirus B4 (CB4)-induced T1D, diabetes development is not due to CB4-directed β-cell death or to molecular mimicry with virus-specific effector T cells (Teffs) recognizing pancreatic antigen.

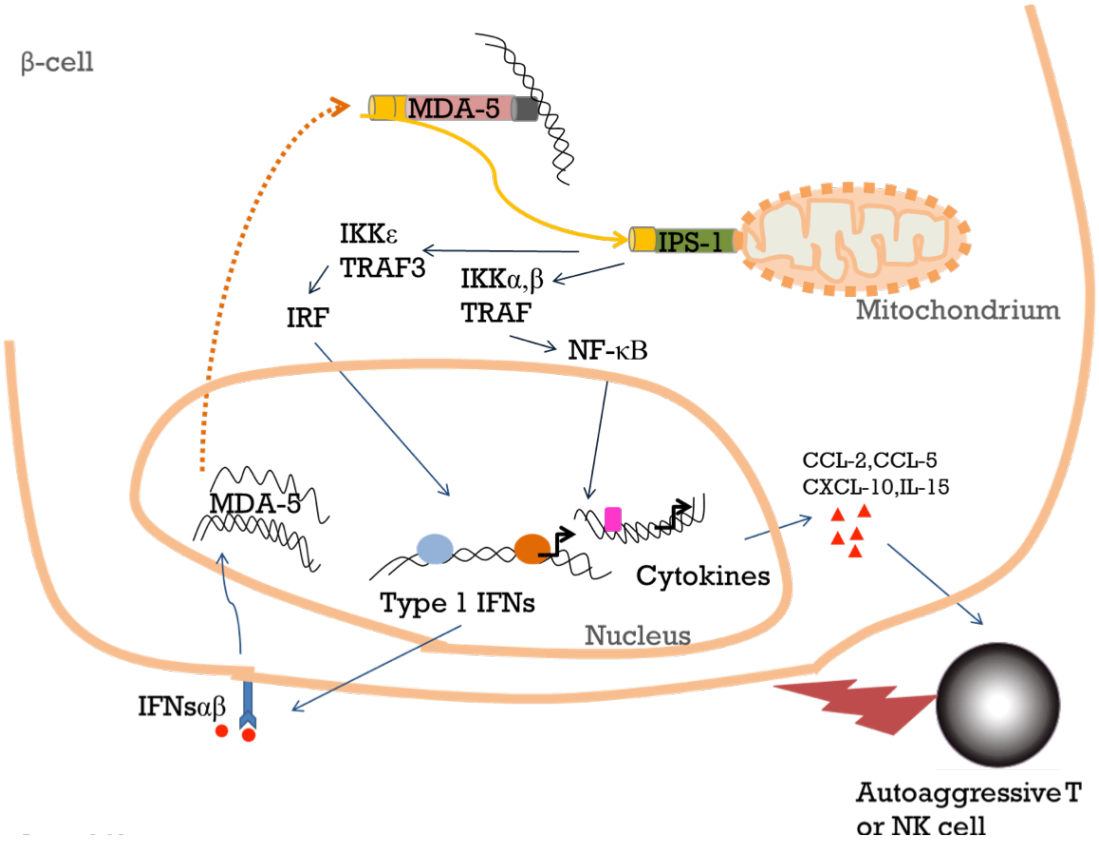


Figure 1.9 Virus signalling in β-cells leads to type 1 interferon, inflammatory cytokine, and chemokine production and the destruction of the β-cell mass. PRR recognition of PAMPs, such as MDA5 recognition of dsRNA, activates the JAK/STAT, MAPK and NK-κB signalling pathways leading to the production of type 1 interferons, chemokines and inflammatory cytokines that can recruit autoaggressive T cells and ultimately lead to the destruction of the β-cell mass and accruing T1D pathogenesis.

Rather, CB4 infects and does not directly destroy β-cells, stimulates cellular stress signaling and recruits resident APCs that engulf the infected β-cells. Previously sequestered antigens including β-cell-specific antigens along with viral antigens are then presented by the APCs to pre-existing autoreactive T cells. The activation of these β-cell-specific T cells leads to the destruction of β-cells and the development of autoimmune diabetes[118]. An important role for APCs in diabetes development has been well established in both human and mouse studies. DCs are important mediators of innate and adaptive responses and specifically, of immune tolerance. As such, the role of DCs in autoimmunity and autoimmune diabetes in particular, has been extensively studied[183].

Owing to their capacity to induce and boost regulatory T cells (Tregs), DCs have been pursued as a therapeutic strategy to restore tolerance in T1D[184].

Macrophages, however, seem to have adverse effects in the development of T1D. In NOD mice, depletion of macrophages reduces spontaneous disease incidence[185]. $CD11b^+$ $CD11c^-$ APCs (containing monocytes and macrophages) isolated from uninfected NOD mice are impaired in their ability to support Treg suppression and maintenance of tolerance. NOD macrophages, dendritic cells, and T cells have also been shown to express low levels of CD86 compared to CD80, CD28 or CD40 expression and compared to cells isolated from diabetes resistant C57BL/6 and BALB/c mice. Low CD86 expression on NOD APCs and T cells has also been shown to be independent of MHC haplotype and diabetes development. NOD APCs and T cells have also shown defective upregulation of an activation-induced CTLA-4/CD28 ratio[186]. "Semi-mature" $CD11b^+$ $CD11c^-$ APCs with low expression of the costimulatory molecule CD40 have reduced inflammatory properties following CB4 infection that allows for an increase in regulatory T cells and protection from CB4-induced T1D[187]. Additionally, studies using blocking antibodies or genetic depletion of either CD40 or its ligand CD154 (CD40L) on macrophages and/or dendritic cells, has shown that deficiency in the CD40/CD154 interaction increases tolerance[188-191].

As APCs are the first cell types to arrive in the islets in human and mouse T1D, they have an important influence on autoreactive responses and the development of T1D. Therapeutic strategies aimed at exploiting their activation propensity and therefore influencing adaptive responses to divert autoreactivity and restore tolerance could serve as effective treatments in autoimmune diseases including T1D.

1.11 Regulatory T cells and type 1 diabetes

Regulatory T cells (Tregs), categorized as such by the expression of the transcription factor Foxp3, are essential mediators in the suppression of immune and autoimmune responses[32, 192-196]. Tregs express various surface adhesion and chemoattractant receptors that allow for their extensive distribution and maintenance of immune

homeostasis across lymphoid and non-lymphoid tissues[197]. Tregs are derived from the thymus (natural Treg, nTreg) and in the periphery (inducible Treg, iTreg) following antigenic exposure. The transcription factor Helios has been previously used as a marker to differentiate nTregs from iTregs, with increased expression commonly associated with nTregs, though several reports have shown expression of Helios in iTregs, rendering its use as an appropriate Treg marker debatable[198-200]. The cell surface molecule, neuropilin-1 (Nrp-1), however has emerged as a new and more reliable marker to distinguish Tregs, where extensive studies have demonstrated that the majority of $Foxp3^{+}$ T reg cells (in NOD and C57BL/6 mice) that express Nrp-1 are thymically derived, whereas Nrp-1^{lo} Tregs represent a peripherally derived T reg cell subset. The Nrp-1^{hi} Treg subset or nTregs from NOD mice also show increased expression of Helios[201]. Nrp-1 has also been shown to contribute to the interaction between dendritic cells and Tregs, which is important in Treg maintenance of self-tolerance[202].

The central role of Tregs in immune homeostasis has been solidified with evidence from human and mouse cases of deletion or loss-of-function mutations in the *Foxp3* gene that cause multiorgan autoimmune disorders[203-205]. Whether a universal mechanism or specific cell programming of suppression mediates Treg function to maintain immune homeostasis is still a matter of debate[206]. The fundamental function of Tregs that we do know is that they suppress immune responses to 'danger' triggers that include self-antigens, commensal microorganisms, and pathogen infections, by interfering with the activation, the T-cell receptor-mediated proliferation and differentiation, of naïve effector T cells (Teffs)[206-209]. Shortly after a pathogenic insult has occurred, Treg suppressive activity must be controlled in order to allow for efficient anti-pathogen responses. Following viral infection, the suppressive function of Tregs can be overturned to allow for effective anti-viral responses[206-209]. Certain bacterial and viral components have been shown to block Treg suppression, either indirectly, through the activation of APCs that produce inflammatory cytokines and cause Teffs to resist Treg suppression, or directly, by targeting Tregs head on, without the involvement of APCs[208, 210-213]. Interestingly, expression of the innate anti-viral sensor MDA5 by Tregs and Teffs, leads to the loss of Treg suppressive function following infection with encephalomyocarditis

virus (EMCV), a picornavirus with diabetogenic potential in mice[208]. This implicates a direct influence of innate anti-viral immunity on adaptive immune responses, which has further propensities in autoimmune diseases like T1D that result in part from the dysregulation of peripheral immune and for which viral infections and anti-viral immunity have been strongly associated with disease etiology.

It has been suggested in the natural immunopathogenesis of T1D that at birth, in healthy individuals, islet-specific autoreactive T cells are restrained from targeting islet cells by immune regulation and with the encounter of some pathogenic factor, immune tolerance can be disrupted and become imbalanced, allowing for the loss in control of autoreactive T cells and the destruction of β-cell mass, ultimately leading to autoimmune diabetes. Autoreactive islet-specific T cells are detected not only in the periphery of patients with T1D, but are also found in healthy individuals. This observation has encouraged the intense investigation of a potential defect in naturally occurring mechanisms that allows for a break in peripheral tolerance, specifically, mechanisms that lead to the impairment of Tregs. Importantly, from observations in immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) patients that have loss-of-functions mutation in the *Foxp3* gene and from extensive studies in the NOD mouse, the mouse model for T1D, we know that a loss of Tregs or profound Treg dysfunction, can lead to T1D even without the influence of other genetic or environmental factors[196, 205]. Though Foxp3 expression strongly contributes to Treg function, other transcription factors such as T-bet, Irf4 and PPARγ, have more recently been implicated in cooperation with Foxp3 to contribute to the Treg phenotype [214]. Additionally, tissue-specific $CD4^+$ T cells lacking Foxp3 that secrete Interleukin-10 (IL-10) have been isolated from patients at risk of T1D, but remain protected[215]. These IL-10 producing $Foxp3^-$ Tregs though 'naturally arising', seem to exert regulatory properties only in cases of severe inflammatory pathology[216]. Nonetheless the specific contribution of Treg dysfunction to T1D development is still a debate, with changes in Treg frequency and suppressive function and Teff resistance to Treg function predominantly implicated.

Studies in prediabetic patients that are islet-cell autoantibody positive have shown that altered or dysfunctional Tregs are present during the early stages of disease preceding clinical presentation. Some researchers have hypothesized that a particular haplotype may be responsible for increasing susceptibility to Treg dysfunction and ultimately T1D. GWAS and functional studies have shown that T1D-associated variants in genes that contribute to the IL-2 signaling pathway (important for Treg fitness and function) such as *IL2RA*, encoding the activation marker CD25, and *PTPN2*, encoding a critical phosphatase in cytokine signal transduction, also lead to impaired Treg function. It must be noted however that healthy individuals from these studies carrying the same variants in the IL-2 pathway genes, also harbored impairments in Treg function, leaving the role of the *IL2RA* and *PTPN2* genes in respect to Treg function in T1D still questionable.

Many recent studies have advocated that in NOD mice, the frequency of Tregs appears stable in the neonatal stage, however, over time, Treg number and function decline as they are accompanied by a rise in type 1 helper T cells (Th1) and Th17 autoreactive T cells at the site of autoimmunity[217-220]. Other studies suggest that Treg function is affected more so by Teffs acquiring resistance to Treg suppression and further that Foxp3 expression dictates Treg function and the potential conversion of Tregs into IFN-γ and IL-17 secreting Teffs[219]. In T1D patients the frequency of Tregs in the blood is similar to healthy individuals, and is concurrent with observations in NOD mice, though the nature of Treg dysfunction in humans is still controversial, owing primarily to a discrepancy in Treg markers and as such, identification of thymic and peripheral Tregs and to inconsistencies in subjects selected by age, sex and HLA typing for controls in patient studies [32, 199, 217, 221-224]. Recent GWAS studies have demonstrated variance between individuals in the expression of genes in Treg-specific regulatory pathways, albeit a particular general Treg signature was associated with T1D, the interindividual variance further complicates the identification of particular factors mediating Treg function[225]. Although the mechanisms influencing Treg function in T1D remain controversial, multiple independent studies have confirmed that a reduction in Treg function is present prior to clinical onset, during the course of disease, and in many subsequent years after diagnosis of T1D[32, 226].

Many studies have alluded to the role of cytokines, present in an already established inflammatory environment within the islets, in the reduction of Treg function. The cytokine milieu, or the presence of IL-2, IL-12, IL-1β, IL-6, IL-10, IL-17, IFN-I, IFN-γ TGF-β or TNF- cytokines, within the islet, has been implicated by many studies in mice as a contributing factor in Treg function, the balance of Th1/Th17 cells to Tregs and the development and pathogenesis of T1D[195, 227].

Primary evidence to substantiate this role of a cytokine milieu in influencing Treg function, spurred from studies in NOD mice demonstrating how reduced secretion of the cytokine interleukin-2 (IL-2), as a result of an *Idd3* T1D susceptibility allele, impacted Treg stability and function and that restoration of IL-2 secretion, either through low dose recombinant IL-2 delivery or IL-2 gene therapy, promotes the expansion and proliferation of Tregs and can even prevent and reverse T1D[217, 228, 229]. Further research in patients with the delivery of IL-2 to patients with immune-mediated diseases has shown success in expansion of Tregs, but in some cases, has not been associated with clinical outcome[230]. Nevertheless, clinical studies administering low-dose IL-2 in T1D patients have reported the successful expansion and activation of Tregs and further functional studies investigating the intrinsic effects of low dose therapy on Treg function, support low dose IL-2 therapy as a T1D therapeutic strategy that evades the commonly administered immunosuppression approach and its associated toxicities[231, 232].

Other investigation of cytokines capable of expanding Tregs and improving their function include studies with the pleiotropic family of cytokines, transforming growth factor-β (TGF-β), known for their potent immune response suppressor functions. Transient pulses of TGF-β in the islets of transgenic TTA/TGF-β NOD mice prior to T cell priming expands Tregs at the site of autoimmunity and prevents diabetes[233]. A study from Richer et al has shown that transgenic overexpression of TGF-β in the pancreas of NOD mice promotes Treg expansion and suppressive capacity leading to the protection from coxsackievirus-mediated T1D [119]. Additionally, Ishigame and colleagues have shown that depletion of thymic TGF-β signaling accelerates T1D in NOD mice transgenic for

the BDC2.5 T-cell receptor (NODBDC2.5) and that disease coincides with a reduction in peripheral Foxp3^{+}Tregs and Th17 cells and an increase in Th1 cells. Moreover, an autocrine/paracrine mechanism of TGF-β signaling in diabetogenic CD4^{+} T cells and not Foxp3^{+}Tregs was demonstrated and was shown to influence T1D susceptibility[234].

Furthermore, a recent study has reported that following RNA virus infection, RIG-I-like receptor (RLR) signaling by the RLR receptors MDA5 and RIG-I that leads to the activation of the signaling mediator IRF3 and downstream expression of IFN-β, also leads to the downregulation of Smad signaling in the TGF-β signaling pathway and consequently inhibits TGF-β responses including TGF-β-induced Treg cell differentiation. Xu et al. also reported that a loss in IRF3 expression rescued TGF-β signaling and allowed TGF-β induction of Tregs even following virus infection[235]. Signaling from RLRs and the induction of IFN-I following acute viral infection has also demonstrated a direct effect of IFN-I on Treg activation and proliferation. Srivastava et al have shown that IFNAR expression on Tregs allows for direct IFN-I-mediated inhibition of Treg function and effective antiviral adaptive responses[236]. These studies provide important crosstalk mechanisms by which antiviral innate responses influence adaptive responses that have significant implications for autoimmune diseases such as T1D for which viral infection and Treg dysfunction are strongly associated with disease susceptibility and pathogenesis.

Genetic and functional studies in humans and mice continue to enrich our understanding of genetic defects, immune components and mechanisms that modulate Treg function and consequently, T1D pathogenesis. Under the modulation of as yet undetermined specific factors, the specificity of the Treg TCR repertoire and impairment in thymic selection, interference in trafficking to the pancreatic lymph nodes at the site of autoimmunity, increased susceptibility to apoptosis and functional instability have all been attributed to the break in peripheral tolerance and improper Treg function leading the development or exacerbation of T1D[32, 39, 193, 195, 196, 206, 220, 237]. Nonetheless, human and mouse studies examining Treg dysfunction have created an avenue of T1D therapeutic

strategies capitalizing on approaches to restore Treg function and promote Treg expansion for the prevention or reversal of autoimmune diabetes.

Accumulating evidence for tolerogenic dendritic cells and their ability to reestablish tolerance through either direct inhibition of autoreactive T cells or the expansion of Tregs is one such avenue explored for Treg therapy[184]. Many strategies to manipulate Tregs including *ex vivo* expansion and transplantation of Tregs to protect beta cells from destruction have been devised with some strategies successfully preventing and reversing T1D in NOD mice as well as recent-onset and diabetic patients [238-242]. Though Treg immunotherapy strategies offer great promise in the treatment and prevention of T1D, they are not without barriers. Treg stability, discrepancies in co-stimulation pathways and the activation of other T cell subsets, the presence of memory T cells and assuring purity and potency of Treg preparations are prominent barriers researchers must surpass in devising Treg therapies[242]. Additionally, several current therapies aimed at expanding Tregs have shown early success in treating autoimmunity, but have been unsuccessful in maintaining protection over time[238, 243]. In light of these challenges, researchers are devising new protocols to improve Treg stability, purity in preparations, storage, and off-target side effects.

1.12 Rationale, hypothesis and aims

Type 1 diabetes (T1D) is a devastating disease involving the autoimmune destruction of insulin-producing pancreatic β-cells. The personal and economic burden of this disease is enormous, therefore simpler and more cost effective therapeutic approaches than those currently available must be explored. Epidemiological and genetic data have associated virus infections and anti-viral type 1 interferon (IFN-I) response genes with T1D. In children at risk for Type 1 diabetes (T1D), a unique IFN-I transcriptional signature precedes islet autoimmunity. Additionally, recent onset of T1D strongly associates with infection by RNA viruses like Coxsackievirus that induce IFN-I and further implicate the importance of the early unique IFN-I signature. In conjunction with genetic and immunological risk factors, T1D risk has been strongly tied to enteroviral infection, particularly coxsackievirus B (CBV) infection. Our lab and others have demonstrated the

diabetogenic potential of the RNA virus CVB type 4 (CB4) in the non-obese diabetic (NOD) mouse.

Genetic variants in the T1D risk locus interferon induced with helicase C domain 1 (*IFIH1)* have been identified by genome-wide association studies (GWAS) to confer resistance to T1D and result in the reduction in expression of the intracellular RNA virus sensor known as melanoma differentiation-associated protein 5 (MDA5).
MDA5 activates in the presence of viral double stranded RNA affecting many cell-signalling pathways that initiate anti-viral responses including most importantly, type 1 interferon (IFN-I) production. IFN-I responses as a result of MDA5 signalling have significant implications in the development and exacerbation of autoimmune disease. Recent genome-wide association studies (GWAS) have linked non-synonymous polymorphisms (nsSNPs) in MDA-5 that confer protection to T1D, therefore making this receptor a candidate for therapeutic maintenance of T1D.

Within an autoimmune prone host, amplified inflammatory responses from viral sensing can be detrimental and instigate autoimmune disease. Finding a threshold between pathogen defense and protection from autoimmune disease relies on a balance in viral sensing and peripheral tolerance. As such, this research has aimed to determine how MDA-5 acts as a susceptibility factor in spontaneous T1D and CB4-mediated T1D.

We hypothesize that there is a direct correlation between the IFN-I signature induced by MDA5 following environmental challenge with the induction of a strong effector T cell (Teff) and a matched regulatory T cell (Treg) response. As patient polymorphisms in MDA-5 retain some level of protein function, we anticipate that a reduction in MDA5 expression, as we have translated onto the NOD mouse, alters IFN-I signaling after CB4 infection in a manner that controls and clears virus and mediates the strength, polarization and regulation of T cell responses in favor of T1D protection. Understanding how MDA5 controls anti-viral signaling and consequently T1D pathogenesis is critical in defining disease progression and developing prophylactic and therapeutic measures.

1.12.1 Aim1

Our first directive was to test the link between protective polymorphisms identified in the human population that result in the reduction of an innate receptor and the susceptibility to T1D following viral infection. We designed experiments in Chapter 2 to test the hypothesis that a reduction in MDA5 expression, as with patients carrying protective *IFIH1* polymorphisms, protects from spontaneous and CB4-induced T1D.

1.12.2 Aim2

After translating a reduction in MDA5 expression on to the non-obese diabetic (NOD) mouse we observed protection from spontaneous and CB4-induced T1D and identified a specific IFN-I signature as a result of reduced (not eliminated) MDA5 sensing of CB4. With adoptive transfer experiments we also observe that the cells responsible for the sustained presence of the signature are $CD11b^{+}CD11c^{+}$ cells. As such, in Chapter 3, we sought to determine whether disease pathogenesis and immununological phenotypes such as the unique type 1 interferon signture observed in $MDA5^{+/-}$ were specific to a reduction in MDA5 signaling and whether the unique IFN-I and T cell responses observed in $MDA5^{+/-}$ were specific to CB4 infection.

1.12.3 Aim3

Our functional and mechanistic studies helped identify a unique IFN-I signature limited to MDA5 that does not waver in the presence of sustained IFN-I stimulation and that is not observed with a reduction in another RNA sensing molecule (TLR3) or T1D-associated receptor (VDR) following CB4 infection. We also demonstrate that disease pathogenesis is linked to the specific IFN-I response induced by the virus as a strain of CB3 failed to modify the IFN-I signature associated with disease. These results suggest that MDA5 signaling is essential in regulating the IFN-I signature that mediates T cell responses following virus infection and determines disease fate.

As such, we sought to characterize the IFN-I transcriptional landscape associated with a reduction in MDA5 following CB4 infection. In Chapter 4, we hypothesized that $MDA5^{+/-}$ infected with CB4 also maintain a unique transcriptome signature at day 3pi from NOD mice retaining full expression and function of MDA5 signaling. With RNAseq technology, we established a transcriptome phenotype at day 3 pi in $MDA5^{+/-}$

PLNs, spleen and pancreas to help identify potential changes in IFN-I and T cell activation associated genes to support our molecular and cell data and further our understanding of the mechanism driving protection in MDA5hets.

Chapter 2: Reduced expression of the RNA virus sensor MDA5 prevents autoimmune diabetes.

2.1 Introduction

Type 1 diabetes (T1D) is a devastating organ-specific disease resulting from the autoimmune destruction of pancreatic beta cells [31]. The events leading to autoimmunity in T1D are complex and unclear, demanding the design of new treatments that consider both the strong genetic influence and environmental stressors linked to the disease. Protective polymorphisms that have been identified in the intracellular virus receptor MDA5 gene *IFIH1* that lead to a reduction in MDA5 urges further investigation of RNA virus sensing in T1D pathogenesis [71, 72, 74].

Understanding how MDA5, a double-stranded RNA (dsRNA) virus sensor expressed from the T1D risk gene *IFIH1,* controls IFN-I and consequently T1D is critical in understanding the events leading to autoimmunity, defining disease progression and developing prophylactic and therapeutic measures.

In children at risk for T1D, an IFN-I transcriptional signature precedes islet autoimmunity[169]. Recent onset of T1D is strongly associated with infection by RNA viruses such as enteroviruses including coxsackievirus further implicating the IFN I signature[91, 171]. MDA5 specifically detects dsRNA intermediates from viruses like coxsackievirus that are produced in the cytoplasm during RNA virus replication. Upon recognition of its dsRNA ligand and proper assembly of a filament structure around the dsRNA stem, MDA5 activates and triggers signaling from IFN-β promoter stimulator-1 (IPS-1, also known as the mitochondrial antiviral signaling protein MAVS), IFN regulatory factor-3 (IRF-3) and IRF-7 molecules to induce the transcription of IFN-β. The specificity and kinetics of MDA5-RNA binding as well as MDA5-induced IFN I responses that result from viral infection have been well studied [244].

Recently it was demonstrated that mice carrying a missense mutation G821S in the *Ifih1* gene disrupts MDA5 responsiveness to dsRNA, yet allows MDA5 to remain

constitutively active and induce lupus-like nephritis and autoimmunity [166]. Funabiki et al also demonstrated constitutive IFN I signaling with the common T1D risk variant A946T[166]. Further, non-diabetogenic mice (C57BL/6 mice) heterozygous at *Ifih1* develop rapid hyperglycemia after infection with the pancreatropic virus encephalmyocarditis virus-D (EMCV-D) due to virus-directed beta cell death [122]. Unlike EMCV-D, coxsackievirus serotype B4 (CB4) infects, and without directly killing pancreatic β cells induces diabetes in NOD mice [118, 120] while more intriguingly has been strongly associated in recent onset T1D patients [6,7,13].

To better our understanding of the immunological consequences following MDA5 activation and their effects on autoimmunity in a susceptible host, we developed a mouse model resonant with *IFIH1* protected patients, by backcrossing mice deficient in Ifih1 (also known as MDA5) onto the accepted mouse model for T1D, NOD/Ltj mice and studying mice heterozygous for the deficient MDA5 allele. We demonstrate that these heterozygote mice ($MDA5^{+/-}$) express roughly half the level of MDA5 protein as wildtype mice ($MDA5^{+/+}$) and when infected with CB4, a clinically relevant stimulator of T1D, the $MDA5^{+/-}$ were protected from T1D, thereby defining the ability of MDA5 to augment autoimmunity and control T1D.

2.2 Materials and methods

Mice. NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $MDA5^{-/-}$ on the C57BL/6 background were a generous gift from Dr. M. Colonna (St.Louis, MO). We successfully backcrossed $MDA5^{-/-}$ mice onto the NOD background and confirmed by SNP analysis (our group and DartMouse, Lebanon, NH) that they carry the full complement of NOD *idd* alleles. More importantly, the ability to develop spontaneous diabetes is strongly indicative that the required susceptibility loci have crossed over and to this end, littermates to the backcrosses that were either heterozygote and wildtype for the $MDA5^{-/-}$ alleles developed spontaneous diabetes. $MDA5^{-/-}$ mice were bred with NOD mice and $MDA5^{+/-}$ and $MDA5^{+/+}$ progeny were bred for further use in experiments. All mice were maintained in the Centre for Disease Modeling (Life Sciences Centre, Vancouver, British Columbia) and kept in a pathogen-free environment.

Diabetes incidence was monitored by nonfasting blood glucose measurements. Disease onset was determined by two consecutive blood glucose levels exceeding 300 mg/dL. Only pre-diabetic mice (blood glucose between 130mg/dL and 200 mg/dL) were used for experiments. All animal work was performed under strict accordance with the recommendations of the Canadian Council for Animal Care. The protocol was approved by the Animal Care Committee (ACC) of the University of British Columbia (certificate numbers: A08-0415, A08-0622, and A130116).

Western blotting. Mice were stimulated by intraperitoneal injection with 100μg of polyinosinic:polycytidylic acid (P1530, Sigma, St. Louis, MO). After 24 hours stimulation, spleens were isolated and homogenized by sonication and tissue homogenates were lysed with CellLytic MT Mammalian Tissue Lysis Reagent (Sigma, St. Louis, MO). Samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene fluoride membranes, blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) probed with monoclonal rabbit anti-MDA5 (Cell Signaling, Danvers, MA) and polyclonal goat anti-tubulin Santa Cruz Biotech, Santa Cruz, CA) primary antibodies and IRDye 800CW and IRDye 680 RD secondary antibodies (LI-COR, Lincoln, NE). Membranes were scanned with the LI-COR Odyssey Scanner (LI-COR, Lincoln, NE). Protein was quantified using LI-COR Odyssey 3.0 Software.

Virus. Ten-to 12-week old mice were infected intraperitoneally with sublethal doses of 400 plaque-forming units (PFUs) of CB4 Edwards strain 2 diluted in DMEM. As there is no gender bias in CB4-mediated T1D, equal numbers of male and female mice were infected with CB4. Virus stocks were prepared as described previously[118].

Virus titer. Free virus particles were detected from tissue homogenates by plaque assay as described previously[118].

Flow cytometry. Pancreatic lymph node and splenic single cell-suspensions were counted and stained with fluorescently conjugated mABs for cell surface markers CD4

(clone L3T4), CD8 (53-6.7), CD25 (clone PC61), CD11b (clone M1/70), CD11c (clone HL3), CD44 (clone IM7), and CD62L (clone MEL-14), intracellular transcription factors Foxp3 (clone FJK-16s) and Helios (clone 22F6) and the inflammatory cytokine IFN-γ (XMG1.2). All mAbs were purchased from eBiosciences (San Diego, CA) with the exception of Helios from BioLegend (San Diego, CA). Stained cells were analyzed by flow cytometry with the BD Biosciences LSR II (San Jose, CA) and Flow Jo vX.0.6 software (TreeStar, Ashland, OR).

Intracellular cytokine staining. Single cell-suspensions from pancreatic lymph nodes and spleens were restimulated for 4 hours at 37°C in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum with 500ng/ml PMA, 10 ng/ml ionomycin and Golgi Plug (BD Biosciences). Cells were stained for surface markers, fixed, permeabilized, stained for inflammatory cytokine γ-interferon (IFN-γ) and analyzed by flow cytometry.

In vitro T cell activation. Splenic $CD4^+$ T cells were isolated from uninfected $MDA5^{+/+}$ and CB4 infected $MDA5^{+/+}$ and $MDA5^{+/-}$ mice at day 7 pi using the EasySep Mouse CD4+ T cell pre-enrichment kit and "The Big Easy" Silver EasySep Magnet (Stemcell, Vancouver, BC). $CD4^+$ T cells (5×10^7 cells/mL) were then enriched for CD25 activation using the EasySep Mouse CD25 Positive Selection Kit (Stemcell, Vancouver, BC). $CD4^+CD25^-$ T cells from uninfected $MDA5^{+/+}$ mice were mixed with $CD4^+CD25^+$ T cells from CB4 infected $MDA5^{+/+}$ or from infected $MDA5^{+/-}$ mice (3×10^6 in 1 ml RPMI-1640 containing 10% FCS, 50 μM 2-mercaptoethanol, and penicillin/streptomycin) in a 96-well plate coated for 24 hours with anti-CD3e mAb (1μg/mL, BioLegend) and anti-CD28 mAb (1μg/mL, BD Pharmingen). Mixed cells were also co-cultured in uncoated wells as a control and all cells were cultured for 72 hours at 37°C. To assess T cell activation, cells were stained with anti-CD4-Pacific Blue and anti-CD25-PE (eBioscience). To assess T cell effector function, cells were stimulated with PMA and ionomycin (Sigma) in the presence of BD Golgi Plug™ (BD Biosciences) for 4 h at 37°C. The cells were then stained with anti-CD4-Pacific Blue and anti-CD25-PE before being fixed and

permeabilized for intracellular staining with anti-IFN-γ-PECy7 (eBioscience). Data were acquired using an LSRII flow cytometer and analyzed with FlowJo software vX.0.6.

Immunohistochemical staining (or islet pathology). Pancreases were fixed in 70% ethanol for 24 hours and paraffin embedded (Wax-IT, Vancouver, British Columbia) as previously described[118]. Serial tissue sections were stained using standard procedures for hemotoxylin and eosin to analyze the anatomical structure and were scored for insulitis according to three-tiered scale.

Cytokine analysis. Cytokines IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFN-γ were measured from serum days 0, 3 and 7 post-CB4 infection in a multiplexed format using a Cytometric Bead Array (mouse Th1/Th2/Th17 cytokine kit; BD Biosciences, Mississauga, ON). Type 1 interferons IFN-α and-β were measured from serum by ELISA using VeriKine Mouse Interferon--α and β ELISA kits (PBL Interferon Source, Piscataway, NJ).

RNA isolation. Organs were removed and immediately snap frozen in TRIzol reagent (Life Technologies Inc, Burlington, ON). Tissues were weighed and organs were homogenized using QIAGEN stainless steel beads and TissueLyser II benchtop homogenizer at 19/s for 10 min. Total RNA was prepared with TRIzol reagent according to the manufacturer's protocol (TRIzol, Life Technologies). RNA was quantified using a NanoDrop-ND-1000 (VERIFY) (Thermo Scientific, Wilmington, DE).

Reverse transcription and quantitative real-time PCR. cDNA was prepared from 1μg of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reverse transcription-PCR was performed with the BioRad T-100 Thermal Cycler.

cDNA was diluted with UltraPure™ DNase/RNase-Free Distilled Water (Life Technologies) and a final RNA concentration equivalent to 10μg/μl was used for real

time (RT)-PCR. Gene expression for MDA5, IFN-β, IFN-α, TLR3 and GAPDH was quantified using the iQ™ SYBR® Green Supermix (BioRad, Mississauga, ON) and specific primers (mouse MDA5 forward 5'- GTGATGACGAGGCCAGCAGTTG -3', reverse 5'- ATTCATCCGTTTCGTCCAGTTTCA-3'; IFN-β forward 5'- GCACTGGGTGGAATGAGACTATTG-3', reverse 5'- TTCTGAGGCATCAACTGACAGGTC-3'; IFN-α forward 5'- TGATGAGCTACTGGTCAGC-3', reverse 5'- GATCTCTTAGCACAAGGATGGC-3'; TLR3 forward 5'- GAGAGAGATTCTGGATGCTTGTGTTTG-3', reverse 5'- GTCTCATAATGGTTTATCATCTACAAA-3'; and GAPDH forward 5'- AGGTCGGTGTGAACGGATTTG-3', reverse 5'- TGTAGACCATGTAGTTGAGGTCA-3'). PCR amplification was performed in 384-well plates with the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). All samples from three independent experiments were evaluated in duplicate amplification reactions. mRNA expression was normalized to GAPDH. The comparative C_t method was used as previously described in Example 5 [245] and data is shown as $-\Delta C_t$ and fold change of $-\Delta C_t$ relative to Wt (NOD) samples [245].

Adoptive transfer studies. Spleens harvested from donor uninfected mice were used to generate single-cell suspensions. Cells were stained with fluorescently conjugated mAbs against CD11b and CD11c and sorted with a FACSAria flow cytometer (BD Biosciences, Mississauga, ON). A total of a hundred thousand $CD11b^+CD11c^-$ and $CD11b^+CD11c^+$ cells, sorted at a purity of 96% and diluted in phosphate buffered saline with 2% fetal bovine serum were adoptively transferred intraperitoneally into uninfected recipient mice. Recipient mice were infected intraperitoneally 24 hours after adoptive transfer with CB4 Edwards strain 2 and spleens, pancreatic lymph nodes, pancreas and serum were harvested at day 7 post-infection for analysis. Control mice did not receive cells and were infected with CB4 Edwards strain 2 at the same time as experimental recipient mice.

Statistical analysis. GraphPad Prism 6.0 software (GraphPad, San Diego, CA) using the Student's t-test (two-tailed distribution) and a P value <0.05 determined statistical

significance. Serum cytokine concentrations were determined with FCAP Array Software (BD Biosciences, Mississauga, ON). Data are presented as means ± SEM.

2.3 Results

2.3.1 A reduction in MDA5 protects NOD mice from T1D

Since patients carrying *IFIH1* protective variants are heterozygous, we followed T1D incidence and pathologies in NOD mice heterozygous for the MDA5 gene ($MDA5^{+/-}$). $MDA5^{+/-}$ were generated by successfully backcrossing C57BL/6 MDA5 knock out (ko,-/-) mice on to the NOD background and confirmed by DartMouse. Western blot confirmed that after stimulation with the RNA mimetic, polyinosinic:polycytidylic acid (poly i:c), $MDA5^{-/-}$ (-/-) were unable to generate MDA5 protein, while $MDA5^{+/-}$ produced 48% less protein than their $MDA5^{+/+}$ littermates (Figure 2.1). MDA5 expression was also markedly reduced in the spleens and in different antigen presenting cell (APC) subsets from the pancreatic lymph nodes (PLNs) of Coxsackievirus B4 (CB4) infected $MDA5^{+/-}$ mice at day 3 post-infection (pi) compared to CB4-infected $MDA5^{+/+}$ mice (Figure 2.1, Figure 2.5). Importantly, we observed that MDA5 deficiency (MDA5 -/-) protected mice from spontaneous disease, while loss of a single MDA5 allele ($MDA5^{+/-}$) reduced the incidence of spontaneous diabetes compared to $MDA5^{+/+}$ littermates (Figure 2.1). These results implicate MDA5 signaling in the development of T1D in NOD mice and justify the study of $MDA5^{+/-}$ as a model for the protective IFIH1 variants found in patients.

CB4 has been isolated from patients with T1D, associated to disease onset, and found to accelerate NOD diabetes. CB4 infects, but does not directly kill pancreatic β cells and instead activates a pre-existing population of autoreactive T cells to elicit autoimmunity[118, 120]. Resident antigen presenting cells (APCs) engulf infected β-cells, present sequestered islet and viral antigens to resting autoreactive T cells and induce a population of T cells directed both at the virus and specific to the pancreatic islets. The β-cell directed T cells then destroy the islets and accelerate insulin loss and T1D disease pathology[118].

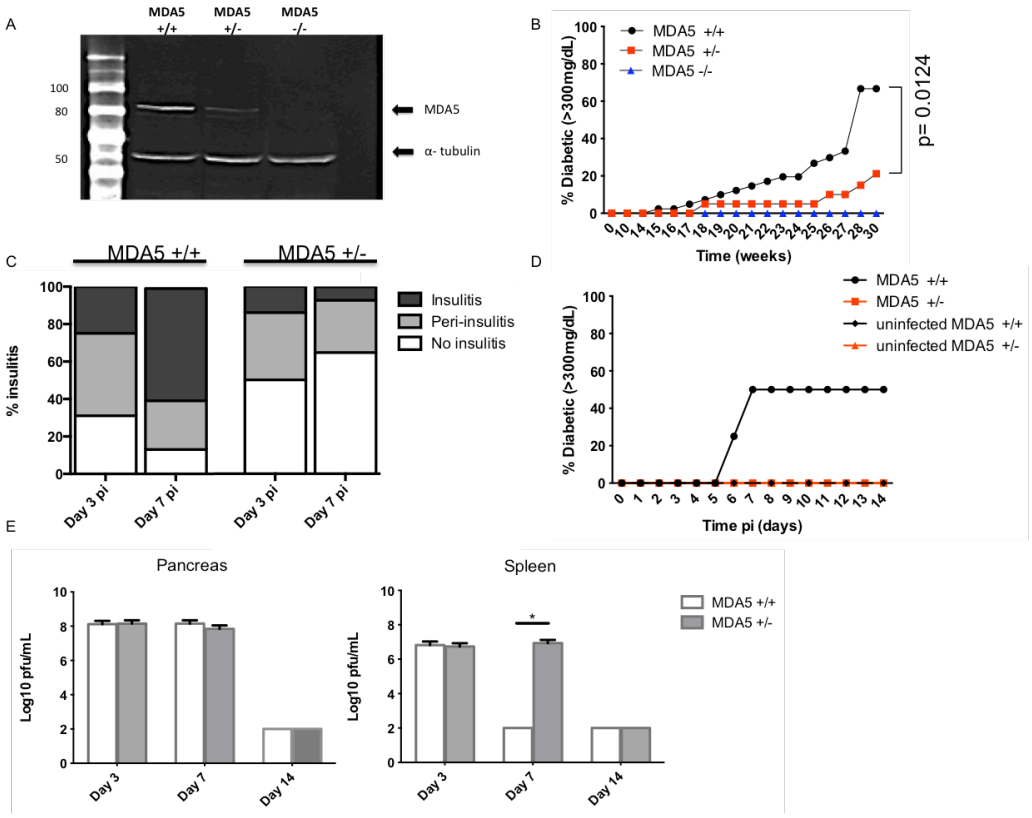


Figure 2.1 A reduction in MDA5 protects NOD mice from spontaneous and virus-mediated type 1 diabetes. (A) MDA5$^{+/+}$ (+/+), MDA5$^{+/-}$ (+/-), and MDA5$^{-/-}$ (-/-) were stimulated with poly i:c as described in Materials and Methods. After 24 hours stimulation, a 48% reduction in MDA5 protein from MDA5$^{+/-}$ spleens compared to MDA5$^{+/+}$ was confirmed by western blot (B) Diabetes incidence was monitored in MDA5$^{+/+}$ (n=41), MDA5$^{+/-}$ (n=23) and MDA5$^{-/-}$ mice (n=20) for 30 weeks. Two consecutive blood glucose levels greater than 300mg/dL determined diabetes incidence. (C) Serial sections of pancreas tissue from MDA5$^{+/+}$ and hets at days 3 and 7 post-CB4 infection were stained for hemotoxylin and eosin and scored for insulitis according to a three-tiered scale (no insulitis, peri-insulitis, insulitis). (D) Infected 10-12 week old MDA5$^{+/+}$ (n=10) and MDA5$^{+/-}$ (n=15) and uninfected MDA5$^{+/+}$ (n=12) and MDA5$^{+/-}$ (n=8) mice were monitored for cumulative diabetes incidence up to 14 days pi and (E) viral titers were quantified by standard plaque assay from pancreas and spleens at 3, 7 and 14 days post-infection (pi). Results are shown as mean ± SEM and statistical significance was determined by student's t test. *p<0.05.

Though strongly linked to T1D, in patients CB4 may only be responsible for a subset of new onset cases as other viruses such as CB1 and rotavirus have more recently been implicated[87]. As CB4 is a clinically relevant inducer of T1D that stimulates MDA5 and accelerates NOD diabetes, it clearly models the events leading to T1D in mice by mimicking environmental influences that lead to T1D. Cumulative diabetes incidence

was monitored following infection with CB4 in 10-12 week old MDA5$^{+/-}$ mice. We observed that MDA5$^{+/-}$ mice were completely protected from the development of T1D compared to infected age-matched MDA5$^{+/+}$ littermates that developed disease incidence at 50% by 14 days post-infection (pi) (Figure 2.1). As expected of mice 10-12 weeks old, uninfected MDA5$^{+/-}$ and MDA5$^{+/+}$ littermates did not develop disease (Figure 2.1). It is astonishing however, that MDA5$^{+/-}$ mice demonstrated sufficient anti-viral responses to clear the virus similarly to MDA5$^{+/+}$ mice (Figure 2.1), though MDA5$^{+/-}$ mice have a reduction in MDA5 expression (Figure 2.1, Figure 2.1 & Figure 2.5). Overall, virus was cleared in both MDA5$^{+/+}$ and MDA5$^{+/-}$ mice by day 14 pi, with an equal rate of clearance in the pancreas, and a slightly slower rate in the spleen for the MDA5$^{+/-}$ mice (Figure 2.1). Despite reduced expression and function of a critical innate immune sensor of CB4, the MDA5$^{+/-}$ mice did not exhibit immunosuppression or a reduced ability to handle virus infection.

As expected, following infection, diabetic MDA5$^{+/+}$ mice exhibited a high degree of pancreatic insulitis reflecting the observed hyperglycemia [118, 120], while MDA5$^{+/-}$ mice at the same time post-infection lacked the same level of insulitis reflecting their protected status (Figure 2.1). While mice with a full complement of MDA5 show dramatic increases in pancreatic islet insulitis from the peak of infection (day 3 pi) until the initiation of diabetes (day 7 pi), MDA5$^{+/-}$ mice show no increase in insulitis. This suggested that reduced MDA5 expression and function altered the ability of effector T cells to home to the pancreatic islets and destroy the pancreatic β cells.

2.3.2 MDA5$^{+/-}$ mice have a unique IFN-I signature after CB4 infection

Following the sensing of dsRNA, MDA5 triggers a signaling pathway leading to the induction of an anti-viral response driven by type 1 IFNs. Analysis of serum levels of IFNs-α, and β showed a significantly different IFN I pattern of expression in the MDA5$^{+/-}$ compared to MDA5$^{+/+}$ mice. At 3 days post-CB4 infection, MDA5$^{+/-}$ showed a significantly greater amount of IFN-β in the serum compared to MDA5$^{+/+}$ (Figure 2.2). By day 7, the MDA5$^{+/-}$ mice had returned to pre-infection levels of IFN-β, where IFN-β

levels in MDA5$^{+/+}$ mice steadily rose over the 7 day time course, with greater IFN-β at day 7 than MDA5$^{+/-}$ mice (Figure 2.2). In both mice, IFNα levels steadily rose post-infection with greater levels in the MDA5$^{+/-}$ mice. The differences in IFN I signature post-infection is best represented as a ratio of IFN-β/IFN-α, where the MDA5$^{+/-}$ mice show a rise at day 3 and return to balanced levels by day 7, while the MDA5$^{+/+}$ mice show a steady increase in the ratio to maximum at day 7 (Figure 2.2). To ask whether a concomitant increase was reflected in mice deficient for the other major sensor of coxsackievirus dsRNA, NOD mice heterozygously deficient for Toll-like receptor 3 (TLR3hets, +/-) were infected and measured for serum IFN I. TLR3het mice show an increase in IFN-β earlier than MDA5$^{+/-}$ by day 2 pi, and a significant rise in IFN-α at day 3 compared to MDA5$^{+/+}$ mice (Figure 2.2).

Further, the variance in IFN I production in the serum of our MDA5$^{+/-}$ mice at day 3 pi was reflected in the level of mRNA expression, where pancreas and spleen tissue from infected MDA5$^{+/-}$ mice, also showed a unique profile of IFN I expression (Figure 2.2). IFN-β expression was elevated in MDA5$^{+/-}$ pancreas whereas IFNα was greater in MDA5$^{+/-}$ spleen at day 3 pi compared to infected MDA5$^{+/+}$. The increase in IFN-β expression in MDA5$^{+/-}$ pancreas also correlated with higher levels of MDA5 expression compared to infected MDA5$^{+/+}$ pancreas. In MDA5$^{+/-}$ spleen, where MDA5 expression was lower than MDA5$^{+/+}$, we observed elevated TLR3 and IFNα expression (Figure 2.2). It is possible that the unique IFN I profile observed in MDA5$^{+/-}$ mice acts in concert with TLR3-induced IFN I responses to control virus infection, yet sustain a level of IFN I production that does not abrogate further inflammation and the onset of autoimmunity.

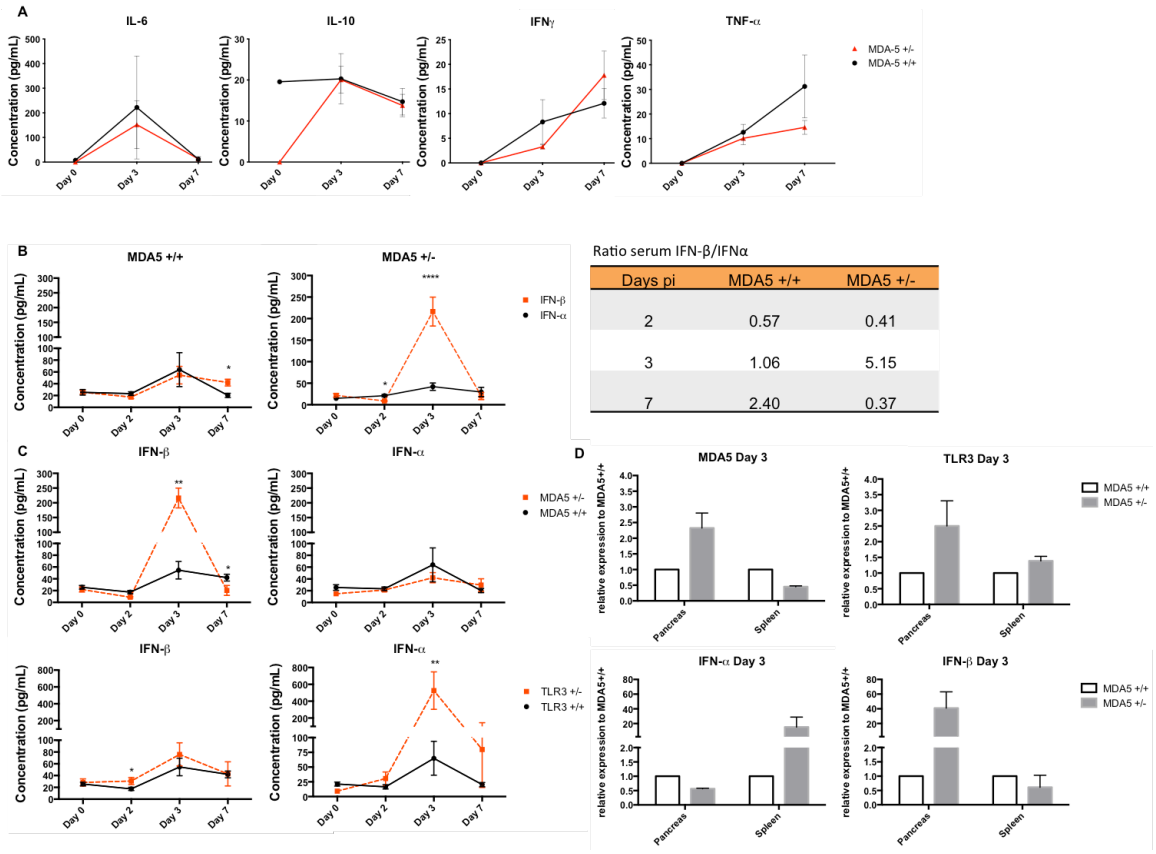


Figure 2.2 MDA5 expression alters the levels of type 1 IFN and not inflammatory cytokines that respond to CB4 infection. Cytokines were measured by FACS bead array (A) and ELISA (B, C) in sera harvested from MDA5$^{+/+}$ (n=8-15) and MDA5$^{+/-}$ mice (n=8-15) before infection (day 0) and days 2, 3 and 7 post-CB4 infection. Statistical significance was determined by Student's t test. *p<0.05, **p<0.001, and ****p<0.0001. (D) Relative mRNA expression levels of MDA5, TLR3 and IFN I from the spleen and pancreas from MDA5$^{+/+}$ (n=8-10) and MDA5$^{+/-}$ mice (n=8-10) at days 0 (not shown), 3, and 7 (not shown) post-CB4 infection were quantified by quantitative real-time PCR and normalized to GAPDH. The comparative C_t method was used to calculate mean relative expression ± SEM against MDA5$^{+/+}$ mice as described in Materials and Methods section. Data shown are from duplicate samples from two independent experiments.

To ask if other cytokines were equally affected following infection under conditions of reduced MDA5 signaling serum was sampled from both mice following infection and measured for inflammatory cytokines and the levels of IL-6, IL-10, TNF-α and IFN-γ, were similar from both pre- and post-CB4 infection when compared between MDA5$^{+/-}$ mice and MDA5$^{+/+}$ mice (Figure 2.2). While the differences in MDA5 innate signaling

altered the IFN I signature, no overall change was observed in other major inflammatory mediators.

2.3.3 A reduction in MDA5 induces regulatory rather than effector immune responses

Type 1 IFNs have diverse immunomodulatory functions that play an important role in many autoimmune diseases including T1D[246]. Dendritic cell (DC) activation and the presentation of sequestered self-antigens to pre-existing autoreactive T cells can be directly affected by IFNs-α, β [247]. To determine whether the changes in MDA5 and IFN I expression within the infected MDA5$^{+/-}$ mice altered APC activation and subsequent T cell polarization leading to protection, we analyzed the expression of major histocompatibility complex (MHC) and costimulatory molecules (CD40, CD80, CD86, F4/80) on APCs (CD11^{b+}CD11^{c+} or CD11^{b+}CD11^{c-} cells) from the spleens and PLNs of MDA5$^{+/-}$ mice and wt mice. At 48 hours post-infection no difference in activation of APCs from both spleen and PLNs were observed between the infected mice (Figure 2.6).

Development of spontaneous diabetes in NOD mice is critically linked to the balance and polarization of effector and regulatory T cells [196]. A growing pancreatic insulitis composed of diabetogenic effector T cells occurs over time and results in the loss and destruction of insulin producing beta cells in the islets. CB4 infection acts to accelerate this insulitis by exposing infected beta cells to the autoreactive T cells causing reactivation[118]. The induction of diabetes is the result in an alteration in the balance between effector autreactive T cells and regulatory T cells forming the pancreatic insulitis. In MDA5$^{+/-}$ mice that show protection from diabetes, an expansion of Tregs (CD4^{+}, CD25^{+}, Foxp3^{+}) is observed by day 7 pi in the pancreas with a concomitant decrease in effector CD4^{+} T cells (CD4^{+}CD44^{hi}CD62^{lo}) as compared to infected MDA5$^{+/+}$ mice (Figure 2.3).

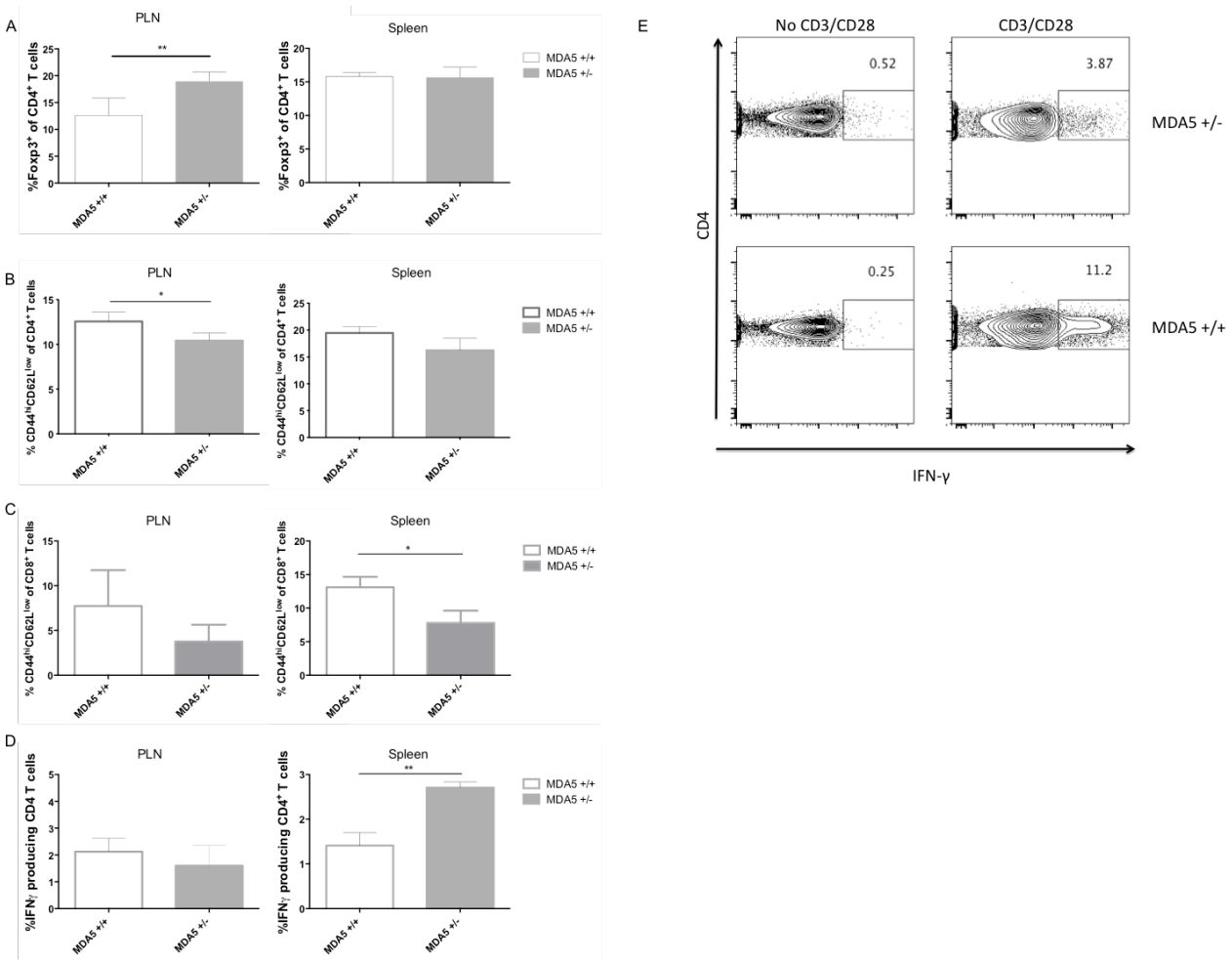


Figure 2.3 Reduced MDA5 expression polarizes a regulatory T cell response. MDA5$^{+/-}$ mice (+/-) have increased levels of (A) regulatory T cells (Foxp3^{+} of CD4^{+} T cells) in the pancreatic lymph nodes (PLNs), (B, C) decreased effector CD4^{+} and CD8^{+} (CD44^{high}CD62^{low}) T cells in both PLNs and spleen, and (D) increased IFN-γ-producing CD4^{+} T cells in the spleen compared to infected MDA5$^{+/+}$ (+/+) mice at day 7 post-CB4 infection. T cells were isolated from MDA5$^{+/+}$ (n=5) and MDA5$^{+/-}$ mice (n=5) at day 7 post-infection and with classical activation and maturation marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from three independent experiments. p-values were determined used Student's two-tailed paired t-test **p<0.01 *p<0.05. (E) Tregs from MDA5$^{+/-}$ mice suppress IFN-γ-production from MDA5$^{+/+}$ CD4^{+} T cells in vitro. CD4^{+} CD25^{-} T cells were isolated from spleens of uninfected MDA5$^{+/+}$ mice and mixed with MDA5$^{+/-}$ or MDA5$^{+/+}$ CD4^{+} CD25^{+} Tregs isolated from CB4 infected mice at day 7 pi as described in Materials and Methods. After 72 h cells were re-stimulated and intracellular staining determined the percent of IFN-γ-producing CD4^{+} T cells. One of two independent experiments that yielded similar results is shown.

A decrease in effector CD4^{+} T cells and a significant decrease in effector CD8^{+} T cells was observed in MDA5$^{+/-}$ spleens compared to infected MDA5$^{+/+}$ (Figure 2.3). Further, at

day 7 pi, CB4 challenged MDA5$^{+/-}$ mice harbor greater numbers of CD4^{+} T cells secreting IFN-γ in their spleens compared to infected MDA5$^{+/+}$ mice that have greater CD4^{+} IFN-γ T cells in the pancreatic lymph nodes (PLNs) (Figure 2.3).

To demonstrate regulatory T cell function, we performed Treg suppression assays by stimulating CD4^{+} CD25^{-} T cells from uninfected MDA5$^{+/+}$ mice and CD4^{+} CD25^{+} Tregs from either infected MDA5$^{+/-}$ or MDA5$^{+/+}$ mice. The Tregs isolated from MDA5$^{+/-}$ mice demonstrated a significantly greater ability to suppress IFN-γ - producing CD4^{+} T cells than Tregs from infected MDA5$^{+/+}$ mice (Figure 2.3). The overall change in the balance of the pancreatic infiltrating effector and regulatory T cells we have observed in MDA5$^{+/-}$ mice and the increased suppressive function of regulatory T cells generated by day 7 pi in these mice likely leads to the observed diabetes resistance.

2.3.4 CD11b^{+}CD11c^{+} cells from MDA5$^{+/-}$ mice induce regulatory T cells.

Polarization of T cells suggested that MDA5 was acting within APCs and to confirm this, APCs were isolated, subdivided (CD11b^{+} CD11c^{+} and CD11b^{+} CD11c^{-}) from either MDA5$^{+/-}$ or MDA5$^{+/+}$ donor spleens and adoptively transferred into MDA5$^{+/+}$ (Figure 2.4), MDA5$^{+/-}$ (not shown) and MDA5$^{-/-}$ (not shown) recipients 24 hours prior to CB4 challenge. After 7 days pi, we observed a significant increase in the percentage of Tregs in the PLNs of infected MDA5$^{+/+}$ recipient mice that received MDA5$^{+/-}$ CD11b^{+} CD11c^{+} cells compared to infected MDA5$^{+/+}$ recipients that did not receive cells prior to infection (no cells, Figure 2.4) and compared to APCs transferred from MDA5$^{+/+}$ donors that did not induce Treg levels in recipient mice, but rather boosted CD4^{+} effector (CD44^{hi} CD62L^{lo}) T cells in the PLN and spleen of MDA5$^{+/+}$ recipients by day 7 pi (Figure 2.7). Both IFN-α and β were at significantly higher detectable levels in the serum of recipient mice(MDA5$^{+/+}$) that received CD11b^{+} CD11c^{+} cells from MDA5$^{+/+}$ as compared to MDA5$^{+/-}$ donors (Figure 2.4). The induction of Tregs and decreased expression of IFN I in recipients(MDA5$^{+/+}$) following transfer of CD11b^{+} CD11c^{+} cells from MDA5$^{+/-}$ donors versus MDA5$^{+/+}$ donors strongly suggests that the cells responsible for the sustained expression of this unique IFN I signature in MDA5$^{+/-}$ mice are CD11b^{+} CD11c^{+} cells.

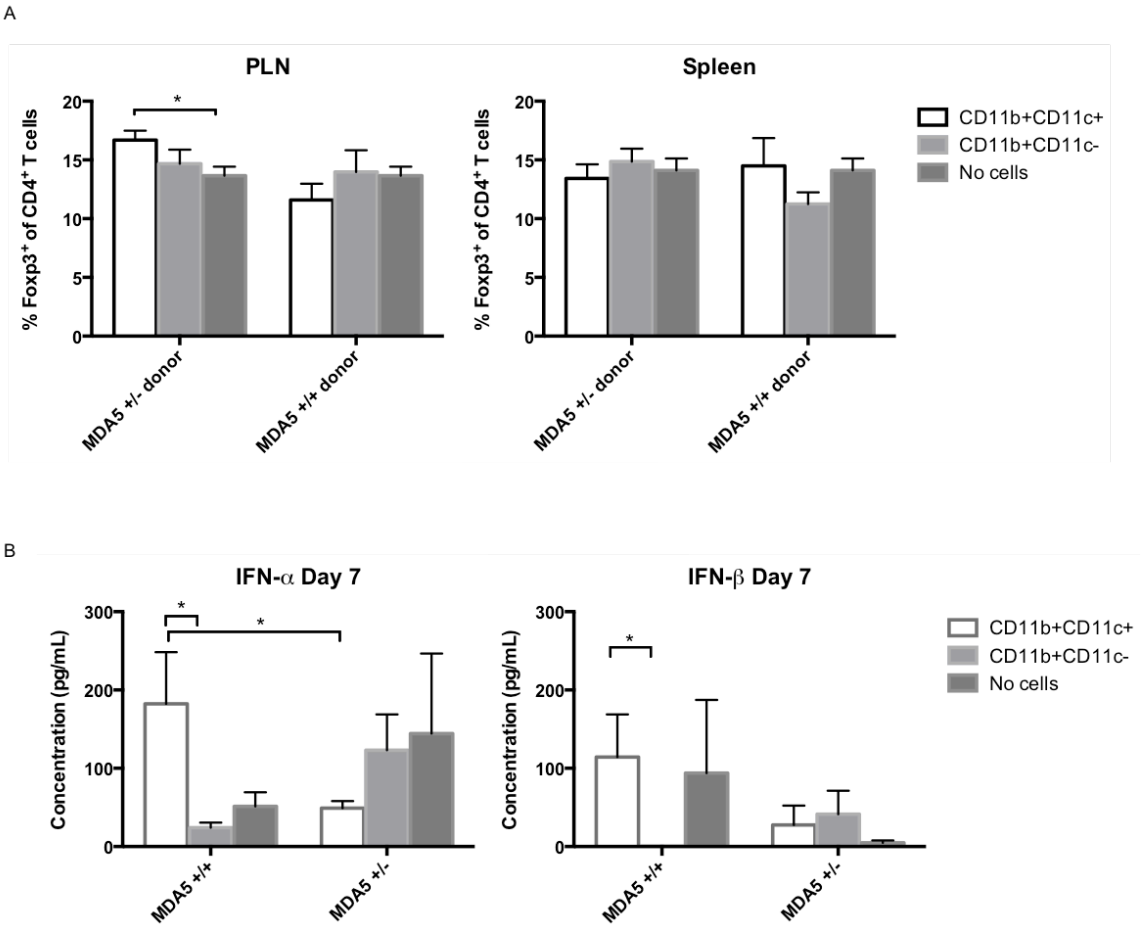


Figure 2.4 CD11b^{+}CD11c^{+} cells from MDA5$^{+/-}$ mice polarize a regulatory T cell response. Antigen presenting cells (CD11b^{+}CD11c^{+} or CD11b^{+}CD11c^{-}) isolated from MDA5$^{+/-}$ or MDA5$^{+/+}$ spleens were adoptively transferred to MDA5$^{+/+}$ (n=10-15), MDA5$^{+/-}$ (not shown, n=10-15) and MDA5$^{-/-}$ (not shown, n=10-15) recipients. After 24 hours, recipient mice including age-matched controls that did not receive cells (no cells) were infected with 400pfu CB4. (A) After 7 days post-infection, lymphocytes from spleen and pancreatic lymph nodes (PLN) of infected mice were stained for FACS analysis. (B) IFN I concentrations in serum from recipient mice were measured by ELISA as described in Materials and Methods. Data shown are mean ± SEM of pooled samples from three independent experiments.p-values were determined used Student's two-tailed paired t-test *p<0.05.

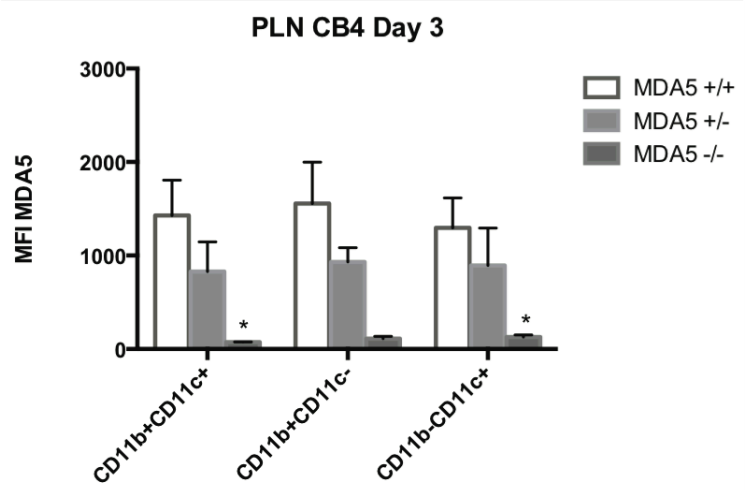


Figure 2.5 MDA5 expression is reduced in APCs from PLNs of CB4-infected MDA5$^{+/-}$ and MDA5$^{-/-}$ mice. MDA5$^{+/+}$ (n=4), MDA5$^{+/-}$ (n=4), and MDA5$^{-/-}$ (n=4) mice were infected with 400pfu CB4. After 3 days pi, lymphocytes from the PLNs of infected mice were isolated and stained for FACS analysis as described in Materials and Methods. Data shown are mean ± SEM of a representative from three independent experiments. *$p<0.05$ relative to MDA5$^{+/+}$.

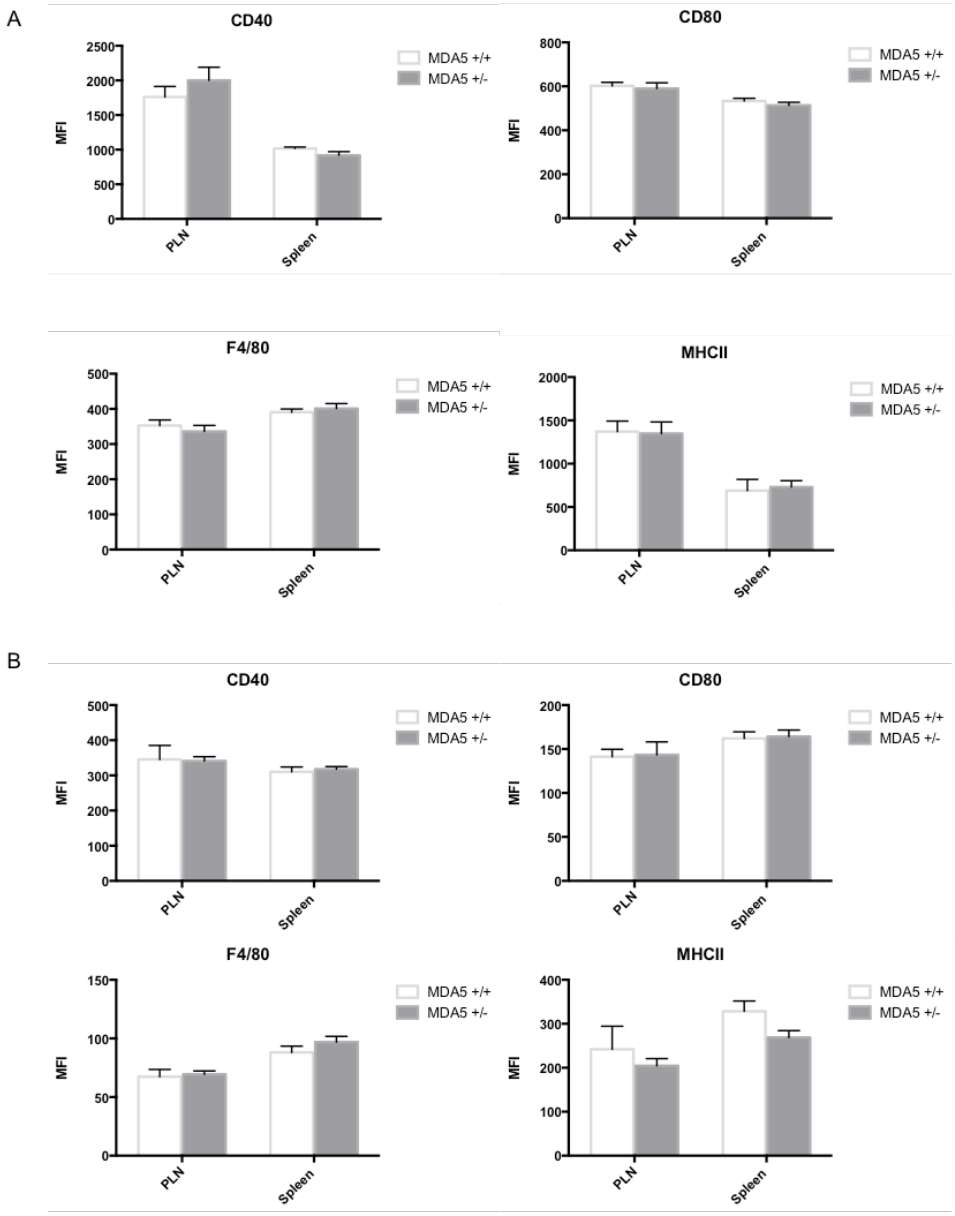


Figure 2.6 Changes in MDA5$^{+/-}$ T cell polarization are not due to alterations in antigen presenting cell activation. CD11^{b+}CD11^{c+} cells (A) and CD11^{b+}CD11^{c-} cells (B) from MDA5het PLNs and spleens show similar activation to MDA5wt by day 2 post-CB4 infection. Data shown are mean ± SEM of a representative from three independent experiments.

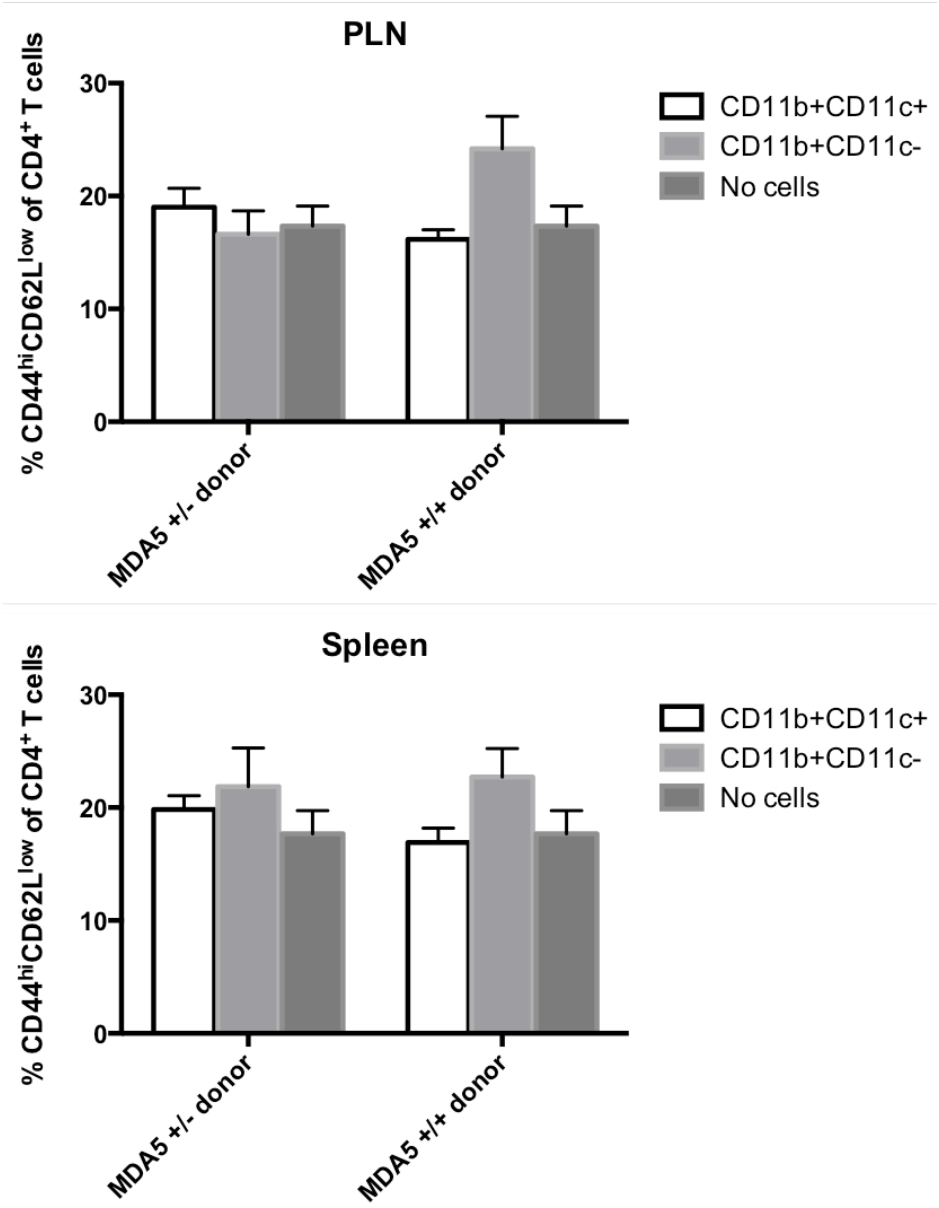


Figure 2.7 CD11b^{+}CD11c^{-} cells from MDA5wt induce CD4^{+} effector T cells. APCs (CD11b^{+}CD11c^{+} or CD11b^{+}CD11c^{-} cells) transferred from MDA5wt or MDA5het spleens prior to CB4 challenge, induce CD4^{+} effector T cells in the PLN and spleen of MDA5wt recipients by day 7 pi. Data shown are mean ± SEM of pooled samples from three independent experiments.

2.4 Discussion

Our findings demonstrate that control of the interferon signature pathway following an environmental insult regulates a critical balance between effector and regulatory T cells thereby influencing disease. In T1D, MDA5 sensing acts as an essential regulator of the diabetogenic T cell response. We have observed that partial loss of MDA5 expression creates a unique IFN I signature, where a burst of IFN I is induced early post-infection, likely to help clear viral infection, and returns to a reduced level as the infection is cleared. This unique IFN I signature likely prevents the triggering of autoimmunity.

MDA5 signaling of IFN-I acts in partnership over the course of infection with another RNA sensor TLR3 to develop a unique pathogen-specific signature. In the case of the pancreatropic virus EMCV, the loss of one MDA5 allele, though on the T1D resistant C57BL/6 mouse background, resulted in transient hyperglycemia due to direct killing of pancreatic beta cells and did not protect from EMCV- induced diabetes[122]. Herein, our work demonstrates the importance of MDA5 signaling kinetics post-infection as the partial loss of MDA5 expression in diabetes susceptible NOD mice protects from the establishment of T1D through a change in the polarization of the T cell response to increase regulation of the autoimmune response. The host response is not significantly diminished in its ability to clear virus infection as CB4 does not replicate out of control and directly kill the pancreatic β-cells. Rather CB4 mimics clinical diabetes onset with the presentation of self-antigen through resident CB4 infected cells in the pancreas [118]. Altering MDA5 sensing simply regulates the autoreactive component of the host response to infection.

Specifically, we observed that a reduction in MDA5 alters IFN-I signaling in a tissue and cell specific manner that allows for Tregs in the PLN, at the site of autoimmunity. Further, we have observed that $CD11b^{+}$ $CD11c^{+}$ cells from $MDA5^{+/-}$ (and not from $MDA5^{+/+}$ donors) induce Tregs and maintain lower levels of IFN I post-infection suggesting that $CD11b^{+}$ $CD11c^{+}$ cells are responsible for driving the unique IFN I signature observed in $MDA5^{+/-}$ mice that protects from T1D. Early intervention of plasmacytoid DC (pDC) function has been recently reported to prevent autoimmune

pathologies in an autoimmune lupus model[248]. Further, children at risk for T1D have shown a unique IFN-I transcriptional signature that precedes islet autoimmunity, recent onset of T1D has been strongly associated with infection by RNA viruses like coxsackievirus and an increase in pDCs and IFN-α production, especially following CB4 infection, have been observed in recent onset T1D patients[92, 99, 169, 171, 249]. These studies and our work suggest an important role for natural IFN I- producing cells like pDCs and their contribution, in response to environmental T1D-associated IFN I stimulators like CB4, to an early IFN I signature in the development of autoimmunity. We have shown here that a reduction in MDA5 creates a unique IFN I signature and leads to development of a regulatory rather than an effector T cell response following infection with a T1D-inducing RNA virus in a T1D susceptible model.

This work identifies MDA5 as an important target for preventative and therapeutic strategies to halt T1D. Our demonstration that a reduction in MDA5 signaling induces regulatory T cell levels at the site of autoimmunity suggests a potential protective mechanism. *Ex vivo* expansion and transplantation of regulatory T cells to protect beta cells from destruction has successfully prevented and reversed T1D in NOD mice and has shown great promise as a clinical intervention in children with recent-onset of T1D[238-240], though current therapies aimed at expanding regulatory T cells to treat autoimmunity have been unsuccessful in maintaining protection over time[238, 243]. Here, by reducing MDA5 gene expression we have uncovered a unique IFN-I signature in NOD mice, that has lead to the expansion of regulatory T cells at the site of autoimmunity and to the protection from T1D, suggesting a new avenue for T1D therapy, in targeting MDA5 that considers both genetic and environmental factors known to alter disease pathogenesis.

Chapter 3: A reduction in MDA5 signals a unique IFN-I signature that mediates autoreactive T cell responses.

3.1 Introduction

The functional consequences of the early detection of viral infection include the establishment of an anti-viral response and the production of type 1 interferons (IFN-I) IFN-α and IFN-β and the eventual activation of a virus-specific adaptive immune response aimed at clearing viral infection[128]. Mounting evidence suggests that deregulation of the innate immune response to viruses may also contribute to the pathogenesis of autoimmune diseases including T1D. In children at risk for T1D, an IFN-I transcriptional signature precedes islet autoimmunity and recent onset of T1D is also strongly associated with infection by RNA viruses including coxsackievirus, which further implicates the IFN-I signature[91, 171, 250, 251].

The endosomal PRR, toll like receptor 3 (TLR3), and the cytoplasmic PRR melanoma differentiation-associated protein 5 (MDA5) recognize double stranded RNA (dsRNA) replication intermediates from specific dsRNA viruses including coxsackievirus B that have important implications in T1D pathogenesis [131, 137, 252]. Sensing of the viral dsRNA induces IFN-I and activates a signaling cascade that involves the adaptor molecule IFN-β promoter stimulator 1 (IPS-1) (also known as MAVS) and ultimately results in the production of antiviral effectors including the production of IFN-I and expression of IFN-stimulated genes (ISGs) [128]. The production of IFN-I can then promote antigen presentation, induce chemokine expression and activate effector lymphocyte responses that can all lead to the induction of autoimmunity [75].

Genome wide association studies (GWAS) have associated the MDA5 gene interferon induced with helicase C domain 1 (IFIH1) with susceptibility to T1D[71, 72]. Genetic variants in the T1D risk locus *IFIH1* have been identified to confer resistance to T1D and result in the reduction in expression of the intracellular RNA virus sensor known as melanoma differentiation-associated protein 5 (MDA5). Recently it was demonstrated that constitutive IFN I signaling can result from the common T1D risk variant A946T in the *Ifih1* in mice and that mice carrying a missense mutation G821S in the *Ifih1* gene

disrupts MDA5 responsiveness to dsRNA and allows MDA5 to remain constitutively active and induce lupus-like nephritis and autoimmunity [166]. These studies along with accumulating evidence of IFN-I having a pathogenic role in autoimmune disease urges further investigation of RNA virus sensing and the consequences of IFN-I in T1D pathogenesis [71, 72, 74, 75, 78].

We have previously translated the reduction in MDA5 observed in patients carrying protective polymorphisms in the *IFIH1* gene on to the non-obese diabetic (NOD) mouse, the mouse model for human T1D, by generating NOD MDA5$^{+/-}$ mice and have observed protection from both spontaneous and Coxsackievirus B serotype 4 (CB4)- induced T1D (Figure 2.1, **see section 2**). The diabetogenic potential of CB4 in NOD mice has been previously demonstrated by our lab and others and represents the best model for the induction of autoimmunity and diabetogenic pathologies[118]. CB4 infection of MDA5$^{+/-}$ mice induces a unique IFN-I signature that we believe contributes to the protective phenotype observed in these mice (Figure 2.2, **see section 2**).

Based on our previous observations of a unique IFN-I signature and increased regulatory T cells (Tregs, Foxp3^{+} CD4^{+} T cells) in the pancreatic lymph nodes (PLNs) of MDA5$^{+/-}$ mice (Figure 2.3, **see section 2**) we believe that a reduction in MDA5 alters IFN-I signaling in a tissue and cell specific manner that allows for a shift in T cell polarity towards Tregs at the site of autoimmunity and protection from CB4 mediated-T1D. We hypothesize that immununological phenotypes including the unique IFN-I signature previously observed in MDA5$^{+/-}$ are specific to a reduction in MDA5 signaling following CB4 infection. This section will show that this unique IFN-I signature in MDA5$^{+/-}$ is limited to a reduction in MDA5 signaling and not a reduction from another RNA sensing molecule TLR3 or reduction of another T1D associated gene, the vitamin D receptor (VDR). That MDA5$^{+/-}$ maintain a regulatory adaptive response in the PLNs unlike MDA5$^{+/+}$ under the conditions of IFN-I stimulation from two different IFN-I inducers: CB4 and the artificial dsRNA mimetic polyinosinic:polycytidylic acid (poly i:c). This section will also demonstrate how disease pathogenesis is linked to the specific IFN-I response induced by the virus as a strain of coxsackievirus B3 (CB3) failed to modify the

IFN-I signature associated with disease. These results suggest that MDA5 signaling is essential in regulating the IFN-I signature that mediates T cell responses following virus infection and determines disease fate.

3.2 Materials and methods

Mice. NOD/ShiLtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MDA5$^{+/-}$ mice were backcrossed from C57BL/6 MDA5$^{-/-}$ mice onto the NOD background as previously described **(see section 2)**. We confirmed by SNP analysis (in house and by DartMouse, Lebanon, NH) that they carry the full complement of NOD *idd* alleles. More importantly, we confirmed that littermates to the backcrosses that were either heterozygote and wildtype for the MDA5$^{-/-}$ alleles mice had the ability to develop spontaneous diabetes which is strongly indicative that the required susceptibility loci had crossed over. TLR3$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, USA) and were backcrossed and maintained on the NOD/ShiLtJ mouse background. NOD TLR3$^{+/-}$ and TLR3$^{+/+}$ progeny were bred for use in experiments. VDR$^{-/-}$ mice on the NOD background were a generous gift from Dr. Chantal Mathieu (University Hospital of Leuven, Belgium). VDR$^{-/-}$ mice were bred with NOD mice and VDR$^{+/-}$ progeny were used for experiments. IFNAR$^{-/-}$ mice on the NOD background were a generous gift from Dr. Helen Thomas and Dr. Kate Graham (The University of Melbourne, Fitzroy, Australia).

Mice were maintained in the Modified Barrier Facility (Pharmaceutical Sciences Building, Vancouver, British Columbia) and kept in a pathogen-free environment. Diabetes incidence was monitored by nonfasting blood glucose measurements. Disease onset was determined by two consecutive blood glucose levels exceeding 300 mg/dL. Only pre-diabetic mice were used for experiments. All animal work was performed under strict accordance with the recommendations of the Canadian Council for Animal Care. The protocol was approved by the Animal Care Committee (ACC) of the University of British Columbia (certificate numbers: A08-0415 and A08-0622).

Western blotting. Mice were stimulated by intraperitoneal injection with 100μg of polyinosinic:polycytidylic acid (P1530, Sigma, St. Louis, MO). After 24 hours

stimulation, spleens were isolated and homogenized by sonication and tissue homogenates were lysed with CellLytic MT Mammalian Tissue Lysis Reagent (Sigma, St. Louis, MO). Samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene fluoride membranes, blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) probed with monoclonal mouse anti-TLR3 (Novus Biologicals, Littleton CO) and polyclonal goat anti-tubulin (Santa Cruz Biotech, Santa Cruz, CA) primary antibodies and IRDye 800CW and IRDye 680 RD secondary antibodies (LI-COR, Lincoln, NE). Membranes were scanned with the LI-COR Odyssey Scanner (LI-COR, Lincoln, NE). Protein was quantified using LI-COR Odyssey 3.0 Software.

Virus. Ten-to 12-week old mice were infected intraperitoneally with sublethal doses of 400 plaque-forming units (PFUs) of CB4 Edwards strain 2 or coxsackievirus group B type 3 (CB3, Nancy Strain) diluted in DMEM. As there is no gender bias in CB4-mediated T1D, equal numbers of male and female mice were infected with CB4. Both male and female mice were infected with CB3. Virus stocks were prepared and free virus particles were detected from tissue homogenates by plaque assay as described previously [118].

Flow cytometry. Pancreatic lymph node and splenic single cell-suspensions were counted and stained with fluorescently conjugated mABs for cell surface markers CD4 (clone L3T4), CD8 (53-6.7), CD25 (clone PC61), CD11b (clone M1/70), CD11c (clone HL3), CD44 (clone IM7), CD40 (clone 3/23), CD62L (clone MEL-14), CD80 (clone clone 16-10A1), CD86 (clone clone GL1) and intracellular transcription factors Foxp3 (clone FJK-16s) and Helios (clone 22F6), intracellular receptors TLR3 (clone 40C1285.6) and MDA5 (Abcam, ab69983) and the inflammatory cytokines IFN-γ (XMG1.2) and IL-17 (clone). All mAbs were purchased from eBiosciences (San Diego, CA) with the exception of Helios from BioLegend (San Diego, CA). Stained cells were analyzed by flow cytometry with the BD Biosciences LSR II (San Jose, CA) and Flow Jo vX.0.6 software (TreeStar, Ashland, OR).

Intracellular cytokine staining. Single cell-suspensions from pancreatic lymph nodes and spleens were restimulated for 4 hours at 37°C in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum with 500ng/ml PMA, 10 ng/ml ionomycin and Golgi Plug (BD Biosciences). Cells were stained for surface markers, fixed, permeabilized, stained for inflammatory cytokine γ-interferon (IFN-γ) and analyzed by flow cytometry.

Cytokine analysis. Cytokines IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFN-γ were measured from serum days 0, 3 and 7 post-CB4 infection in a multiplexed format using a Cytometric Bead Array (mouse Th1/Th2/Th17 cytokine kit; BD Biosciences, Mississauga, ON). Type 1 interferons IFN-α and-β were measured from serum by ELISA using VeriKine Mouse Interferon--α and β ELISA kits (PBL Interferon Source, Piscataway, NJ).

RNA isolation. Organs were removed and immediately snap frozen in TRIzol reagent (Life Technologies Inc, Burlington, ON). Tissues were weighed and organs were homogenized using QIAGEN stainless steel beads and TissueLyser II benchtop homogenizer at 19/s for 10 min. Total RNA was prepared with TRIzol reagent according to the manufacturer's protocol (TRIzol, Life Technologies). RNA was quantified using a NanoDrop-ND-1000 (VERIFY) (Thermo Scientific, Wilmington, DE).

Reverse transcription and quantitative real-time PCR. cDNA was prepared for 1μg of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reverse transcription-PCR was performed with the BioRad T-100 Thermal Cycler.

cDNA was diluted with UltraPure™ DNase/RNase-Free Distilled Water (Life Technologies) and a final RNA concentration equivalent to 10μg/μl was used for real time (RT)-PCR. Gene expression for MDA5, IFN-β, IFN-α, TLR3 and GAPDH was

quantified using the iQ™ SYBR® Green Supermix (BioRad, Mississauga, ON) and specific primers (mouse MDA5 forward 5'- GTGATGACGAGGCCAGCAGTTG -3', reverse 5'- ATTCATCCGTTTCGTCCAGTTTCA-3'; IFN-β forward 5'- GCACTGGGTGGAATGAGACTATTG-3', reverse 5'- TTCTGAGGCATCAACTGACAGGTC-3'; IFN-α forward 5'- TGATGAGCTACTGGTCAGC-3', reverse 5'- GATCTCTTAGCACAAGGATGGC-3'; TLR3 forward 5'- GAGAGAGATTCTGGATGCTTGTGTTTG-3', reverse 5'- GTCTCATAATGGTTTATCATCTACAAA-3'; and GAPDH forward 5'- AGGTCGGTGTGAACGGATTTG-3', reverse 5'- TGTAGACCATGTAGTTGAGGTCA-3'). PCR amplification was performed in 384-well plates with the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). All samples from three independent experiments were evaluated in duplicate amplification reactions. mRNA expression was normalized to GAPDH. The comparative C_t method was used as previously described in Example 5 [245] and data is shown as $-\Delta C_t$ and fold change of $-\Delta C_t$ relative to Wt (NOD) samples [245].

Statistical analysis. GraphPad Prism 6.0 software (GraphPad, San Diego, CA) using the Student t test (two-tailed distribution) and a P value <0.05 determined statistical significance. Serum cytokine concentrations were determined with FCAP Array Software (BD Biosciences, Mississauga, ON). Data are presented as means ± SEM.

3.3 Results

3.3.1 A reduction in MDA5 sensing maintains a regulatory adaptive response in the PLNs despite stimulation from two IFN-I inducers.

From our previous work, we know that CB4 infection of MDA5$^{+/-}$ induces a temporal IFN-I response (Figure 2.2, **see section 2)** with a unique IFN-I signature observed at day 3 post-infection (pi) that subsides by day 7 pi. As humans encounter various pathogenic insults from their environment, it is likely that frequent bursts of IFN-I signaling, produced as a part of innate immune responses, help clear pathogenic insults, albeit as a

consequence of this clearance, may also frequently alter the IFN-I signature in the host and as such, alter immune homeostasis.

To test whether the IFN-I signature we observe in MDA5$^{+/-}$ and subsequent protective adaptive responses we observed in CB4-infected MDA5$^{+/-}$ are maintained even following interference with another IFN-I stimulator, we stimulated CB4-infected MDA5$^{+/-}$ and wt mice 3 and 5 days subsequent to CB4 infection with the dsRNA mimetic poly i:c. Following stimulation with poly i:c at days 3 and 5, we expected that IFN-I levels would remain elevated by day 7 pi and consequentially would alter adaptive T cell responses in favor of an effector rather than regulatory T cell response.

In CB4-infected MDA5$^{+/-}$ unstimulated with poly i:c, elevated IFN-β expression in the pancreas and elevated concentrations in the serum at day 3 pi drop by day 7pi to below levels relative to IFN-α and to those detected in infected wt mice (Figure 3.1 & Figure 2.2, **see section 2**). Following poly i:c stimulation, at day 7 pi, MDA5$^{+/-}$ maintain lower IFN-β levels similarly to unstimulated mice, but have an increase in IFN-α production compared to untreated CB4-infected MDA5$^{+/-}$ (Figure 3.1). Poly i:c treatment significantly affected MDA5$^{+/+}$, where both IFN-α and β levels in the serum at day 7pi are higher than untreated wt mice (Figure 3.1).

CD40 is a costimulatory protein expressed on the surface of APCs that interacts with its ligand CD154 (CD40L) on the surface of T cells during the course of a normal immune response. This interaction between CD40 and CD154 induces T cell proliferation, cytokine production and further APC maturation and several studies have supported a role for CD40-CD154 interactions in tolerance and in the induction of autoimmunity[253-255]. CD40 expression is not restricted to APCs as expression of CD40 has been found on B and T cells. CD40 has also been recognized as a marker of autoreactive T cells in mouse models of T1D[256, 257]. In NOD mice, targeting the CD40-CD154 interaction can prevent and reverse autoimmune diabetes[191, 255].

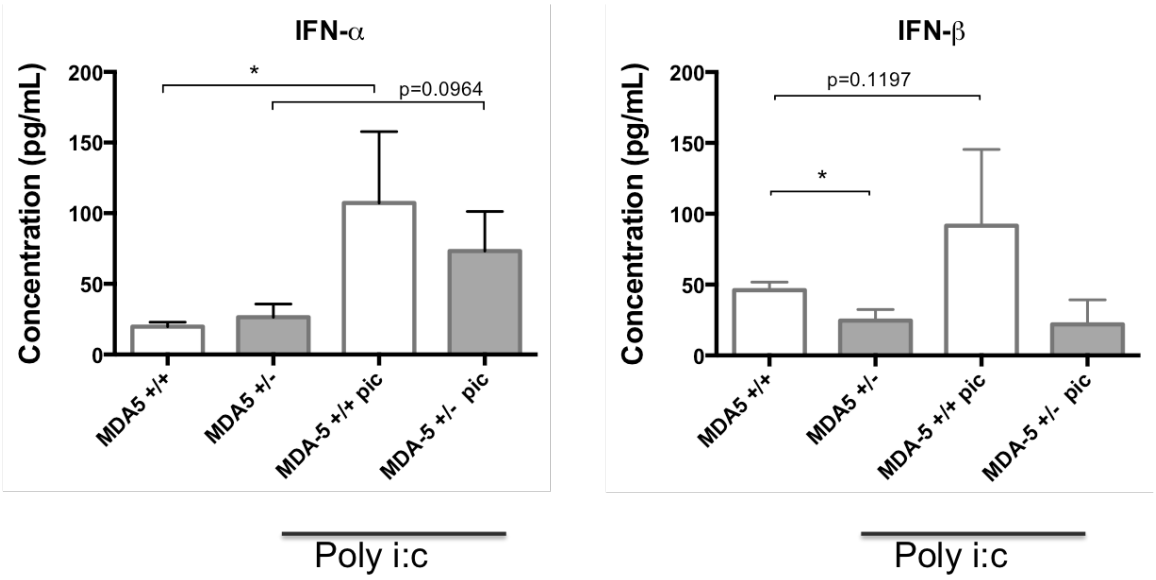


Figure 3.1 Poly i:c treatment following CB4 challenge does not significantly alter the IFN-I signature in MDA5$^{+/-}$. Concentrations of IFN-I were measured by ELISA from the serum of CB4 infected MDA5$^{+/+}$ (n=5) and MDA5$^{+/-}$ (n=5) mice that were either treated at days 3 and 5 pi with poly i:c or left untreated after infection. Results are shown as mean ± SEM of a representative from two independent experiments. Student's t-test determined significance. *p<0.05.

Further, CD11b+CD11c- APCs adoptively transferred from NOD mice deficient for CD40 increase Tregs in the PLNs and pancreas of recipient NOD mice by day 7 following CB4 infection [187]. The expression of the co-stimulatory molecules CD80 and CD86 on APCs or CD28 on T cells is necessary to maintain regulatory cells in peripheral lymphoid tissues and evade spontaneous exacerbation of autoimmunity[194, 258].

In our MDA5$^{+/-}$mice, after CB4 infection and poly i:c treatment, APCs isolated from PLNs and spleen express similar levels of CD40 and CD86 compared to untreated CB4-infected MDA5$^{+/-}$ (Figure 3.2, 3.3), suggesting that APC activation in MDA5$^{+/-}$ is unaffected by additional IFN-I stimulus and potential alterations in the IFN-I signature. Further, the number of Tregs (Foxp3^{+}CD4^{+} T cells) remains high in the PLN relative to the number of effector CD4^{+} T cells (Teff, CD44^{hi} CD62L^{lo} CD4^{+} T cells) (Figure 3.4, 3.5) after poly i:c treatment in CB4 infected MDA5$^{+/-}$ similarly to untreated CB4-infected

MDA5$^{+/-}$mice (Figure 3.5). In poly ic treated MDA5$^{+/+}$ mice, CD86 expression on APCs is also unchanged, however, CD11b+CD11c+ cells have lower CD40 expression (Figure 3.2), which may be responsible for the increase in Tregs in the MDA5$^{+/+}$ spleens (Figure 3.4), but was not sufficient in protecting Treg numbers in the PLNs as after poly i:c treatment, the percentage of Tregs in the PLNs of MDA5$^{+/+}$ significantly drops compared to untreated CB4-infected MDA5$^{+/+}$ mice. These results emphasize the strength of the regulatory protective phenotype observed in MDA5$^{+/-}$mice and not MDA5$^{+/+}$ mice and despite challenge from two IFN-I inducers (CB4 and poly i:c), a reduction in MDA5 maintains low IFN-I levels at day 7 and the generation of a protective Treg response at the site of autoimmunity.

3.3.2 IFN-I signaling and MDA5 expression are important for polarizing a regulatory T cell response

Previous reports have established an important role for CD11b^{+}CD11c^{-}cells and their maturation in the induction of T1D following CB4 infection in NOD mice[187, 252]. Further, TLR3 signaling from CD11b^{+}CD11c^{-}cells is critical for protection from CB4 infection in NOD mice. As such, we asked whether APCs, subdivided CD11b^{+}CD11c^{+} or CD11b^{+}CD11c^{-} cells, from MDA5$^{+/-}$ were responsible for polarization of a regulatory T cell response in MDA5$^{+/-}$ following CB4 infection and demonstrated with adoptive transfer studies that CD11b^{+}CD11c^{+} were responsible for maintaining the IFN-I signature and regulatory T cell response unique to CB4-infected MDA5$^{+/-}$ (Figure 2.4, **see section 2**). To explore the mechanism responsible for the protective phenotype 1n MDA5$^{+/-}$ further, we then asked whether IFN-I signaling from APCs was sufficient to induce T cell polarization.

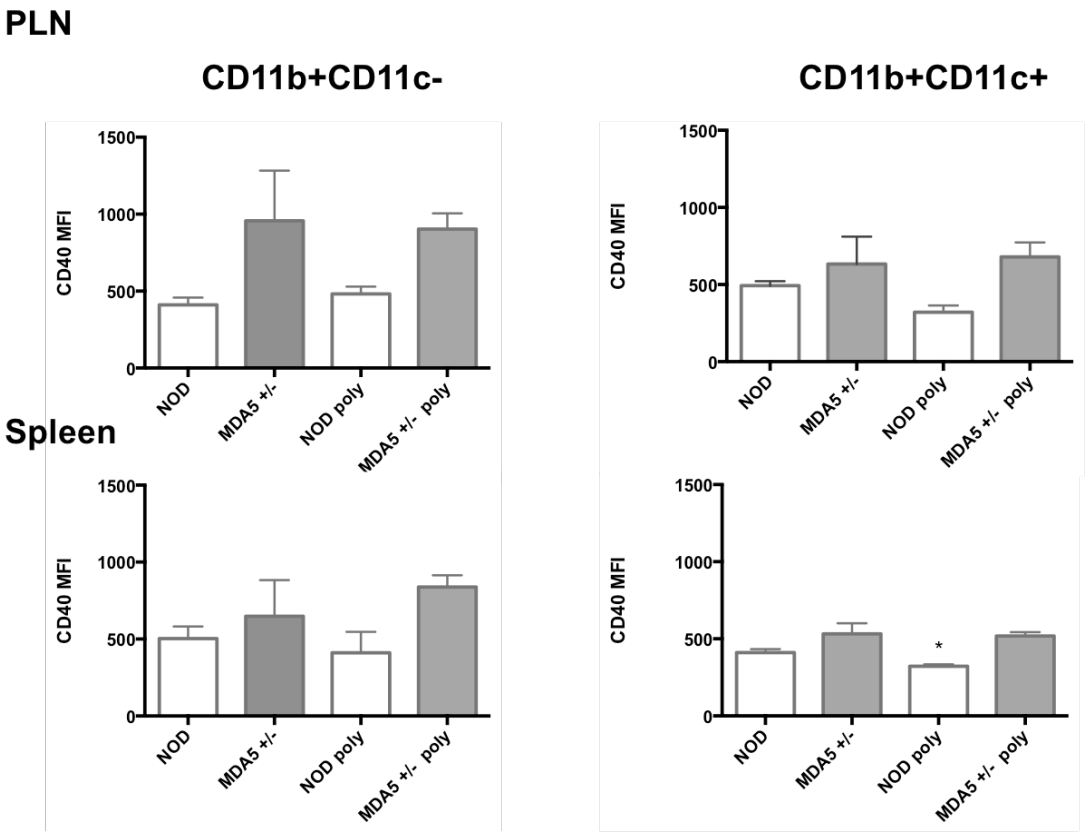


Figure 3.2 Poly i:c treatment does not alter CD40 expression in MDA5$^{+/-}$, but reduces expression of CD40 in wt CD11b^{+}CD11c^{+} cells. Lymphocytes from the pancreatic lymph nodes (PLNs) and spleens of CB4 infected MDA5$^{+/+}$ (n=5) and MDA5$^{+/-}$ (n=5) mice that were either treated at days 3 and 5 pi with poly i:c or left untreated after infection were isolated at day 7pi. Cells were stained with fluorescently labelled anti-CD40 antibody and mean fluorescence intensity (MFI) was measured by FACS analysis. Results are shown as mean ± SEM of a representative from two independent experiments. Student's t-test determined significance. *p<0.05.

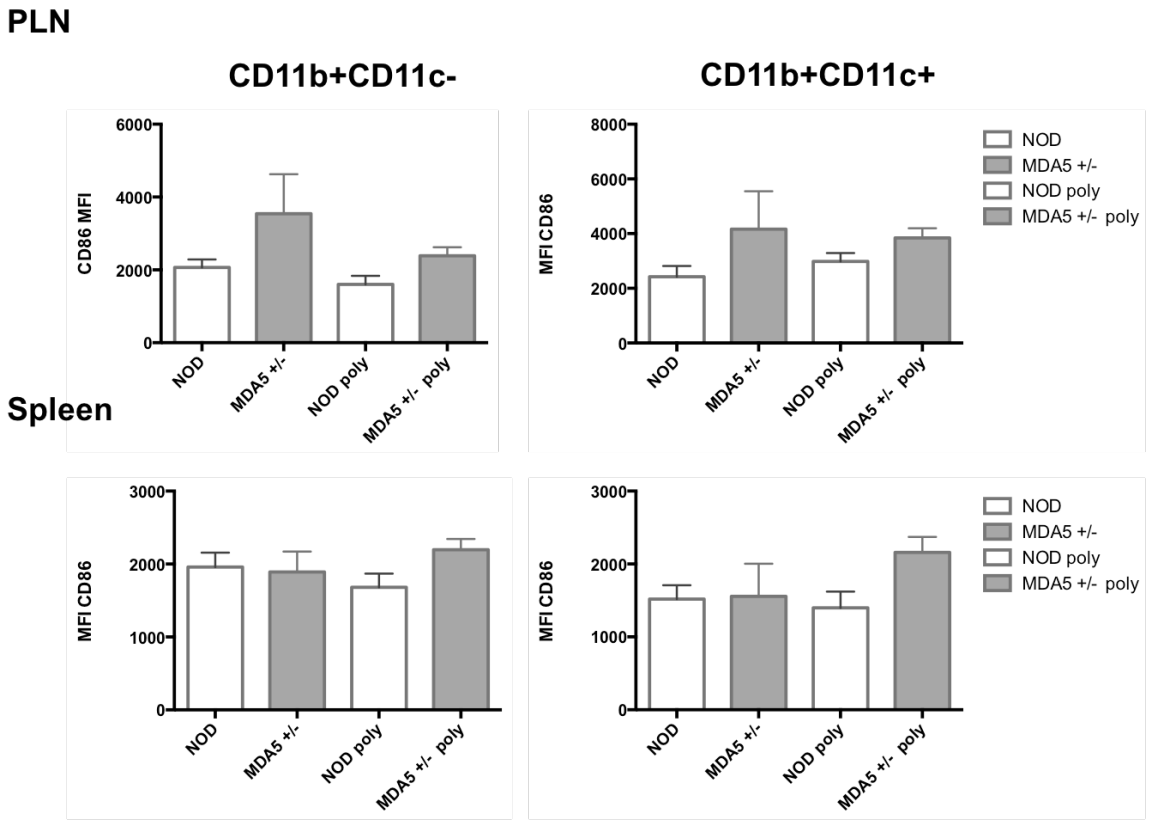


Figure 3.3 Poly i:c treatment does not alter CD86 expression on APCs from treated MDA5$^{+/-}$ or from treated MDA5$^{+/+}$.
Lymphocytes from the pancreatic lymph nodes (PLNs) and spleens of CB4 infected MDA5$^{+/+}$ (n=5) and MDA5$^{+/-}$ (n=5) mice that were either treated at days 3 and 5 pi with poly i:c or left untreated after infection were isolated at day 7pi. Cells were stained with fluorescently labelled anti-CD40 antibody and mean fluorescence intensity (MFI) was measured by FACS analysis. Results are shown as mean ± SEM of a representative from two independent experiments. Student's t-test determined significance.

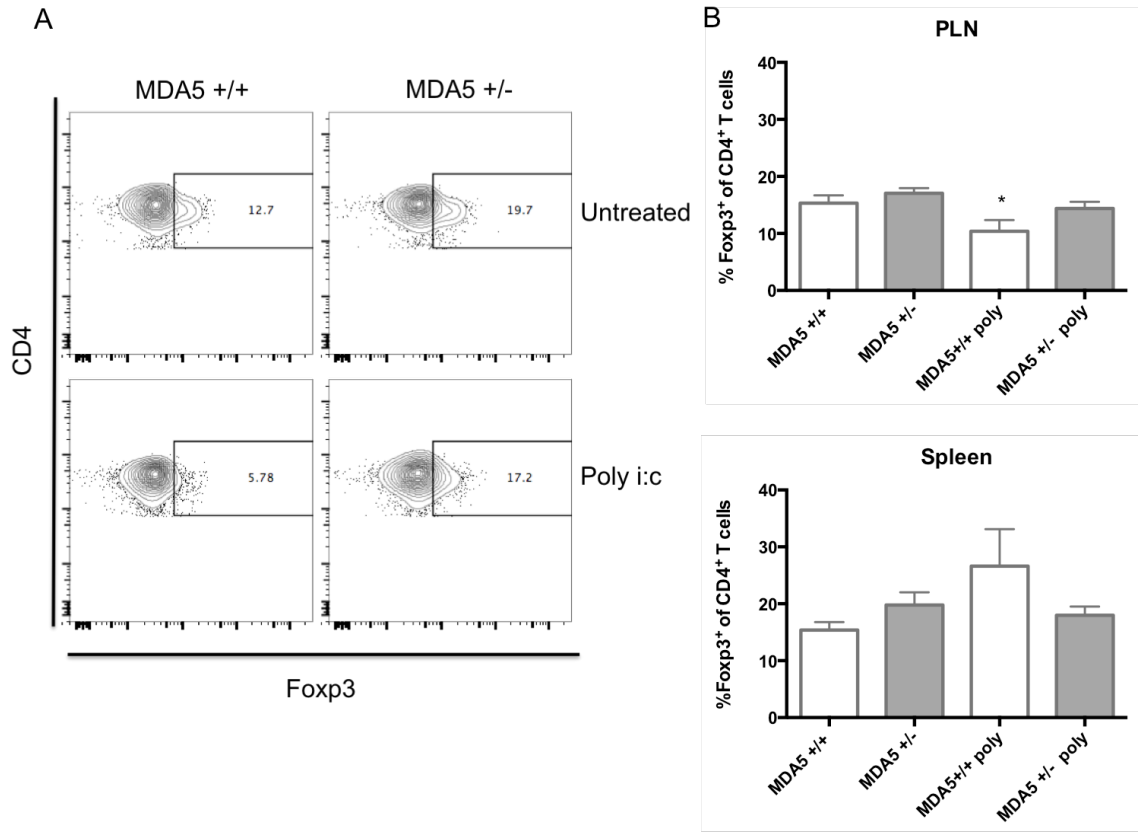


Figure 3.4 A reduction in MDA5 sensing maintains a regulatory phenotype despite IFN-I stimulation with CB4 and poly i:c. MDA5$^{+/-}$ mice have high levels of regulatory T cells (Foxp3^{+} of CD4^{+} T cells) in the pancreatic lymph nodes (PLNs) after CB4 infection and followed by two doses at day 3 and 5 pi of poly i:c compared to infected and poly i:c treated MDA5$^{+/+}$ (+/+) mice at day 7 post-CB4 infection. T cells were isolated from MDA5$^{+/+}$ (NOD, n=5) and MDA5$^{+/-}$ (n=5) at day 7 post-infection and were stained with classical Treg marker (CD25, Foxp3, CD4) antibodies for FACS analysis. Results are shown as FACS plots (A) and quantified in mean ± SEM (B) of a representative from two independent experiments. Student's t-test determined significance. *p<0.05.

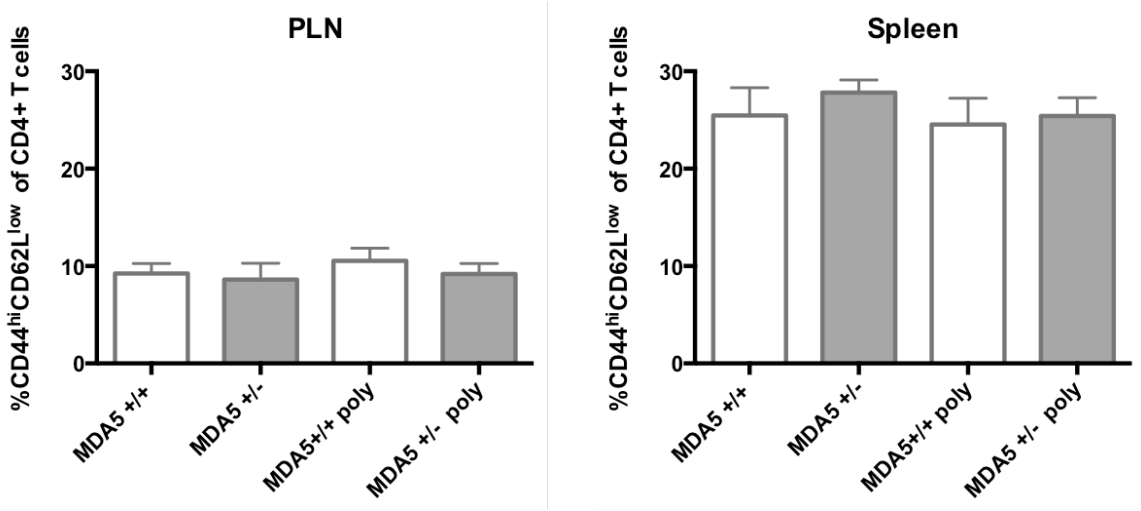


Figure 3.5 MDA5$^{+/-}$ maintain controlled numbers of effector CD4^{+} (CD44^{high}CD62^{low}) T cells in the PLNs despite poly ic treatment. The percent of effector CD4 T cells in MDA5$^{+/-}$ mice is unchanged in the pancreatic lymph nodes (PLNs) after CB4 infection and followed by two doses at day 3 and 5 pi of poly i:c compared to untreated mice at day 7 post-CB4 infection. T cells were isolated from MDA5$^{+/+}$ (NOD, n=5) and MDA5$^{+/-}$ (n=5) at day 7 post-infection and were stained with CD4, CD44, and CD62L effector T cell marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from two independent experiments. Student's t-test determined significance.

We isolated APCs, subdivided (CD11b^{+} CD11c^{+} and CD11b^{+} CD11c^{-}) from NOD mice deficient in type 1 IFN receptors (NOD.IFNAR1$^{-/-}$) donor spleens and adoptively transferred into NODIFNAR$^{+/+}$ recipients 24 hours prior to CB4 challenge. After 7 days pi, we did not observe a significant increase in the percentage of Tregs in the PLNs of infected NODIFNAR$^{+/+}$ recipient mice that received either NODIFNAR$^{-/-}$ CD11b^{+} CD11c^{+} or CD11b^{+} CD11c^{-} cells compared to infected NODIFNAR$^{+/+}$ recipients that did not receive cells prior to infection (no cells, Figure 3.6). The percentage of CD4^{+} effector (CD44^{hi} CD62L^{lo}) T cells (Teffs) in the PLNs were unaffected by the APC transfers though they were increased in the spleens of recipient mice from both cell types transferred (Figure 3.6). These results suggest that IFN-I is important for CD11b+CD11c+ for polarizing a regulatory T cell response, as APCs from NOD mice lacking the IFN-I receptor IFNAR failed to boost Tregs and lower Teffs in the PLNs. Further, APCs transferred from NOD.IFNAR1$^{-/-}$ into NOD mice deficient in MDA5 (MDA5$^{-/-}$) resulted in lower Tregs and increased Teffs in both the PLNs and spleen

(Figure 3.6). This demonstrates the importance of MDA5 signaling and IFN-I in the polarization of regulatory T cell response following CB4 infection.

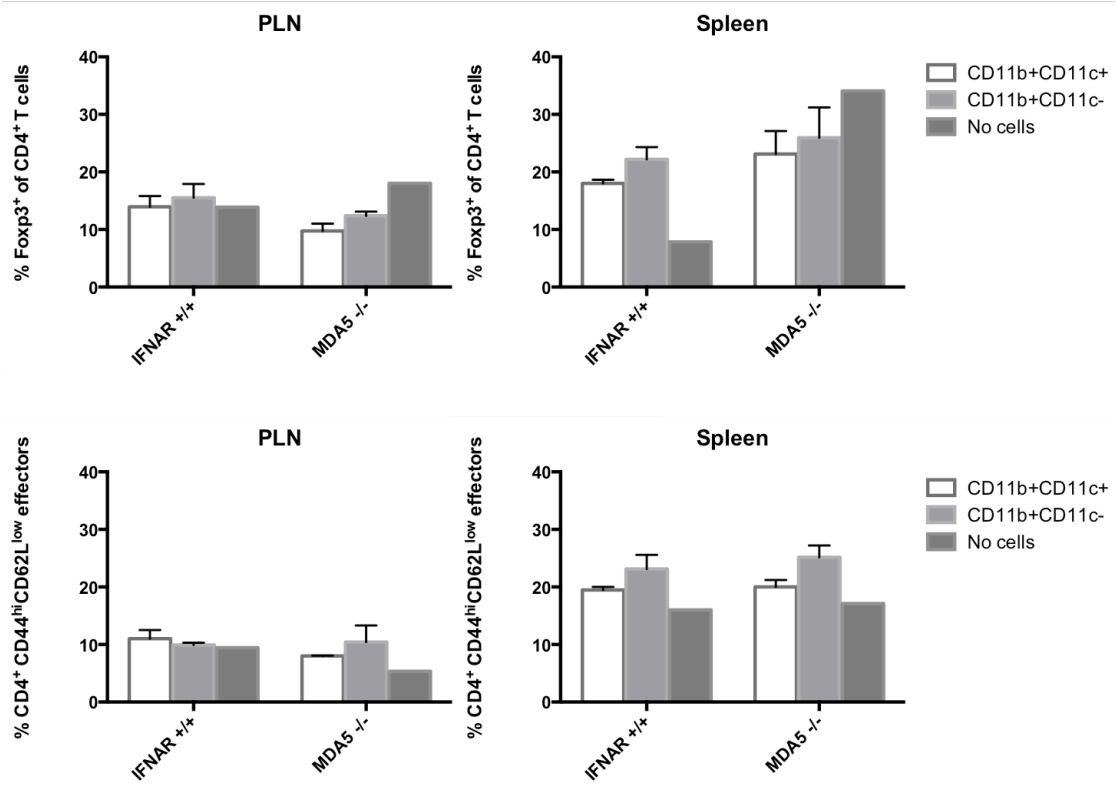


Figure 3.6 CD11b^{+}CD11c^{+} cells from IFNAR$^{-/-}$ mice do not polarize a regulatory T cell response in wt or MDA5 deficient recipients. Antigen presenting cells (CD11b^{+}CD11c^{+} or CD11b^{+}CD11c^{-}) isolated from female IFNAR$^{-/-}$ spleens were adoptively transferred to female IFNAR$^{+/+}$ (n=3-5) and female MDA5$^{-/-}$ (n=3-5) recipients. After 24 hours, recipient mice including age-matched controls that did not receive cells (no cells, n=1) were infected with 400pfu CB4. After 7 days post-infection, lymphocytes from spleen and pancreatic lymph nodes (PLN) and spleens of infected mice were stained for FACS analysis. Data shown are mean ± SEM from one independent experiment.

3.3.3 IFN-I signaling from MDA5 and not from another dsRNA sensor TLR3 maintains a regulatory T cell response in the PLNs following CB4 infection

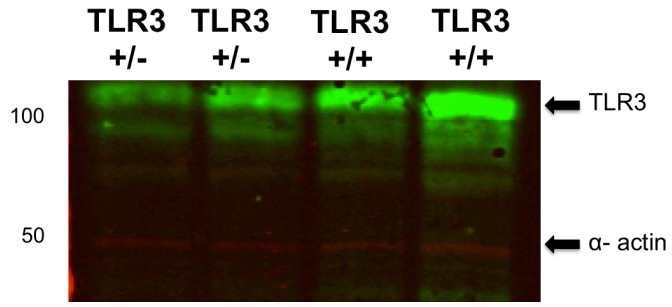
After affirming the strength of the IFN-I signature and regulatory phenotype observed in MDA5$^{+/-}$ to withstand supplemental IFN-I stimulation, and the importance of IFN-I signaling in maintaining the IFN-I signature and protective adaptive responses following

CB4 infection, we then sought to confirm whether the phenotype observed in MDA5$^{+/-}$ is specific to a reduction in MDA5 signaling or could also be induced with a reduction in TLR3- another dsRNA sensor that detects CB4.

First, we determined whether TLR3 expression was reduced in the spleen in TLR$^{+/-}$ similarly to the reduction in MDA5 protein we observed in the spleens of MDA5$^{+/-}$. Following poly i:c stimulation for 24 hours, western blot confirmed that TLR3$^{+/-}$ only have a 28% reduction in TLR3 expression in the spleen compared to poly i:c stimulated wt (Figure 3.7). This suggests that despite lacking a functional allele, TLR3$^{+/-}$ retain expression of TLR3 in the spleen following poly i:c stimulation. Quantification of TLR3 mRNA expression following CB4 infection by qPCR, however, shows a significant reduction in TLR3 expression in the spleen and protein expression (Figure 3.7).

Next we investigated whether CB4 challenged TLR3$^{+/-}$ mice demonstrated altered inflammatory cytokine responses to TLR3$^{+/+}$ as CB4-infected MDA5$^{+/-}$ mice show similar inflammatory cytokine (apart from IFN-I) responses to MDA5$^{+/+}$ mice at days 0, 3, and 7 pi. Interestingly, after CB4 infection, we observed changes in Th1, Th17 and IFN-1 cytokines at day 3 pi in TLR3$^{+/-}$ mice that were not observed with a reduction in MDA5 compared to infected wild type (wt) controls. At day 2 pi, TLR3$^{+/-}$ have a rise in IL-17 and at day 3pi a burst in IL-6, IL-10 and IFN-α (Figure 2.2 & Figure 3.7, 3.9). This molecular signature is not observed with CB4-infected MDA5$^{+/-}$ that have a significant increase in IFN-β at day 3 pi (Figure 2.2, **see section 2**) and not other inflammatory cytokines compared to infected MDA5$^{+/+}$ mice. In terms of the IFN-I signature, TLR3$^{+/-}$ also differ from MDA5$^{+/-}$. In the pancreas TLR3$^{+/-}$ have altered IFN-I expression compared to MDA5$^{+/-}$ at day 3pi, where IFN-β is reduced and IFN-α is increased in TLR3$^{+/-}$ and IFN-β is greater in MDA5$^{+/-}$ and IFN-α is reduced compared to wt controls. Expression of IFN-I in the spleen of TLR3$^{+/-}$ in respect to wt mice is however similar to MDA5$^{+/-}$ at day 3 pi (Figure 3.7).

A.



B.

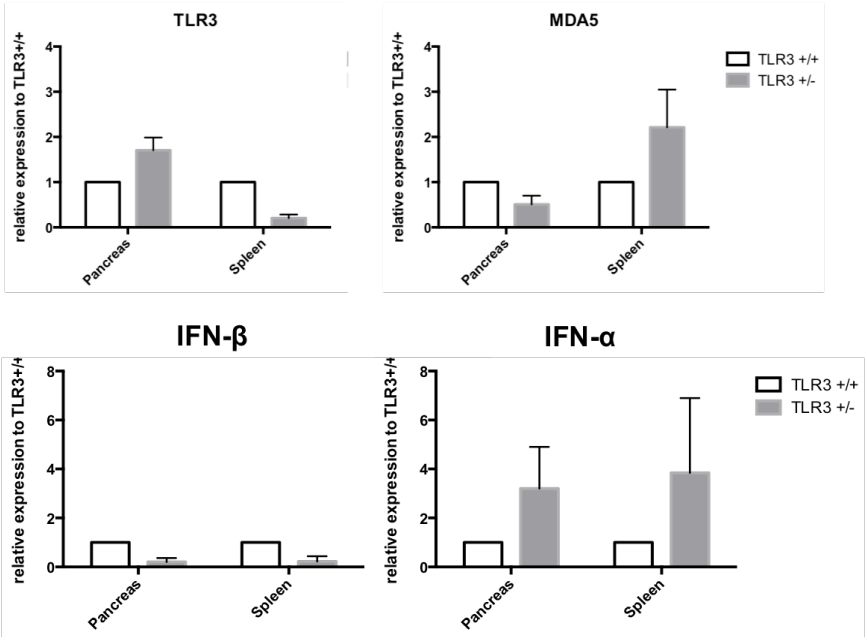


Figure 3.7 TLR3$^{+/-}$ have distinct spatial expression of TLR3 and MDA5 following poly i:c and CB4 infection.(A) TLR3$^{+/+}$ (+/+), TLR3$^{+/-}$ (+/-), and TLR3$^{-/-}$ (-/-) were stimulated with poly i:c as described in Materials and Methods. After 24 hours stimulation, a 48% reduction in MDA5 protein from MDA5$^{+/-}$ spleens compared to MDA5$^{+/+}$ was confirmed by Western Blot. (B) Relative mRNA expression levels of TLR3, MDA5 IFN-α and β from the spleen and pancreas from TLR3$^{+/+}$ (n=8-10) and TLR3$^{+/-}$ mice (n=8-10) at day 3 post-CB4 infection were quantified by quantitative real-time PCR and normalized to GAPDH. The comparative C_t method was used to calculate mean relative expression ± SEM against MDA5$^{+/+}$ mice as described in Materials and Methods section. Data shown are from duplicate samples from two independent experiments.

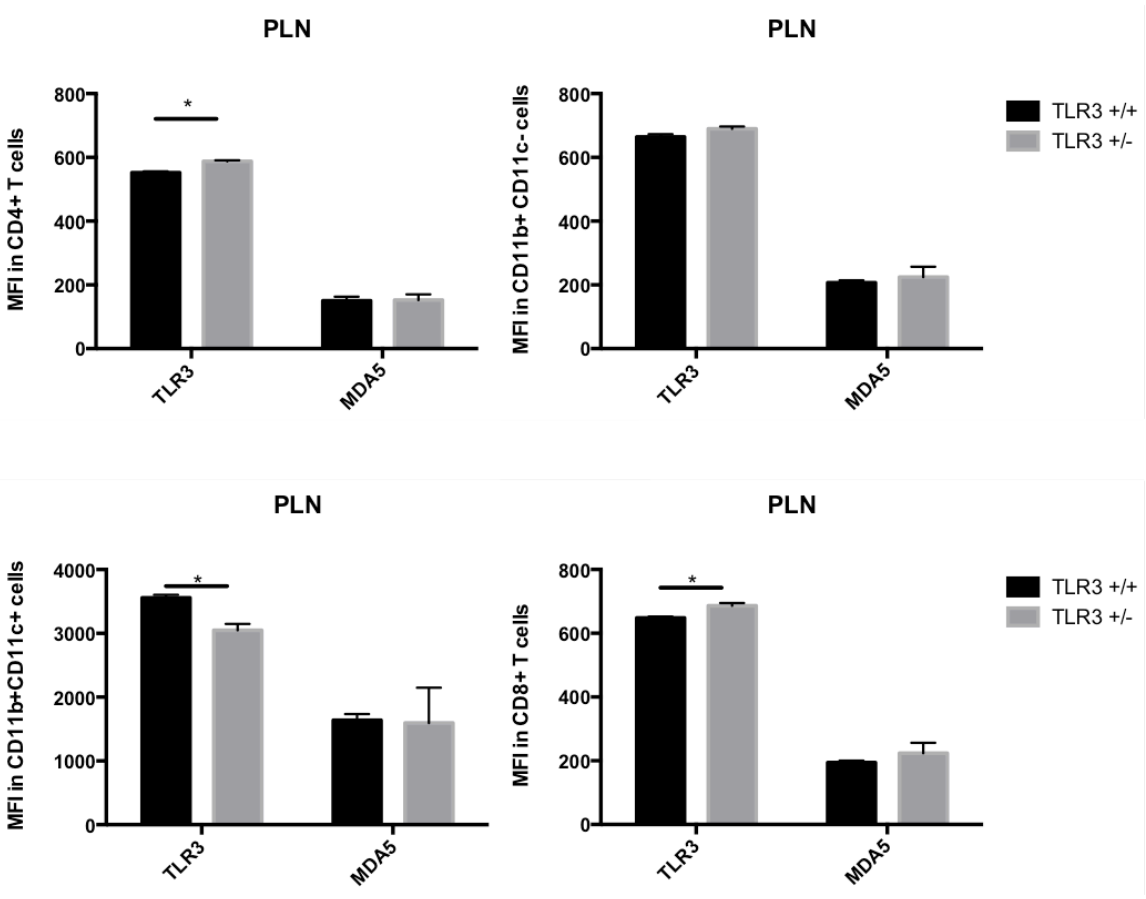


Figure 3.8 TLR3 and not MDA5 expression varies in TLR3$^{+/-}$ and varies according to immune cell type following CB4 infection. TLR3$^{+/-}$ mice (+/-) have increased TLR3 expression in CD11b^{+}CD11c^{-}, CD4^{+} and CD8^{+} T cells in the pancreatic lymph nodes (PLNs), but reduced TLR3 expression in CD11b^{+}CD11c^{+} cells compared to infected TLR3$^{+/+}$ mice at day 7 post-CB4 infection. T cells were isolated from TLR3$^{+/+}$ (n=2) and TLR3$^{+/-}$ mice (n=2) at day 7 post-infection and with classical activation and maturation marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from one experiment. *$p<0.05$.

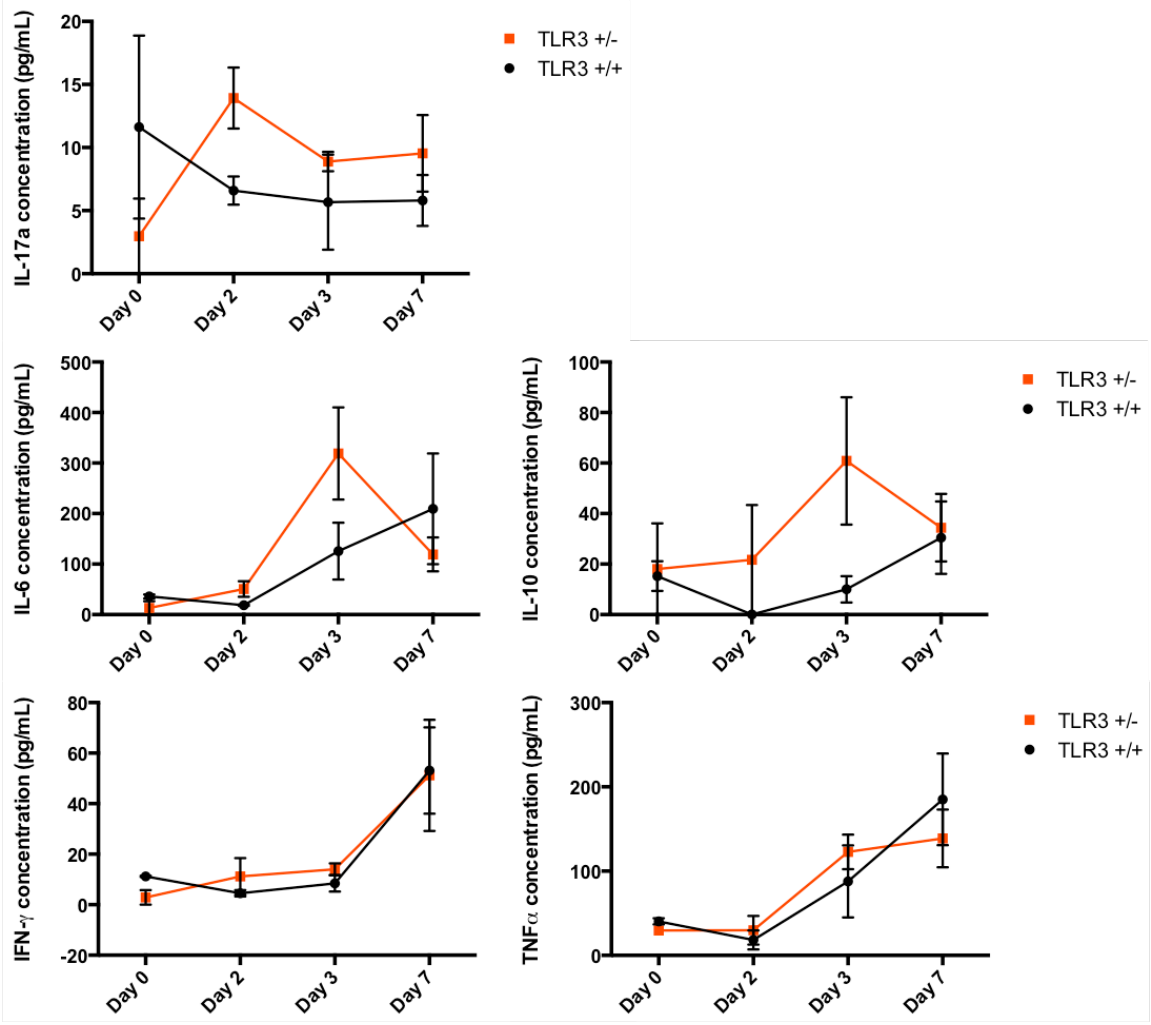


Figure 3.9 TLR3 expression alters inflammatory cytokines that respond to CB4 infection.
Cytokines were measured by FACS bead array in sera harvested from TLR3$^{+/+}$ (n=3-8) and TLR3$^{+/-}$ mice (n=3-8) before infection (day 0) and days 2, 3 and 7 post-CB4 infection. Statistical significance was determined by Student's t test. *p<0.05, **p<0.001.

Next, we examined whether TLR3$^{+/-}$ mice have greater Tregs in the PLN at day 7 pi similar to MDA5$^{+/-}$ Treg responses at day 7pi (Figure 3.10, **see section 2**). Intriguingly, TLR3$^{+/-}$ mice also show higher numbers of Tregs in the PLNs compared to wt controls, though not as significantly as MDA5$^{+/-}$ and Tregs in the spleens of CB4-infected TLR3$^{+/-}$ mice are lower than wt controls in contrast to the levels of Tregs in the spleens of CB4-infected MDA5$^{+/-}$ (Figure 3.10). Also dissimilar from MDA5$^{+/-}$ mice, TLR3$^{+/-}$ have more effector T cells (Teffs) in the PLN compared to wt controls at day 7 pi (Figure 3.10).

TLR3$^{+/-}$ mice also have significantly more IFN-γ producing CD4^{+} T cells in the PLN pi and not in the spleen, where IFN-γ producing CD4^{+} T cells were greater in MDA5$^{+/-}$ at day 7 pi (Figure 3.10). The cytokine and T cell responses observed in TLR3$^{+/-}$ mice pi do not correlate with a protective phenotype as we have observed in MDA5$^{+/-}$ mice and rather suggests that MDA5 and not TLR3 signalling of IFN-I is critical in polarizing a regulatory and protective response that controls CB4 infection and T1D.

To help explain the changes in T cell polarity observed for TLR3$^{+/-}$ following CB4 infection, we examined potential spatial and quantitative alterations in TLR3 and MDA5 expression by flow cytometry and qPCR. TLR3 and MDA5 expression was analyzed by flow cytometry in CD4^{+}, CD8^{+}, CD11b^{+}CD11c^{+}, and CD11b^{+}CD11c^{-} cells from the PLNs of infected TLR3$^{+/-}$ and TLR3$^{+/+}$ mice. At day 7 pi TLR3$^{+/-}$ show higher TLR3 expression in CD11b^{+}CD11c^{-}, CD4^{+} and CD8^{+} cells and significantly less TLR3 expression in CD11b^{+}CD11c^{+} cells than infected TLR3$^{+/+}$ mice, whereas MDA5 expression remained comparable in infected TLR3$^{+/-}$ to TLR3$^{+/+}$ mice (Figure 3.8). qPCR analysis demonstrated spatial changes in MDA5 and TLR3 expression in MDA5$^{+/-}$ and MDA5$^{+/+}$ at day 3 pi, where TLR3 expression compensated the lack of MDA5 expression in the spleen of MDA5$^{+/-}$ and expression of both RNA sensors remained greater in the pancreas of MDA5$^{+/-}$ relative to MDA5$^{+/+}$ (Figure 3.7). In TLR3$^{+/-}$, TLR3 expression and not MDA5 expression was greater than wt in the pancreas, but in the spleen, TLR3 expression was reduced and MDA5 expression was increased compared to wt. The expression of TLR3 in TLR3$^{+/-}$ pancreas and not in the spleen suggests that TLR3$^{+/-}$ only need TLR3 for controlling virus in pancreas. These results imply that a reduction in virus sensing induces tissue specific changes in TLR3 and MDA5 expression at day 3 post-CB4 infection and that in concert with the T cell responses we observed in TLR3$^{+/-}$ it is likely that TLR3 is more important for the anti-viral response and MDA5 signaling as a factor in the autoreactive response.

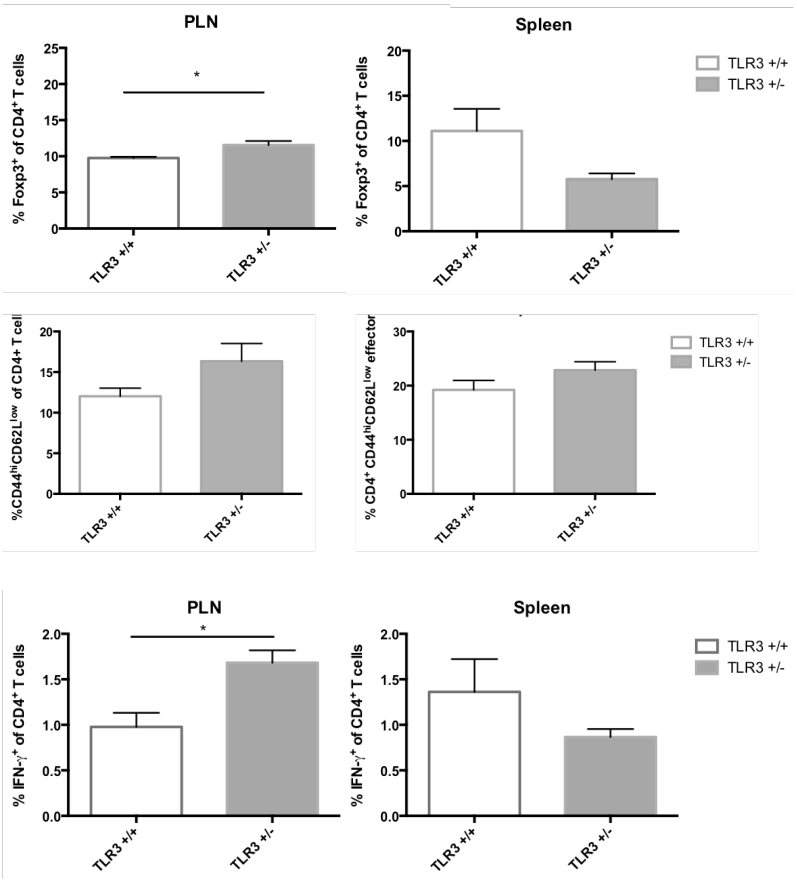


Figure 3.10 Reduced TLR3 expression induces regulatory T cells in the PLNs, but does not reduce effectors at the site of autoimmunity. $TLR3^{+/-}$ mice (+/-) have increased levels of (A) regulatory T cells ($Foxp3^+$ of $CD4^+$ T cells) in the pancreatic lymph nodes (PLNs), (B, C) increased effector $CD4^+$ and $CD8^+$ ($CD44^{high}CD62^{low}$) T cells in both PLNs and spleen, and (D,E) increased IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the PLNs and spleen compared to infected $TLR3^{+/+}$ (+/+) mice at day 7 post-CB4 infection. T cells were isolated from $TLR3^{+/+}$ (n=5) and $TLR3^{+/-}$ mice (n=5) at day 7 post-infection and were stained with classical activation and maturation marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from three independent experiments. *$p<0.05$.

3.3.4 Reduced expression of MDA5 and not of another T1D-associated receptor the VDR polarizes protective T cell responses

Another intracellular receptor, though not a dsRNA receptor like MDA5 and TLR3, but an important immunomodulating factor in innate immune defenses and in T1D susceptibility, is the vitamin D receptor (VDR). The active form of vitamin D (VD) known as $1,25(OH)_2D_3$ (1,25VD) and its affiliated signalling receptor the VDR, a

receptor expressed on all immune cells, have important immune modulating effects that have been closely tied to T1D [259-266]. It has been previously demonstrated that $VDR^{-/-}$ on the NOD background develop spontaneous T1D similarly to wt littermates however, T1D incidence and the immunological consequences of VDR deficiency in a virus-induced model have not been explored. To determine the immunological consequences of a reduction in the VDR after CB4 infection and whether they are akin to the immune responses observed with a reduction in MDA5 that lead to protection from T1D, we infected $VDR^{-/-}$, $VDR^{+/-}$ and wt mice with 400 pfu CB4 (Edwards 2) and examined disease incidence, inflammatory cytokines and T cell responses pi.

In contrast to the spontaneous model from Mathieu and colleagues, where $VDR^{-/-}$ develop disease similarly to wt littermates, we have observed that a reduction in the VDR increases diabetes incidence compared to wt, with $VDR^{+/-}$ developing disease most rapidly by day 7 pi (Figure 3.11). This suggests that expression of the VDR is critical in protection from CB4-induced T1D, contrary to what we have observed in $MDA5^{+/-}$ that are protected from T1D past day 7pi (Figure 2.1, **see section 2**).

Also unlike CB4-infected $MDA5^{+/-}$, infected $VDR^{+/-}$ did not show a significant increase in regulatory T cells in the PLNs by day 7 pi. Rather, $VDR^{+/-}$ have an increase in the number of effector $CD4^+$ and $CD8^+$ T cells in both the PLN and spleen tissues compared to infected wt (Figure 3.12). In the spontaneous model, Mathieu and colleagues demonstrated that complete loss of the VDR results in less regulatory T cells in the PLNs and disease incidence similar to wt littermates, an opposing effect to what we observe with complete and partial loss of MDA5 (Figure 2.1, 2.3, **see section 2,** Figure 3.12) [40]. $VDR^{+/-}$ T cell responses also differ from those of $MDA5^{+/-}$ in numbers of IFN-γ-producing $CD4^+$ T cells, where CB4-infected $VDR^{+/-}$ have similar percentages of IFN-γ-producing $CD4^+$ T cells compared to wt in the PLNs and spleen (Figure 3.12) and $MDA5^{+/-}$ PLNs show an increase in IFN-γ- producing $CD4^+$ T cells (Figure 2.3, **see section 2)**. These results further emphasize the distinctive role of a reduction in MDA5 and not other dsRNA or T1D-associated receptors in establishing an immunoenvironment that polarizes a regulatory T cell response and protects from T1D after CB4 infection.

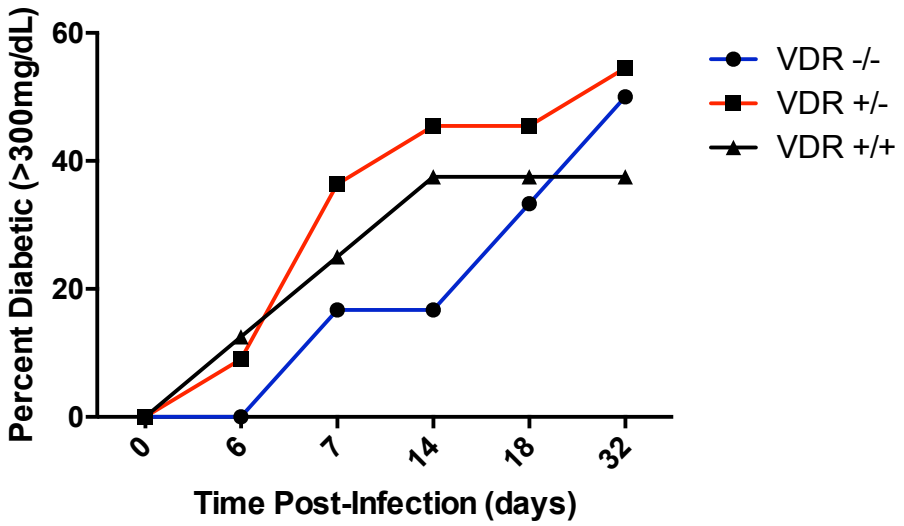


Figure 3.11 A reduction in VDR expression alters diabetes incidence following CB4 infection. VDR NOD +/+, VDR NOD +/-, and VDR NOD -/- mice (n=5-7) were infected with 400 pfu of CB4 and monitored for diabetes onset. Diabetes was determined by two consecutive blood glucose readings >300 mg/dL. Data represent one of two independent experiment.

3.3.5 The IFN-I signature and protective regulatory phenotype observed in MDA5$^{+/-}$ is specific to CB4 infection

IFN-I signaling by MDA5 can be stimulated detection of other coxsackieviruses and although CB4 is strongly linked to T1D, in patients CB4 may only be responsible for a subset of new onset cases as other viruses such as coxsackievirus B1 have more recently been implicated[87]. It is therefore important to consider whether the immunological responses observed in CB4 infected MDA5$^{+/-}$ are a virus-specific phenotype.

To test whether the IFN-I phenotype observed in infected MDA5$^{+/-}$ is a result of immunological responses to virus infection and replication in the islets rather than indirect IFN-I stimulation from outside the islets, we challenged MDA5$^{+/-}$ with another T1D-associated coxsackievirus serotype, coxsackievirus B3 (CB3).

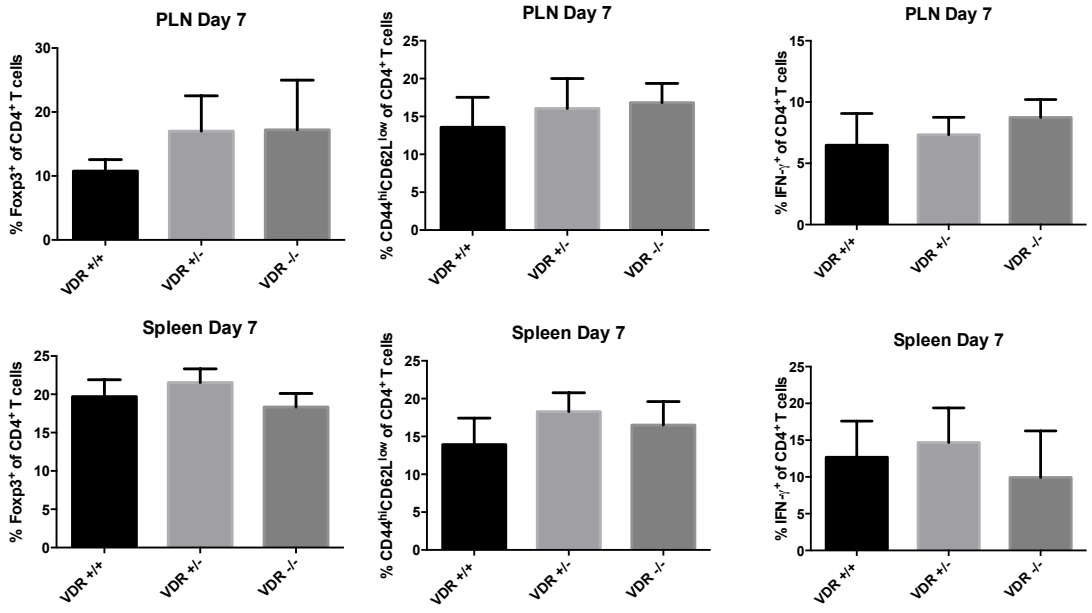


Figure 3.12 A reduction in VDR does not promote protective regulatory T cell responses following CB4 infection. $VDR^{+/+}$ (n=5), $VDR^{+/-}$ (n=4), and $VDR^{-/-}$ mice (n=2) were infected with 400 pfu of CB4. (A) regulatory T cells ($Foxp3^+$ of $CD4^+$ T cells), (B) effector $CD4^+$ ($CD44^{high}CD62^{low}$) T cells, and (C) IFN-γ-producing $CD4^+$ T cells were isolated from PLNs and spleens at 7 pi, stained with their respective surface and intracellular markers and analyzed by flow cytometry. Results are shown as mean ± SEM of a representative from two independent experiments.

The strain of CB3 we used to infect $MDA5^{+/-}$ replicates and infects primarily the pancreas and secondly the heart similarly to CB4 in mice. Both serotypes share the same receptor, the coxsackievirus-adenovirus receptor (CAR) that is expressed by the islets of the pancreas, though CB3 does not infect pancreatic islets[267, 268]. Rather, CB3 infection outside the islets initiates pancreatitis and not insulitis as we observe for CB4 infected NOD mice[269].

By infecting $MDA5^{+/-}$ with CB3, we anticipated two distinct scenarios that would help decipher where MDA5 signaling of IFN-I is important to maintain virus control and still induce regulatory adaptive responses to protect from autoimmunity. We expected that if MDA5 signaling outside of the islets dictates virus control and a regulatory phenotype, we would expect CB3 infected $MDA5^{+/-}$ to have similar IFN-I and T cell responses as we

see with CB4 infection. If MDA5 signaling from within the islets is critical, we expect CB3 infected MDA5$^{+/-}$ to have IFN-I and T cell responses that are not in favor of protection from autoimmunity and rather are similar to wt controls (unlike with CB4 infection).

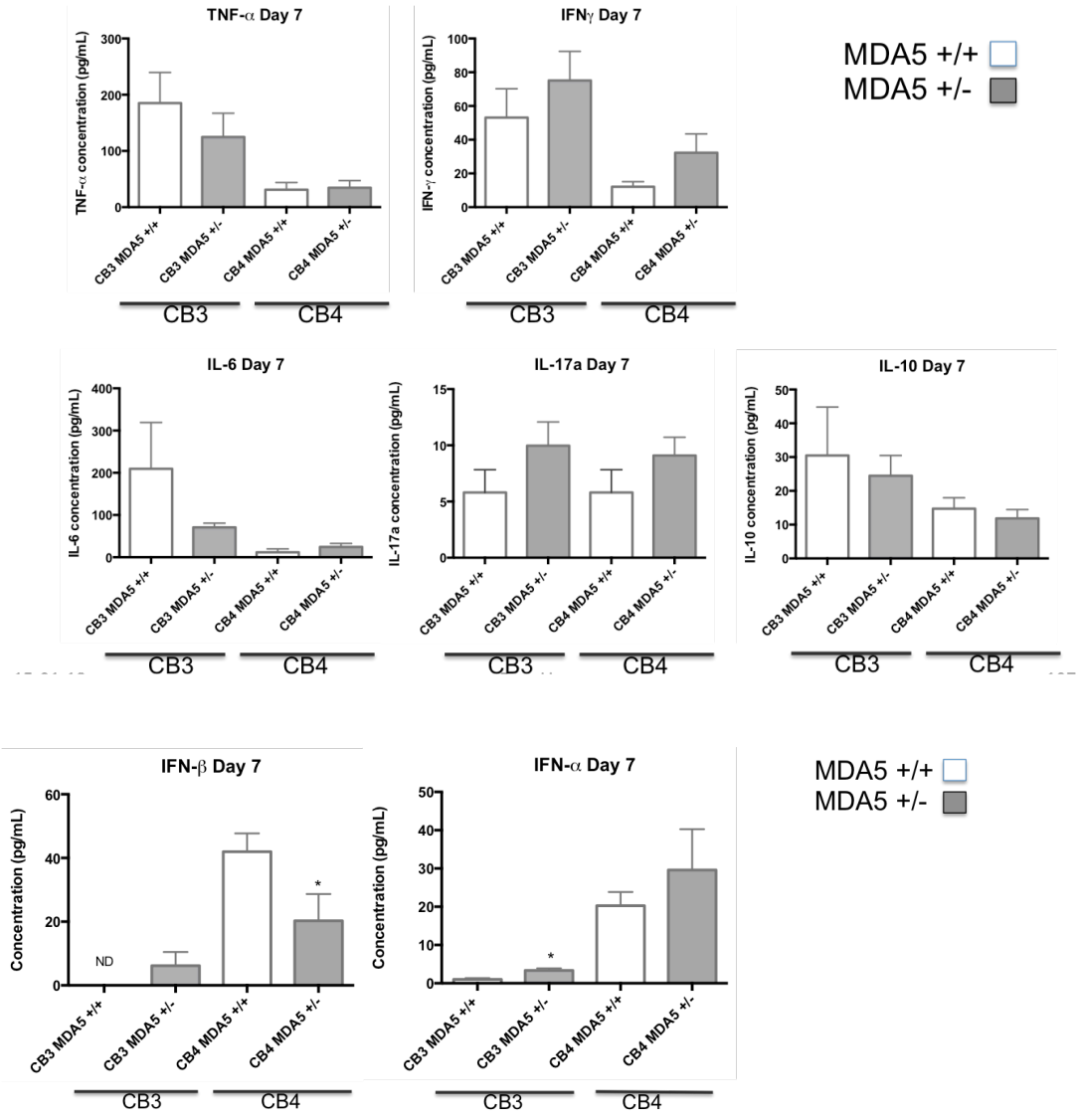


Figure 3.13 CB3 infection of MDA5$^{+/-}$ induces similar inflammatory cytokine responses as CB4 infection. Cytokines were measured by FACS bead array (A) and ELISA (B) in sera harvested from MDA5$^{+/+}$ (n=3-8) and MDA5$^{+/-}$ mice (n=3-8) at day 7 post-CB3 and CB4 infection. Statistical significance was determined by Student's t test.

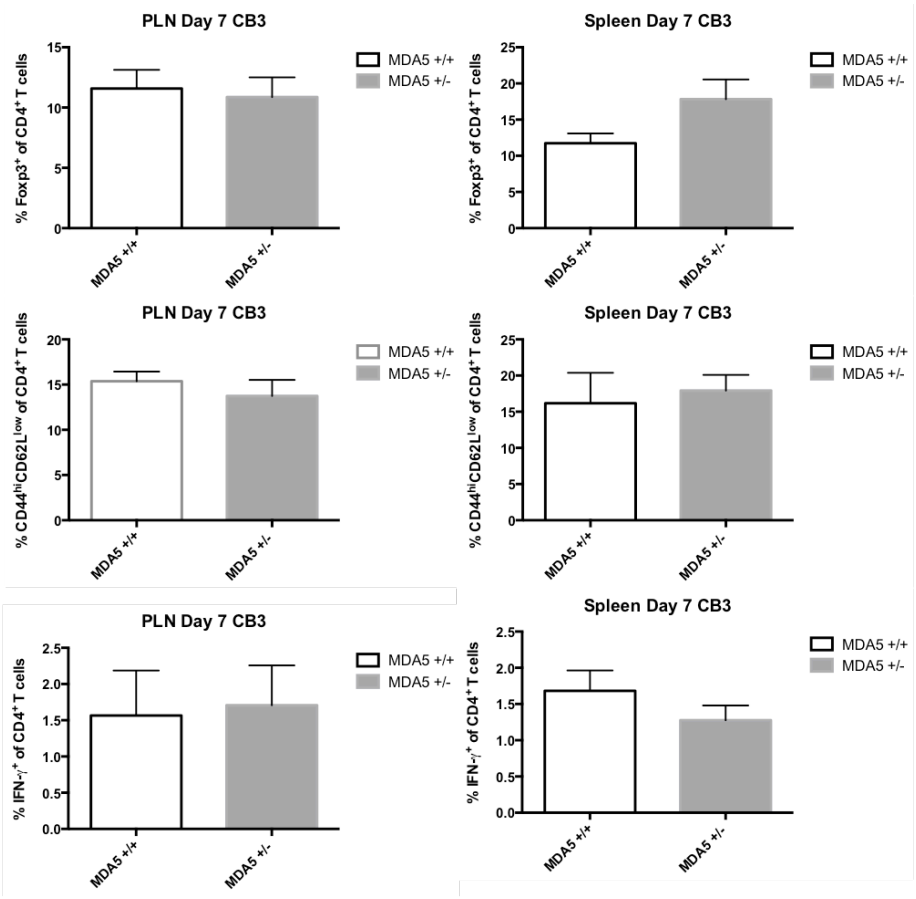


Figure 3.14 Reduced expression of MDA5 does not induce a regulatory T cell response after CB3 infection. (A) regulatory T cells (Foxp3$^+$ of CD4$^+$ T cells), (B) effector CD4$^+$ (CD44^{high}CD62^{low}) T cells, and (C) IFN-γ-producing CD4$^+$ T cells were isolated from PLNs and spleens at 7 pi, stained with their respective surface and intracellular markers and analyzed by flow cytometry. T cells were isolated from MDA5$^{+/+}$ (n=3-5) and MDA5$^{+/-}$ mice (n=3-5) at day 7 post-infection and were stained with classical activation and maturation marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from two independent experiments.

First, looking at inflammatory cytokine levels in the serum, CB3-infected MDA5$^{+/-}$ have similar responses to wt mice at day 7 pi (Figure 3.13) and follow a trend previously observed for CB4 infection (Figure 2.2, **see section 2**). However, in measuring IFN-I concentrations in the serum at day 7 pi, we observe that both CB3-infected MDA5$^{+/-}$ and wt have lower concentrations of IFN-I in the serum compared to CB4 infected mice though IFN-I levels are higher in CB3 infected MDA5$^{+/-}$ compared to CB3-infected wt, where IFN-β was not even detected (ND) in the serum (Figure 3.13), and in comparison

to the trend observed with CB4 infection where IFN-I in MDA5$^{+/-}$ are lower than wt. This IFN-I signature observed for CB3-infected MDA5$^{+/-}$ at day 7 pi differs from the IFN-I concentrations we see with CB4 infection (Figure 2.2, **see section 2**) suggesting that a reduction in MDA5 is linked to the specific IFN-I response induced by the virus. The strain of CB3 used here signals IFN-I, but failed to modify the IFN-I signature associated with protection from disease and points to the possibility that infection of the islets is important for establishing an IFN-I signature that promotes a regulatory phenotype as we see with CB4 infection in MDA5$^{+/-}$.

To further address whether virus replication at the site of autoimmunity in the PLN is critical in altering T cell polarity and shifting adaptive responses in favor of a regulatory T cell phenotype, we analyzed T cell responses in CB3 infected MDA5$^{+/-}$ and wt mice at day 7 pi. After CB3 infection, we observe distinct T cell responses from what we have previously seen following CB4 infection (Figure 2.3, **see section 2** & 3.14). Though the percent of Tregs in the PLNs of CB3-infected MDA5$^{+/-}$ is higher than the percent shown in the PLNs of infected wt (Figure 3.14), similar to the trend we observe after CB4 infection, CB3-infected MDA5$^{+/-}$ also have higher numbers of effector CD4^{+} T cells in the PLNs (Figure). With CB4 infection, effector T cells (CD4^{+} and CD8^{+}) are significantly reduced in MDA5$^{+/-}$ PLNs and spleen (Figure 2.3, **see section 2**). CB3-infected MDA5$^{+/-}$ also have reduced IFN-γ-producing CD4^{+} T cells in the spleen compared to CB3-infected wt, contrary to the increase we have previously observed in the spleens of CB4-infected MDA5$^{+/-}$ (Figure 2.3, **see section 2**, Figure 3.14). These results suggest that a reduction in MDA5 signaling induces a regulatory response that protects from T1D following virus infection and replication in the islets, at the site of autoimmunity. This implicates the consequences of MDA5 signaling within the islets not only as a critical factor in T1D susceptibility, but as a critical mediator of protection against islet targeting T1D-inducing agents like CB4.

3.4 Discussion

Tremendous evidence in both the NOD mouse and with human disease implicates a breakdown in peripheral tolerance, specifically the failed suppression of autoreactive T

cells by regulatory T cells, in the initiation and progression of T1D. The interplay of innate immune responses to environmental insults with consequential adaptive immune responses is increasingly proposed as a mechanism ultimately determining the fate of β cells and the pathogenesis of autoimmune diabetes[270]. Innate responses to viral infection help direct appropriate adaptive immune responses, albeit essential in anti-viral immunity, innate responses are also capable of inducing autoreactive adaptive responses that can lead to autoimmunity. As such, the influence of innate immune responses on autoimmunity pathogenesis has been scrutinized, especially in the development of T1D. As essential pathogen recognition receptors (PRRs) of the innate immune system, dsRNA receptors like MDA5 trigger inflammatory immune responses upon activation. On a predisposed genetic background, IFN-I signaling from these receptors could trigger islet inflammation followed by progressive β cell destruction ultimately leading to T1D.

GWAS and functional studies that have identified protective polymorphisms in the *IFIH1* gene, leading to a reduction in expression of its encoded protein, the RNA virus sensor MDA5, have helped converge the influence of anti-viral immunity on autoimmunity in T1D[78]. We have previously translated a reduction in MDA5, as seen with patients carrying protective polymorphisms, onto the NOD mouse. In NOD mice heterozygous for MDA5 ($MDA5^{+/-}$), we have demonstrated protection from spontaneous and coxsackievirus B4-induced T1D as well as a unique IFN-I signature and the polarization of regulatory T cells in the PLNs that ensues following CB4 infection and that $CD11b^{+}CD11c^{+}$ cells from $MDA5^{+/-}$ are responsible for maintaining the IFN-I and protective phenotype 1n $MDA5^{+/-}$. We have used CB4 in our experiments to induce T1D as it is strongly linked to human T1D and has been successfully modeled by our lab and others to accelerate T1D in the non-obese diabetic (NOD) mouse. Also, CB4 is an RNA virus whose replicative RNA products activate MDA5. Upon recognition of specific RNA ligands MDA5 signals through adaptor molecules leading ultimately to the production of IFN-I. These initial anti-viral interferon responses are critical for clearing viral infections and rely on virus recognition from sensors like MDA5, however in a genetically susceptible host, exacerbated inflammatory responses could be detrimental

and trigger the onset of autoimmunity and such, reduction of IFN-I signaling by reducing MDA5 should adhere exacerbation of autoimmunity following antiviral immunity.

In this section we have carried out further investigation of the functional consequences of MDA5 deficiency, particularly whether IFN-I and T cell polarization are inherent to $MDA5^{+/-}$, in a CB4-induced T1D model. We have demonstrated first that despite additional IFN-I stimulation from poly i:c after CB4 infection, $MDA5^{+/-}$ maintain a regulatory adaptive response in the PLNs unlike poly i:c treated $MDA5^{+/-}$.

Many studies have demonstrated the pleiotropic effects of poly i:c on disease, including the direct effects on islets and APCs that can lead to diabetes. Poly i:c can stimulate APC maturation and the production of cytokines including IL-12, IL-15, and IFN-I. In C57BL/6-rat insulin promoter-B7.1 mice that are resistant to developing diabetes, poly i:c is recognized in the pancreatic islets, promotes the maturation of APCs to activate islet-directed T cells and induces diabetes [271]. Also, though C57BL/6 mice do not harbor anti-insulin autoantibodies or develop spontaneous anti-insulin T cell reactivity, poly i:c treatment prior to insulin immunization can induce diabetes and as such, suggests in the C57BL/6 model that poly i:c acts first as an environmental insult, triggering the release of islet antigens and priming APC and T cell responses leading to autoimmunity. Interestingly, poly i:c administration at a dose of 5μg/g body weight in NOD mice protects from T1D, where [272]but accelerates disease in the BioBreeding (BB) rat model. Low-dose injections (0.05μg/g body weight) of poly i:c in the BB rat model protect rather than accelerate disease, mostly attributing to the induction of suppressor T cell activity[273]. The increase in immunoregulatory cell activity with low dose poly i:c suggests that mechanisms to maintain a low IFN-I signal, such as the reduction in IFN-I signaling components, could afford diabetes sparing activity and avoidance of autoimmunity.

In mouse models of autoimmune cholangitis, injection of poly i:c following immunization of mice with autoantigen exacerbates autoimmunity with the increased induction of infiltrating autoreactive $CD8^+$ T cells, as well as proinflammatory

cytokines[274]. This study along with many others demonstrating the potent ability of poly i:c to induce IFN-I responses and exacerbate autoimmunity, supports the notion that in addition to a breakdown in tolerance, a second insult inducing IFN-I during the breakdown process can regress tolerogenic mechanisms and progress disease pathogenesis to autoimmunity. As such, we would expect that supplemental IFN-I stimulation with poly i:c addition at days 3 and 5 post-CB4 infection would abrogate the the IFN-I signature and polarization of T cell responses typically observed at day 7pi in MDA5$^{+/-}$ and we would observe higher levels of IFN-I in the serum and effector rather than regulatory CD4^{+} T cells would dominate in the PLNs.

It has been shown that poly i:c inoculation before infection with a strain of coxsackievirus B3 that expresses the immunosuppressive cytokine IL-4 (CVB3-mIL4), stimulates an anti-viral IFN-I response sufficient to suppress viral replication in the pancreas despite expression of the immunosuppressive cytokine. As IFN-I are primarily produced by the β cells in the pancreas and intraislet IFN-I production has shown to prevent CB4 replication[275, 276], it would be expected that anti-viral responses triggered to control coxsackievirus replication would alter adaptive responses accordingly. However, in MDA5$^{+/-}$, following CB4 infection and despite addition IFN-I stimulation with poly i:c, a steady IFN-I signature and regulatory T cell responses in the PLNs are maintained. The maintenance of this unique phenotype 1n MDA5$^{+/-}$ was also seen following challenge with a strain of CB3 that does not infect the pancreatic islets.

We challenged MDA5$^{+/-}$ mice with CB3 to test whether the phenotype observed in CB4-infected MDA5$^{+/-}$ was specific to the virus or whether it was inherent to a reduction in MDA5. Although CB4 is strongly linked to T1D, in patients CB4 may only be responsible for a subset of new onset cases as other viruses including other coxsackieviruses have more recently been implicated and should be considered as other potential IFN-I and T1D inducers [87, 277, 278]. Studies examining the effects of CB3 in MDA5 deficient C57Bl/6 mice show no significant increase in mortality over MDA5 competent mice following infection and CB3 infection of C57BL/6 mice deficient for one MDA5 allele with the pancreatropic virus EMCV results in transient hyperglycemia due

to direct killing of pancreatic beta cells [122, 137]. CB3 infection of NOD mice induces myocardial autoantibodies and autoimmune myocarditis pathologies, though in the context of TGFβ expression in the pancreas with transgenic NODTGFβ mice, reduced amounts of autoantibodies and acute rather than chronic disease pathologies are observed[279, 280]. Recent clinical studies have shown that CB3 infection is associated with a decrease in risk for autoimmune diabetes, but likely as a result of cross-protection from the diabetogenic effects of a previous coxsackievirus B1 infection[278]. Functional studies by Kemball et al. have demonstrated that CB3 interferes with proper antigen presentation, selectively the major histocompatibility complex class I (MHC-I) pathway and further work by Mukherjee et al. has showed that CB3 can cleave a critical molecule in the MDA5 signaling pathway with its 3Cpro cysteine protease and evade innate immune defenses[281-283]. As many studies have pointed out, CB3 infection induces various immunological consequences including repercussions in the MDA5 signaling pathway and as such, we would expect that infection of MDA5$^{+/-}$ with CB3 would induce dissimilar immune responses from CB4 infection.

Since the strain of CB3 we used to infect MDA5$^{+/-}$, replicates and infects primarily the pancreas, outside the islets, initiating pancreatitis and not insulits as we observe for CB4 infected NOD mice[269], we anticipated that CB3 infection would help decipher whether infection of the islets was important for IFN-I signaling and T cell responses in MDA5$^{+/-}$. We observed that CB3 infection changed the IFN-I signature in MDA5$^{+/-}$, by increasing serum IFN-I levels by day 7pi compared to CB3 infected wt suggesting that the strain of CB3 used here signals IFN-I, but failed to modify the IFN-I signature associated with protection from disease as we observe in CB4-infected MDA5$^{+/-}$. We also observe after CB3 infection, distinct T cell responses from what we have previously seen following CB4 infection where CB3-infected MDA5$^{+/-}$ have higher effector rather than regulatory T cells in the PLNs. These results suggest that a reduction in MDA5 signaling induces a regulatory response that is specific to virus infection and the location of replication, in the islets at the site of autoimmunity. This implicates MDA5 signaling in the endocrine pancreas not only as a critical factor in T1D susceptibility, but as a critical mediator of protection against T1D-inducing agents like CB4.

Though MDA5 is an important sensor of coxsackievirus B, another intracellular receptor, TLR3, also critically detects coxsackievirus B dsRNA and signals IFN-I as a part of innate immune responses. In many animal models, a distinct role for TLR3 has been shown in diabetes[284, 285]. TLR3 signaling from macrophages is also critical to the survival of NOD mice following CB4 infection[252]. In diabetes resistant C57BL/6 mice, MDA5 and TLR3 are both required to prevent diabetes following infection with a pancreatropic virus encephalomyocarditis virus strain D (EMCV-D) that induces diabetes through the destruction of β cells rather than T cell–mediated autoimmunity as we see with CB4 infection. Additionally with EMCV-D infection in C57BL/6 mice, MDA5 and TLR3 exert different IFN-I response kinetics, with IFN-I responses detected in $MDA5^{-/-}$ at 15 hours pi and at a later time in $TLR3^{-/-}$ mice[122]. With this potential cooperative role in IFN-I signaling (at least in response to EMCV-D) and the previously demonstrated critical role of TLR3 signaling to protect from CB4 infection in NOD mice in mind, it is interesting that with a reduction in MDA5 we see such a distinct IFN-I signature and T cell polarity phenotype that protects from CB4 infection and T1D. It would be expected that TLR3 signaling would compensate for the reduction in MDA5 in $MDA5^{+/-}$ and would still emit IFN-I responses as a part of antiviral immunity to protect against CB4, but as a result, exacerbate autoimmunity.

Interestingly, in CB4-infected $TLR3^{+/-}$ we observe a different IFN-I signature from CB4-infected $MDA5^{+/-}$ that does not mediate an increase in regulatory T cells and decrease in effector T cells associated with the protective phenotype observed in $MDA5^{+/-}$. In $TLR3^{+/-}$ we observe increased Th1, Th17 and IFN-α at day 3 pi and effector rather than regulatory T cell responses at day 7pi, suggesting in comparison to phenotype observed in $MDA5^{+/-}$ that MDA5 and not TLR3 signaling is a critical factor in polarizing a regulatory and protective response and rather TLR3 is more important for the anti-viral response and MDA5 signalling as a factor in the autoreactive response.

A distinctive role for the reduction in MDA5 and not another T1D-associated factor was also emphasized with our observations of NOD mice lacking the vitamin D receptor

($VDR^{+/-}$). $VDR^{+/-}$ mice were not protected from CB4-induced T1D and failed to generate protective T cell responses pi.

In conclusion we suggest that a reduction in MDA5 allows for a unique IFN-I signature that establishes an appropriate immunoenvironment to polarize a regulatory T cell response and protect from T1D after CB4 infection. Targeting IFN-I signaling by a reduction in MDA5 may be an effective strategy in reestablishing tolerance by boosting regulatory T cells that have increased suppressive capacity and/or by depleting the number of available autoreactive T cells. This research has allowed further insight into the interactions between viruses and components of the innate immune system that ultimately lead to the development of autoimmunity. Understanding these interactions and the functional consequences of these interactions will in turn allow the design of therapeutics aimed at preventing the development of autoimmune diseases following viral infection.

Chapter 4: MDA5 signals a unique transcriptional signature following coxsackievirus B4 infection.

4.1 Introduction

Although it is widely accepted that Type 1 diabetes (T1D) is the result of the autoimmune destruction of insulin-producing beta cells in the pancreas little is known about the events leading to islet autoimmunity. Epidemiological and genetic data have associated virus infections and anti-viral type 1 interferon (IFN-I) response genes with T1D**[286].** Genetic variants in the T1D risk locus interferon induced with helicase C domain 1 (*IFIH1)* have been identified by genome-wide association studies (GWAS) to confer resistance to T1D and result in the reduction in expression of the intracellular RNA virus sensor known as melanoma differentiation-associated protein 5 (MDA5).

Our translational studies, modeling the reduction in *IFIH1* gene expression that results in protection from T1D have demonstrated that mice heterozygous at the *Ifih1* gene ($MDA5^{+/-}$) express less than half the level of MDA5 protein, which leads to a unique anti-viral IFN-I signature and adaptive response following virus infection that protects from T1D (**see Chapter 2**)[81]. $MDA5^{+/-}$ mice have a regulatory rather than effector T cell response at the site of autoimmunity, the IFN-I signature induced after viral infection is specific to coxsackievirus B4 and not observed in CB4-infected NOD mice heterozygous for TLR3 ($TLR3^{+/-}$), but maintains polarization of a regulatory T cell response despite additional IFN-I stimulation from the RNA mimetic poly i:c. This research presented in Chapters 2 and 3 supports a role for MDA5 expression as an essential regulator of the diabetogenic T cell response, providing a potential mechanism for patients carrying *IFIH1* protective polymorphisms, and reveals a unique IFN-I signature that leads to the induction of protective adaptive responses following CB4 infection.

It is well established however, that autoimmunity that ensues in T1D is a T-cell mediated process. In NOD mice, it has also been well established that CB4 infection accelerates an ongoing autoimmune process directed at the pancreatic β cells by targeting β cells for infection. The infected cells are engulfed by resident antigen presenting cells (APCs) resulting in the presentation of previously sequestered antigens to the pre-existing

population of autoreactive T cells[118]. Autoreactive T cells that recognize islet antigens then attack the islets, inducing insulitis, loss of insulin production and autoimmune diabetes. The activation or 'maturity status' of APCs during the autoimmune process induced by CB4 is also important in the fate of disease. CD40 expression has been specifically implicated in the type of adaptive immune response that is signaled following CB4 infection and also in the context of transgenic TGF-β expression in the pancreas[119, 187].

Innate immunity is critical for primary detection of pathogens, such as viruses, and for initiating the first wave of defenses [126, 127]. A thorough understanding of virus detection by the innate immune system is critical not only for understanding innate immune mechanisms, but is essential to anticipate viral pathogenesis. The functional consequences of this early detection of viral infection results in the establishment of an anti-viral response and contributes to the eventual activation of a virus-specific adaptive immune response aimed at clearing viral infection that can have significant implications in the development of autoimmunity [128]. Detection of viral RNA produced during replication of certain viruses like coxsackieviruses by the RNA sensors MDA5 and TLR3 leading to the production of IFN-I is one such innate response that can influence T1D susceptibility.

As T1D is a complex, multifactorial autoimmune disease, the events leading to autoimmunity remain difficult to decipher, rendering our capacity to determine possible moments in the disease process for successful intervention a challenge. Many current and emerging high-throughput transcriptome profiling technologies are being used as systems biology approaches[287]. Whole transcriptome sequencing or RNAseq is a robust screening approach that has allowed the identification of genes and transcripts expressed in the events leading to and during autoimmune diabetes [287].

Examination of the transcriptional and cellular landscape of uninfected NOD mice using micro array and RNAseq methods has revealed distinct and chronological phases of spontaneous T1D progression[288]. From 2 weeks of age to the onset of spontaneous

autoimmune diabetes (20-30wks), NOD mice undergo transcriptional changes dissimilar from genetically identical NOD mice lacking an adaptive immune response (NOD *Rag1*$^{-/-}$), and two diabetes resistant strains (C57BL/6 and B6.NOD-H2^{g7} strains). The first phase of significant transcriptional changes in NOD mice is observed as early as 6 wks, where genes most highly expressed in NOD mice are IFN-inducible transcripts. This early IFN-signature increased over time, preceded transcriptional changes in T cell activation, and was exclusive to NOD mouse development. From 8-18wks of age, a transcriptional profile consistent with inflammatory and autoreactive T cell responses marks a second phase of transcriptional changes in NOD mice and, in diabetic NOD mice, a final phase of transcriptional changes is characterized by an enrichment of NF-κB inducible transcripts, consistent with cellular destruction and an ongoing diabetogenic process[288].

RNAseq provides high sensitivity and single-base resolution to help identify significant changes in expression profiles including the identification of alternatively spliced isoforms of genes and tissue-specific alternative splicing. Recent whole transcriptome studies in human pancreatic islet cells and in NOD mice have identified specific molecular changes that are present in the T1D process[175, 288, 289]. Transcriptional profiling allows unbiased investigation of samples on a genome-wide scale, which is essential in deciphering complex interactions between immune system components in autoimmune diseases like T1D.

Based on our previous observations of a distinct IFN-I expression signature that ultimately led to adaptive T cell responses in favor of protection from T1D in MDA5$^{+/-}$ following CB4 infection (**see Chapter 2**) [81], we hypothesized that a reduction in MDA5 induces inflammatory changes in the NOD mouse at the transcriptome level and that inflammatory events marked by transcriptional signatures following CB4 infection in MDA5$^{+/-}$ could be traced using high throughput sequencing methods. There have been only a few studies to date that have analyzed molecular changes occurring in pancreatic islets, in islet infiltrating leukocytes, and in pancreatic lymph nodes using RNAseq. Here we present the first examination of the transcriptional profiles across tissues at the site of

autoimmunity following CB4 infection in the NOD mouse and in the context of a reduction in an essential CB4 receptor, MDA5.

4.2 Materials and methods

Mice. NOD/ShiLtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $MDA5^{+/-}$ mice were backcrossed from C57BL/6 $MDA5^{-/-}$ mice onto the NOD background as previously described **(see section 2)**. We confirmed by SNP analysis (in house and by DartMouse, Lebanon, NH) that they carry the full complement of NOD *idd* alleles. More importantly, we confirmed that littermates to the backcrosses that were either heterozygote and wildtype for the $MDA5^{-/-}$ alleles mice had the ability to develop spontaneous diabetes which is strongly indicative that the required susceptibility loci had crossed over.

Mice were maintained in the Modified Barrier Facility (Pharmaceutical Sciences Building, Vancouver, British Columbia) and kept in a pathogen-free environment. Diabetes incidence was monitored by nonfasting blood glucose measurements. Disease onset was determined by two consecutive blood glucose levels exceeding 300 mg/dL. Only pre-diabetic mice were used for experiments. All animal work was performed under strict accordance with the recommendations of the Canadian Council for Animal Care. The protocol was approved by the Animal Care Committee (ACC) of the University of British Columbia (certificate numbers: A08-0415 and A08-0622).

Virus. Ten-to 12-week old mice were infected intraperitoneally with sublethal doses of 400 plaque-forming units (PFUs) of CB4 Edwards strain 2 or coxsackievirus group B type 3 (CB3, Nancy Strain) diluted in DMEM. As there is no gender bias in CB4-mediated T1D, equal numbers of male and female mice were infected with CB4. Both male and female mice were infected with CB3. Virus stocks were prepared and free virus particles were detected from tissue homogenates by plaque assay as described previously [118].

Flow cytometry. Pancreatic lymph node and splenic single cell-suspensions were counted and stained with fluorescently conjugated mABs for cell surface markers CD4

(clone L3T4), CD8 (53-6.7), CD25 (clone PC61), CD11b (clone M1/70), CD11c (clone HL3), CD44 (clone IM7), CD40 (clone 3/23), CD62L (clone MEL-14), Nrp-1 (R&D, AF566) and intracellular transcription factors Foxp3 (clone FJK-16s), intracellular receptors TLR3 (clone 40C1285.6) and MDA5 (Abcam, ab69983). Stained cells were analyzed by flow cytometry with the BD Biosciences LSR II (San Jose, CA) and Flow Jo vX.0.6 software (TreeStar, Ashland, OR).

RNA isolation and library prep. Pancreatic lymph nodes, pancreas and spleen were removed and immediately snap frozen in TRIzol reagent (Life Technologies Inc, Burlington, ON). Tissues were weighed and organs were homogenized using QIAGEN stainless steel beads and TissueLyser II benchtop homogenizer at 19/s for 10 min. Total RNA was prepared with TRIzol reagent according to the manufacturer's protocol (TRIzol, Life Technologies). RNA was quantified using a NanoDrop-ND-1000 (VERIFY) (Thermo Scientific, Wilmington, DE). RNA was further purified using RNeasy Mini Kit from Qiagen. RNA integrity and quantification was further validated using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Indexed sequencing libraries were constructed using the TruSeq RNA sample Prep Kit v2 (Illumina Inc. San Diego, CA) and sequenced at 50 cycles, single read on an Illumina HiSeq 2000 platform.

3' RNA sequencing and analysis. When working with degraded sample 3'-seq can be employed to allow sequencing and differential expression data from rescued samples. Samples are entered into Illumina TruSeq mRNA sample prep with normal polyA selection, and an adapted fragmentation time in order to create fragments of 250bp. Samples are then selected again using polyA which will mainly capture the first 250bp of the 3' segment. The selected 3'segment then follows the rest of the truseq mRNA sample prep protocol. Sequencing only the 3' end gives total sample gene counts, which will later be normalized by the total number of reads per sample and allow for differential expression analysis. 75x75 Pair End sequencing on Illumina Miseq was used on generated 3' fragments.

Sequenced raw reads are aligned to the genome using mm10 ref-sequence in TopHat. Aligned sequences are then mapped to transcripts with mm10 using Partek Genomics Suite, which outputs a reads on transcripts that are then normalized by Transcript Reads per Million Sample Reads (RPM). To quantify differential expression for a specific gene between $MDA5^{+/-}$ and $MDA5^{+/+}$ we used a "z-score", calculated as z = (a-b)/sqrt(a+b). a represents the RPM value for the given gene in a specific sample from the $MDA5^{+/-}$ and b as the RPM value for the given gene in a specific sample from the $MDA5^{+/+}$ to give a positive (upregulated) or negative (downregulated) differential expression for that gene in the $MDA5^{+/-}$ relative to the $MDA5^{+/+}$ [290, 291].

The z-score gives a sense of the highest impacted genes. The top impacted genes by z-score are then issued to Ingenuity Pathway Analysis (IPA) and Partek Gene Ontology based on this normalized differential expression value. Ingenuity and Partek Gene Ontology then organizes these transcriptional changes based on previously published literature. The organized data can be used to demonstrate network, disease, or processes that are changing most and most often.

Network Analysis. Gene lists featuring top impacted genes generated from 3'sequencing were also issued to IPA for network analysis. Predicted networks generated by IPA are based on molecular relationships, interactions, and pathway associations. (Blue) is predicted to be downregulated, whereas (orange) represents genes potentially upregulated. Direct interactions are marked by direct arrows, whereas indirect associations are represented by dotted lines.

4.3 Results

4.3.1 MDA5 expression mediates specific cellular responses in response to coxsackievirus B in NOD mice.

In order to further describe the role of MDA5 signaling in response to coxsackievirus B4 (CB4) in NOD mice we challenged NOD mice heterozygous ($MDA5^{+/-}$) and deficient in MDA5 ($MDA5^{-/-}$) with CB4 and a strain of CB3. NOD mice retaining full expression of

the MDA5 gene ($MDA5^{+/+}$, wt) were used as controls. The strain of CB3 we used to infect $MDA5^{+/-}$, replicates and infects primarily the pancreas, and secondly the heart similarly to CB4 in mice. Both serotypes share the same receptor, the coxsackievirus-adenovirus receptor (CAR) that is expressed by the islets of the pancreas, though CB3 does not infect pancreatic islets[267, 268]. Rather, CB3 infection outside the islets initiates pancreatitis and not insulitis as we observe for CB4 infected NOD mice[269].

We examined the cellular changes in the pancreatic lymph nodes (PLNs) and spleen at day 3 post-infection (pi) by staining cells for classical regulatory CD4 T cell (Treg, $Foxp3^{+}CD4^{+}$), effector CD4 T cell (Teff, $CD44^{hi}CD62L^{lo}$), and antigen presenting cell (APC, $CD11b^{+}CD11c^{+}$, $CD11b^{+}CD11c^{-}$) activation markers and analyzing stained cell suspensions by flow cytometry. We observed at day 3pi specific cellular responses with a reduction in MDA5 expression compared to wt mice following CB4 infection and compared to CB3 infection. In the PLNs, both $MDA5^{+/-}$ and $MDA5^{-/-}$ mice have reduced percentages of Teffs following CB4 infection, whereas in response to CB3 infection only $MDA5^{-/-}$ mice show a reduction compared to infected wt. In response to CB3 infection, $MDA5^{+/-}$ have an increase in Teffs compared to wt mice and this increase is more significantly observed in the spleen (Figure 4.1). An increase in Teffs in both the PLNs and spleen in $MDA5^{+/-}$ following CB3 infection is also observed at day 7pi (Figure 3.14, **see section 3**). Interestingly, the percent of Tregs in the PLNs of CB3-infected $MDA5^{+/-}$ is markedly reduced compared to CB3-infected wt (Figure 4.1), however, we have previously observed that the number of Tregs increases to levels above those seen in infected wt by day 7 pi (Figure 1.3, **see section 3**). There are no significant changes in the number of Tregs in the PLN or spleen compartments of $MDA5^{+/-}$ or $MDA5^{-/-}$ following CB4 infection at day 3 (Figure 4.1), though a significant increase in Tregs compared to CB4-infected wt is observed in $MDA5^{+/-}$ at day 7 pi (Figure 2.3, **see section 2**).

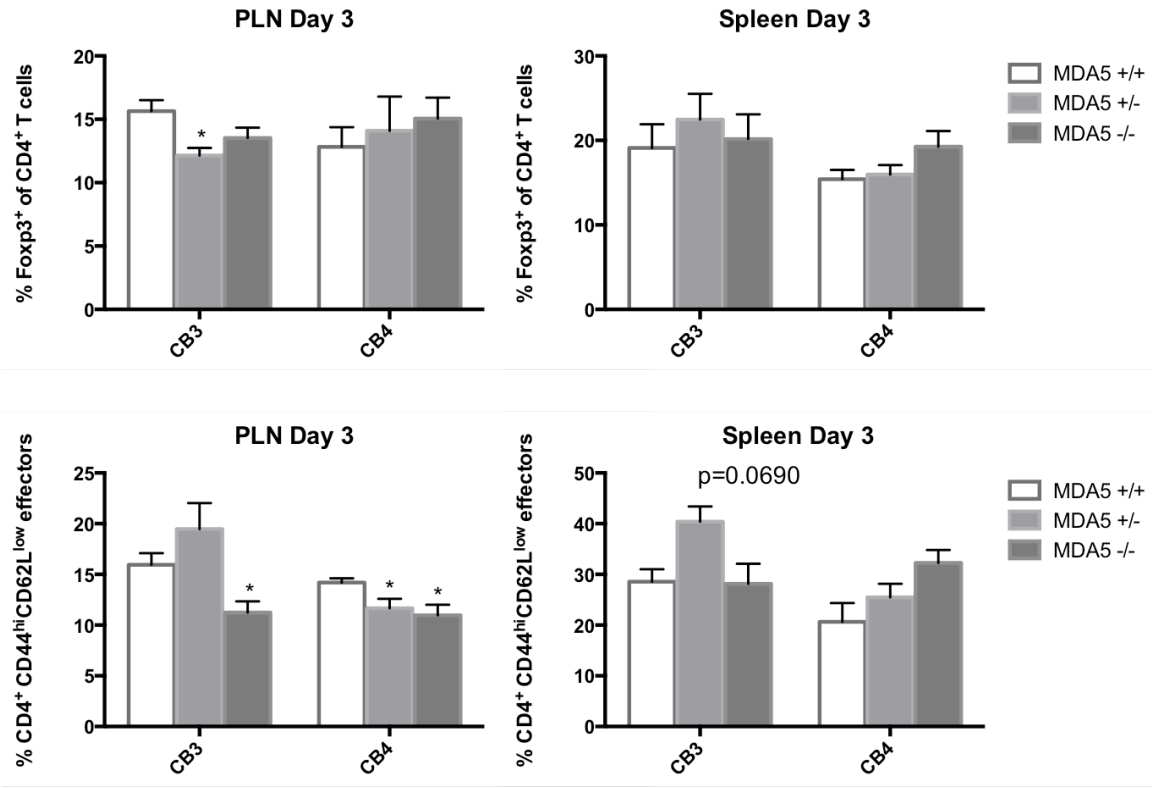


Figure 4.1 A reduction in MDA5 induces virus specific adaptive CD4 T cell responses at day 3 following coxsackievirus B infection. $CD4^+$ T cells were isolated from PLNs and spleens at 3 pi, stained with their respective surface and intracellular markers (regulatory T cells ($Foxp3^+$ of $CD4^+$ T cells) and effector $CD4^+$ ($CD44^{high}CD62^{low}$) T cells), and analyzed by flow cytometry. T cells were isolated from $MDA5^{+/+}$ (n=3-5), $MDA5^{+/-}$ (n=3-5), and $MDA5^{-/-}$ mice (n=3-5) at day 7 post-infection and were stained with classical activation and maturation marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from two independent experiments.

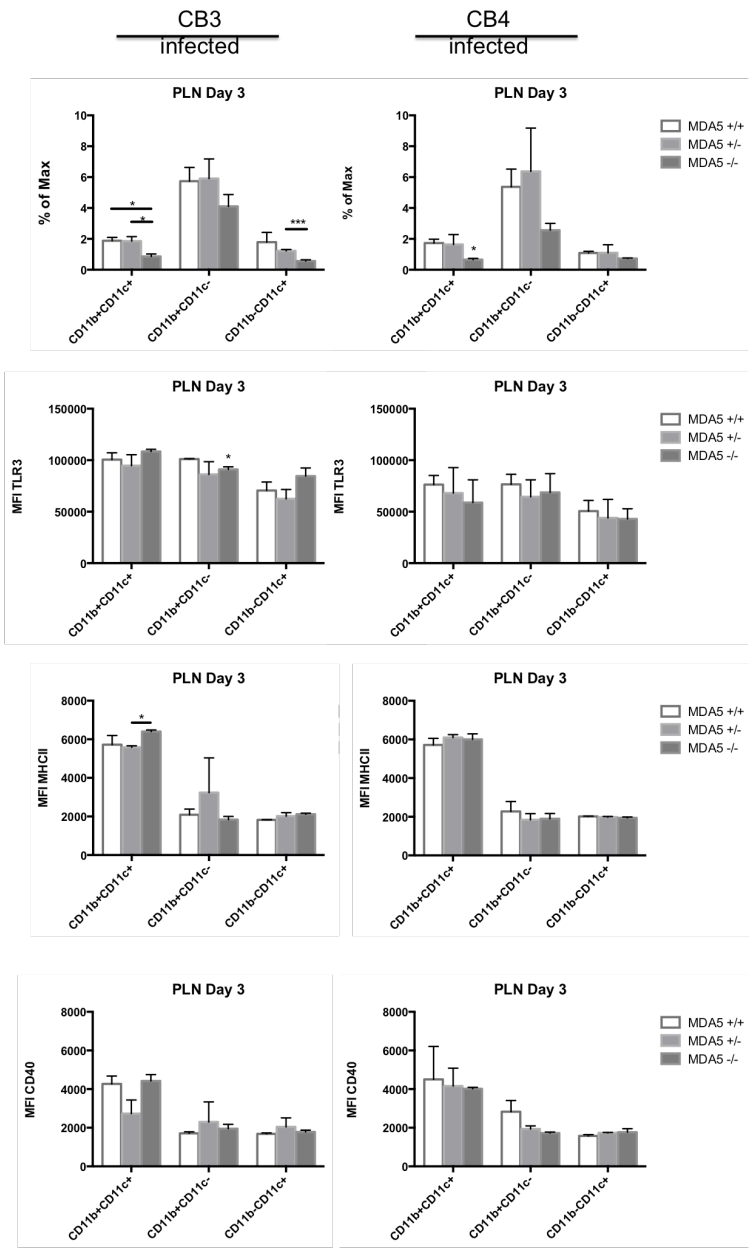


Figure 4.2 The number and activation of antigen presenting cells (APCs) from MDA5$^{+/-}$ is not virus specific and is similar to MDA5$^{+/+}$ mice following coxsackievirus B infection.

CD11b^{+}CD11c^{+} cells, CD11b^{+}CD11c^{-} , and CD11b^{-}CD11c^{+} cells were isolated from the PLNs and spleens of CB3 and CB4 infected MDA5$^{+/+}$, MDA5$^{+/-}$ and MDA5$^{-/-}$ mice at day 3 pi and were stained for CD11b, CD11c, MHC II, CD40, TLR3 expression and analyzed by flow cytometry. Results are shown as mean ± SEM of a representative from two independent experiments.

Similar numbers of APCs and TLR3, MHCII and CD40 expression are observed in the PLNs of CB4 and CB3-infected MDA5$^{+/-}$ compared to wt mice at day 3 pi (Figure 4.2). However, a significant reduction in MDA5 expression is detected in the PLNs at day 3 pi of CB4-infected MDA5$^{+/-}$ (Figure 2.5, **see section 2**). Although there are no changes in the activation or number of APCs found in the PLNs of CB4-infected MDA5$^{+/-}$, the observations of virus-specific changes in adaptive CD4 T cell responses as early as day 3 pi along with a unique IFN-I signature observed in MDA5$^{+/-}$ at this early time point post-CB4 infection (Figure 2.2, **see section 2**) suggests the potential for inflammatory events that can shape the type of adaptive response induced by CB4 infection, occurring at the transcriptional level rather than what can be observed at the cellular level.

4.3.2 A reduction in MDA5 induces unique transcriptional signatures following CB4 infection

Extending our previous observations of a unique IFN-I and adaptive T cell phenotype in CB4-infected MDA5$^{+/-}$, we hypothesize that a distinct transcriptional landscape results from a reduction in MDA5 and directly influences IFN-I and T cell responses following CB4 infection. We believe there is a direct correlation between the IFN-I signature induced following environmental challenge with the induction of a strong effector T cell (Teff) and a matched regulatory T cell (Treg) response that can ultimately determine T1D pathogenesis. We therefore decided to investigate the transcriptional profile of MDA5$^{+/-}$ mice in comparison to MDA5$^{+/+}$ mice following CB4 infection and identify potential transcriptional signatures unique to a reduction in MDA5 that are involved in the development of T1D.

RNA from PLNs, pancreas and spleen of CB4-infected MDA5$^{+/-}$ was used to study transcriptional signatures that were induced with a reduction in MDA5 and following CB4 infection. As we were using samples from CB4 infected mice we anticipated that the integrity of tissues for RNA sampling would be somewhat compromised due to viral replication and cellular destruction. As such, though RNA-Seq is an effective method to study the transcriptome, it can be difficult to apply to scarce or degraded RNA from fixed

samples. In standard RNAseq protocols, oligo (dT) is used to isolate polyadenylated (polyA) RNA, and deplete highly abundant ribosomal RNA (rRNA). Although this is a powerful technique, it excludes many non-polyadenylated transcripts and for partially degraded RNA, oligo (dT) selection will only isolate the most 3′ portion of each transcript[292].

RNA was extracted from 3 replicates of each tissue (pancreatic lymph nodes (PLNs), pancreas and spleen) from MDA5$^{+/-}$ and compared to 3 replicates of each tissue of MDA5$^{+/+}$ mice at day 3 pi. Library preparation and sequencing was run for three PLN and pancreas samples and one spleen sample from each genotype. Since our RNA isolated from infected tissue was partially degraded we implemented additional purification and quality control methods and had to resort to 3' end sequencing. Sequencing only the 3' end gives total sample gene counts which will later be normalized by the total number of reads per sample and allow for differential expression analysis.

Principal Components Analysis (PCA) is a data reduction method that visually represents sample grouping in a 3D scatter plot and groups samples according to their transcriptome similarity. Using PCA we observe that separation along the x-axis or first principal component (PC1) describes 50.6% of the variance in the PCA plot (Figure 4.3) and divides the PLN (upper left) and pancreas samples (upper right) from the spleen samples. The PCA results in Figure 4.3 show a gap between the samples. This gap indicates gene expression changes between the samples. The clustering of the individual PLN and pancreas samples suggests they are similar. We see no clustering or dissimilarity for the spleen samples as there was very low sequencing done for these samples (no replicates) (Figure 4.3).

A comprehensive comparison of transcriptomes revealed 16,863 gene reads in the PLNs, 15,827 in the pancreas and 15,217 in the spleen. Since we performed 3'RNAseq we did not have to account for expression bias due to transcript length- as would be expected for normalizing and generating reads per kilobase of transcript per million reads (RPKM) expression values.

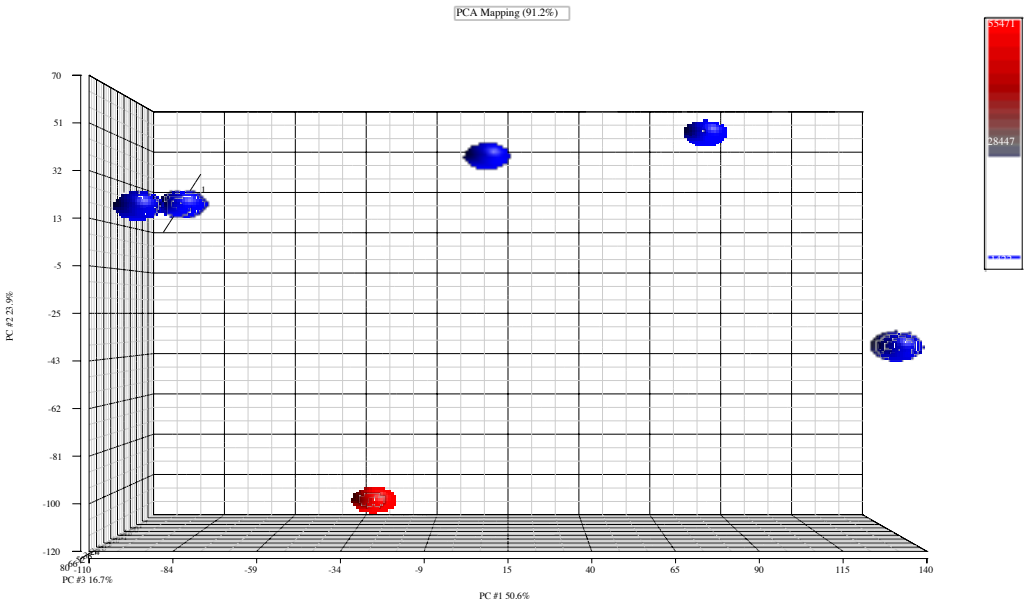


Figure 4.3 PCA analysis of general transcriptome similarity of tissues following 3'RNAseq. PCA analysis offers a visual representation of sample grouping in a 3D scatter plot. RNA from pancreatic lymph node (PLNs, n=2 for each genotype) (upper left), pancreas (upper right, n=3 for each genotype) and spleen (lower center and right, n=1 of each genotype) was isolated from MDA5$^{+/-}$ and MDA5$^{+/+}$ at day 3 post-CB4 infection and sequenced via 3' RNAseq. Data is a representative of one of two independent RNAseq runs.

Rather each sample transcript was normalized and assigned a value based on the number of mapped sequence coverage according to a quantitative gene expression value known as the reads per million reads. (RPM) (using the formula described in Methods). Pooled RPM values for replicates of MDA5$^{+/-}$ and MDA5$^{+/+}$ were compared and subjected first to z-score analysis, where an absolute z-score (>=2) was calculated (described in Methods) for each gene expressed to determine the overall gene expression pattern in MDA5$^{+/-}$ versus the expression pattern in MDA5$^{+/+}$ for each tissue sample. Z-scores evaluate the number of standard deviations that separate a gene expressed in a sample from the mean expression of that gene in all samples. A Chi-squared test was also used on the differential expression data set created by the z-score analysis and assigned p-values for each gene.

We then issued the top impacted genes determined by z-score analysis to Ingenuity Pathway Analysis (IPA) and Partek Gene Ontology (Partek) based on the normalized differential expression value or z-score. Ingenuity and Partek Gene Ontology organizes the transcriptional changes based on previously published literature and the organized data can then demonstrate networks or processes that are changing most and most often. Genes from total gene lists generated after IPA and Partek organization for the PLNs, pancreas and spleen of MDA5$^{+/-}$ and MDA5$^{+/+}$ mice were then sorted and ranked in descending order according to their respective z-scores >=2. This filtered list rendered 190, 4574, and 8224 differentially expressed genes (z-score >=2), in the PLNs, pancreas and spleen respectively, between MDA5$^{+/-}$ and MDA5$^{+/+}$ mice (data not shown). Changes in gene expression in MDA5$^{+/-}$ in respect to MDA5$^{+/+}$ can be seen across the PLN, pancreas and spleen tissues at day 3pi (Table 4.1). The raw z-scores indicate whether a gene from MDA5$^{+/-}$ was downregulated (z-score < 0) or upregulated (z-score >0) relative to the expression of that gene in MDA5$^{+/+}$.

Though not in the top 40 genes differentially expressed, changes in particular genes involved in APC and T cell activation, the IFN-I signaling pathway and an endogenous retrovirus sequence (*xpr1*) associated with NOD stimulation can also be seen in the PLN, pancreas and spleen tissues of CB4-infected MDA5$^{+/-}$ relative to MDA5$^{+/+}$ at day 3 pi (Table 4.2). The most impacted of the genes involved in IFN-I signaling and APC and T cell activation in MDA5$^{+/-}$ relative to wt (z-score >=2) were found to be more concentrated in the spleen. Based on raw and not absolute z-scores for differential expression of genes in the spleen, though representative of n=1 for each genotype, we observe that Foxp3 is upregulated (z-score= 2.788043611) in the spleens of MDA5$^{+/-}$ at day 3, as is more significantly CD86 expression (z-score=8.424400764), whereas the expression of the inflammatory cytokine IL-6, appears to be downregulated (z-score= -3.263351463). However, due to the low sample number (n=1 for each genotype) for the spleen tissues, the differential expression of genes in the spleen are representative of low reproducibility and should be considered as preliminary trends and cannot be compared to the expression changes seen with the PLN and pancreas tissues for which sample numbers (n=3) and reproducibility was much higher.

It is interesting though that the *ifih1* gene coding for MDA5 was not differentially expressed in MDA5$^{+/-}$ relative to wt for any of the tissues. MDA5 expression measured by qPCR in pancreas and spleen tissues was varied in the MDA5$^{+/-}$ relative to the expression measured in MDA5$^{+/+}$ (Figure 2.2, see Chapter 2). A lack in differential expression across tissues was also observed for the co-stimulatory APC marker CD80 and many T cell activation and immunomodulatory cytokine signaling genes including IFN-I pathway genes (Table 4.2).

The natural Treg marker Nrp1 was most differentially expressed only in the PLN and the IFN-I signaling gene *ifit1* was most differentially expressed in both PLN and spleen tissues. Intriguingly, expression of the gene xpr1that encodes an entry receptor expressed by xenotropic endogenous gammaretroviruses in several mouse strains, showed significant differential expression in the spleen of MDA5$^{+/-}$ relative to wt [293]. The tissue-specific variance, though quite low between pancreas and PLN tissues for key regulatory T cell and IFN-I pathway genes, does offer insight into specific transcriptional changes in the diabetogenesis process that result in MDA5$^{+/-}$ relative to wt mice following CB4 infection.

Table 4.1 The top 40 differentially expressed genes in the PLNs, pancreas and spleen of MDA5+/- mice relative to MDA5+/+ at day 3 post-CB4 infection. Common gene names and raw z-scores are shown.

PLN		Pancreas		Spleen	
gene	z-score	gene	z-score	gene	z-score
Lars2	-53.33962623	Amy2a5	-16.9621166	Lars2	-302.0216348
Amy2a5	32.48456001	Ctrb1	-14.56567233	Hbb-b1	138.4270229
Ctrb1	-30.65458147	Try4	12.56004695	Hba-a1	125.6637424
Amy2b	27.27781709	Clps	11.7030465	Hbb-bs	100.5696701
Cela2a	-25.10550837	Prss2	-12.28468396	Hba-a2	95.07624947
Cela3b	-23.55629995	Cela2a	-8.914478624	Amy2a5	-80.07701724
Glycam1	19.79617546	Cela1	-8.10240796	Hbb-b2	64.56348825
Try5	-18.82081975	Pnlip	-7.6808282	Hbb-bt	64.56348825
Hba-a1	-16.98981688	Try5	-6.475786813	Tmsb4x	57.27094794
Hba-a2	-16.42264532	Sycn	-5.514856627	Igj	52.89731312
Pnlip	12.91645229	Cela3b	-5.470698245	Vim	-47.96026226
Cpb1	12.75246536	Cpa1	4.364200951	Rps14	47.44841877
Hbb-b1	-12.86624792	Cpb1	4.739003523	Pnlip	-44.79988272
Ddx3y	-10.70775988	Zg16	4.430636117	Cd74	38.93857493
Ctrc	-10.95032391	Ctrl	-4.359253065	H2-Aa	38.51281038
Prss2	9.425275636	2210010C04Rik	4.205332299	Prss2	-37.94344201
Ctrl	-10.02963781	Ctrc	4.05321078	Rps27rt	37.89838184
Cel	9.126049018	Rnase1	3.870677371	2210010C04Rik	-35.81380367
Hbb-bs	-9.519675484	Cel	3.891161377	Rps27	35.76058719
Pnliprp1	8.632090536	Amy2b	3.844706038	B2m	35.04183718
Mir6236	-8.594397427	Pnliprp1	-3.910910936	Hsp90ab1	-35.02731942
Cpa2	7.911204086	Reg1	3.320244082	Ctrb1	-34.50041486
Sycn	-7.971330784	Cpa2	3.320597391	Cpb1	-34.36785766
Xist	7.37525376	Ins2	3.134728489	Lgals1	-33.42985402
Actn4	5.967160633	Klk1	3.128667129	Lyz2	32.93993275
Nfkbia	5.836260443	Pla2g1b	3.019623843	Psap	32.69032172
Eif5b	5.839768465	Hba-a1	3.129190337	Try4	-32.47533307
Cela1	-6.390267106	Spink3	2.959106559	Cpa1	-32.30292558
C3	5.543694735	Hbb-b1	2.529863351	Rps3	31.82211168
Hbb-b2	-5.617093185	Hba-a2	2.889891893	Cela1	-31.27038691
Hbb-bt	-5.617093185	Gp2	2.879234886	Try5	-30.45456237
Prkcb	5.44206909	Pnliprp2	-2.930014544	Igfbp5	-30.17000957
Igj	5.413517137	Tff2	-2.949074058	Hspa8	-29.98936069
Fabp4	5.267257039	Amy1	2.810565893	Snord22	-29.5771042
Ins2	5.164794105	Nupr1	-2.686114369	Cd52	28.77311455
Foxp1	5.019106529	Hbb-bs	-2.856001097	Rps15a	28.62861737
Cpa1	-5.645509106	Rn45s	2.530697312	Cela2a	-28.15873299
G3bp1	4.990160116	Rpl41	-2.620702068	Coch	27.6059868

Gp2	4.826412298	Rps14	-2.847066243	Cel	-27.22298341
Pfn1	-5.11179214	Mt1	2.325214019	Ptprc	27.13981923

Table 4.2 Differential expression of genes, manually curated, that are in involved in T-cell activation, IFN-I signaling and an endogenous retrovirus sequence following CB4 infection. Common gene names and absolute z-scores are shown. Significant differential expression of genes (z-score>=2) is highlighted.

	PLN	Pancreas	Spleen
CD40	0.433441583	0.399413867	3.837841437
CD86	0.126719192	0.311965455	8.424400764
CD80	0.110881623	0.197179155	1.142166726
CD44	0.23775637	0.62578804	7.072919123
CD62L	0.875222651	1.437153896	7.494372749
Foxp3	0.016785031	0.338539223	2.788043611
Nrp1	2.163761348	0.263467065	1.280939273
tgfb	0.559098333	0.453329983	1.768490995
il10	0.099036531	0.267327451	0.966345042
Ifng	0.282370732	0.231512364	0.683309132
il6	0.149697321	0.247268788	3.263351463
stat1	1.368249861	0.331850322	13.20822087
ifnar2	0.279935171	0.362263971	8.281630374
ifnar1	1.16673443	0.010790122	0.683169228
irf3	0.588612652	0.017434936	1.303097625
irf7	1.699399672	0.863952653	3.705978049
nfkb2	0.052843628	0.639381479	0.184612078
nfkb1	1.192166122	0.661600628	2.356803324
tlr3	0.673973287	0.009120773	4.24301293
ifih1	0.99817191	0.344700136	0.52799827
mavs	0.177340508	0.167717903	1.230565604
ifit1	2.290685454	1.171863235	4.05218804
ifit3	1.655485924	1.959653963	5.848789384
cxcr4	0.903890397	1.13288955	7.649062404
oas1a	0.25329741	0.371486564	4.064058254
isg15	1.586798871	1.118339751	4.520224974
mx1	0.234276537	0.661787855	0.703656161
ifi27	0.195869597	0.28242456	2.84727444
cxcl10	0.75267663	0.575869493	1.183794499
xpr1	0.594314446	0.270970932	3.299879497

To explore the transcriptional changes in MDA5$^{+/-}$ from a broader perspective and to focus more on relevant biological and immune functions impacted with a reduction in MDA5 we interrogated z-score filtered gene lists of each tissue, PLNs, pancreas and spleen, with IPA and Partek, restricting analysis of genes with a p-value greater than 0.001 as a second filter. Functional enrichment analysis from IPA and Partek for genes from each tissue were categorized according to over-represented gene ontology terms and assigned an enrichment score. A higher value of enrichment score indicated that the functional group was over-represented or significant in the gene list.

Looking at genes organized according to biological process we see the majority of differentially expressed genes between CB4 infected MDA5$^{+/-}$ and MDA5$^{+/+}$ at day 3 pi in the PLNs, pancreas and spleen with enrichment scores of 15.55, 4.33, and 5.5 respectively, are involved in metabolic processes (Figure 4.4). The enrichment of genes in metabolic processes compared to other biological processes is most striking in the PLNs, at the site of autoimmunity in T1D. This is suggestive already of an alteration in transcriptional expression between MDA5$^{+/-}$ and MDA5$^{+/+}$ following CB4 infection. It is also interesting to note from the biological process grouping that a small though representative portion (7.27%) of genes expressed in the spleen, and not pancreas or PLNs, are affiliated with cell death (Figure 4.4). Investigating further in to potential transcriptional changes in immune response genes we see tissue-specific changes in the expression of genes associated with T cell activation and signal transduction (Figure 4.5). Genes associated with T cell activation are more differentially expressed (enrichment score > 2) between MDA5$^{+/-}$ and wt in the PLNs (score of 8.9) and pancreas (score of 19) compared to the spleen, where genes associated with B cell activation are more differentially expressed (score of 18). There is also a slight change in the genes differentially expressed in the pancreas versus the PLNs with respect to lymphocyte proliferation and natural killer (NK) cell activation. These results lend support for a potential separate mechanism by which a reduction in MDA5 polarizes distinct adaptive responses in the diabetogenesis of T1D.

As we are comparing gene expression profiles following viral infection and in the context of a reduction in a critical virus sensor, we looked more closely at genes involved in virus signaling pathways. Importantly, we observe that differential gene expression between MDA5$^{+/-}$ and wt of genes expressed in intracellular receptor signaling pathways are most prominent in the PLNs and pancreas and not in the spleen. This supports the notion that intracellular receptor signaling, such as MDA5 signaling, influences immune responses during diabetogenesis in the NOD mouse and in response to CB4 infection. Tissue-specific changes between MDA5$^{+/-}$ and wt are also observed for genes in the IFN-I signaling pathway (Table 4.2, Figure 4.6). Predicted networks based on molecular relationships, interactions, and pathway associations were generated by IPA. Notably, expression of the interferon α/β receptor (IFNAR) is predicted to be downregulated (blue) in the PLN and pancreas, whereas it is predicted to be upregulated (orange) in the spleen. Direct interactions are marked by direct arrows, whereas dotted lines are indirect associations.

Differential expression of genes following CB4 infection in MDA5$^{+/-}$ relative to infected wt mice predict tissue specific differences in IFN-I signaling. Groups of genes expressed in PLNs, pancreas and spleen from CB4-infected MDA5$^{+/-}$ mice at day 3pi have been compared to expression of genes in MDA5$^{+/+}$ mice. Predicted networks are generated by IPA and are based on molecular relationships, interactions, and pathway associations. (Blue) is predicted to be downregulated, whereas (orange) represents genes potentially upregulated (Figure 4.6).

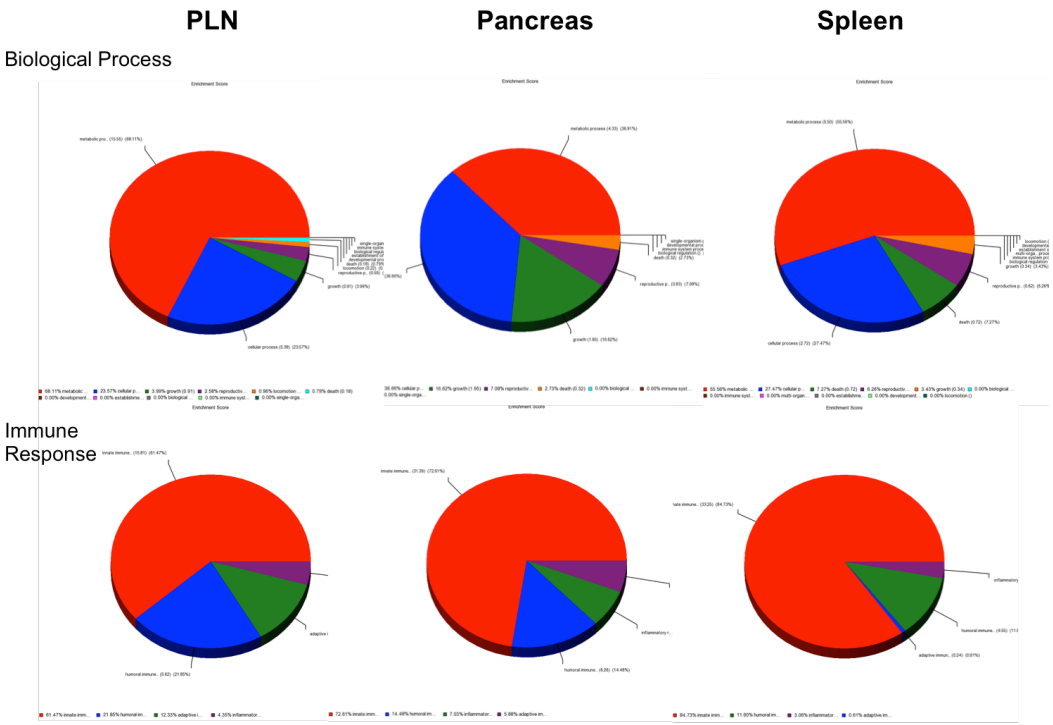


Figure 4.4 MDA5$^{+/-}$ have tissue-specific differential expression of genes in biological and immune response processes at day 3pi relative to wt mice after CB4 infection. A selection of genes was assigned to biologically meaningful gene ontology (GO) categories using GO enrichment analysis (Partek. Groups of genes of gene-associated biological and immune processes in PLNs, pancreas and spleen from CB4-infected MDA5$^{+/-}$ mice at day 3pi have been compared to expression of genes in MDA5$^{+/+}$ mice (fold change cut off is 2.0). **Pancreas**- **biological process**: metabolic process (red), cellular process (blue), growth (green), reproductive (purple), death (orange), **immune process**: adaptive (purple), inflammatory (green), innate immune (red), humoral immune (blue). **PLN biological process**: metabolic process (red), cellular process (blue), growth (green), reproductive (purple), locomotion (orange), death (turquoise). **Immune process:** adaptive (green), inflammatory (purple), innate immune (red), humoral immune (blue). **Spleen- biological process:** death (green), growth (orange), metabolic process (red), cellular process(blue), reproductive (purple). **Immune process:** adaptive (blue), humoral (green), inflammatory (purple), innate immune (red). *A high value of enrichment score indicates that the functional group is over-represented in the gene list.

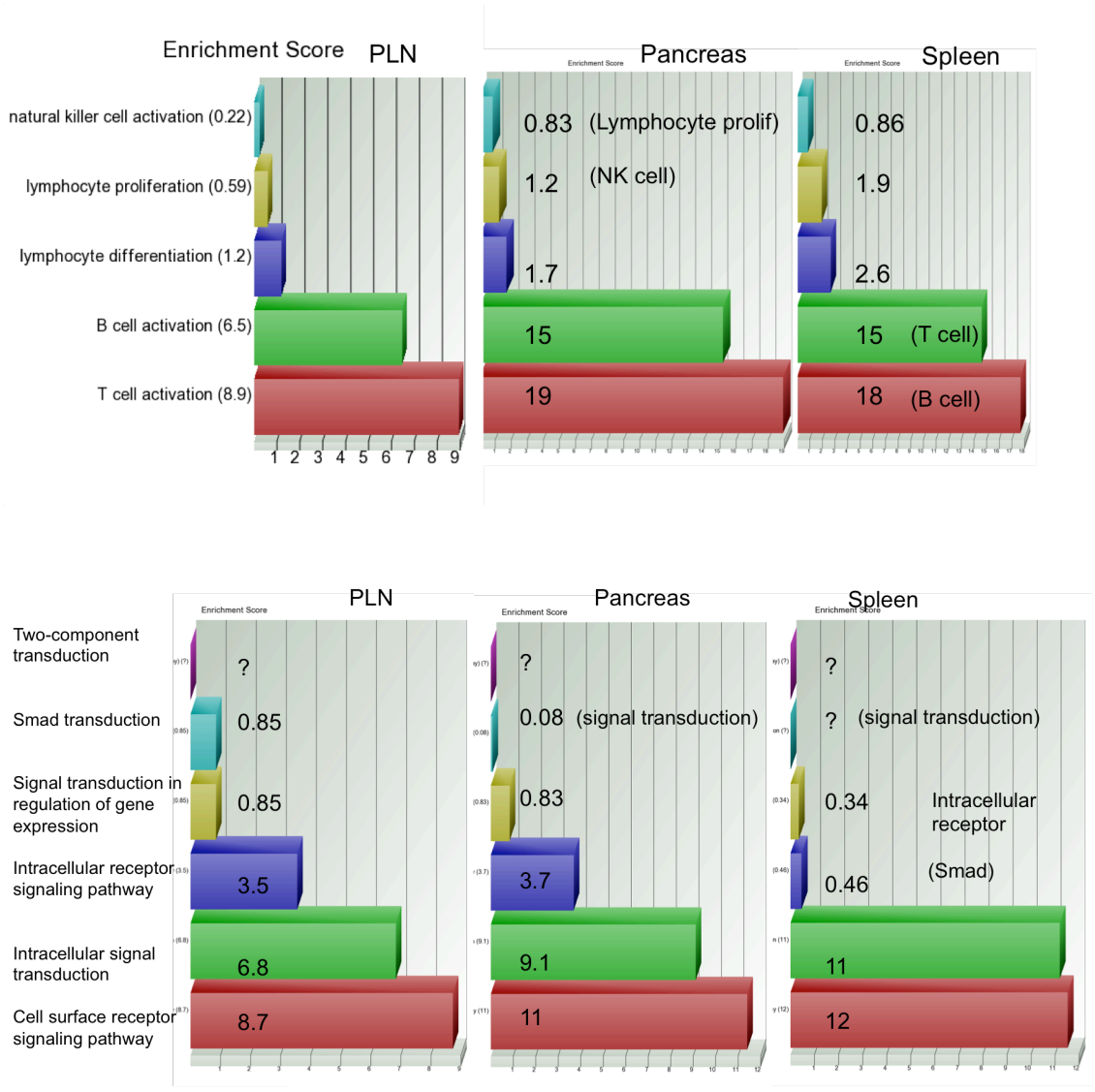


Figure 4.5 MDA5$^{+/-}$ have differential expression of genes involved in lymphocyte activation and signal transduction at day 3pi relative to wt mice after CB4 infection. A selection of genes was assigned to biologically meaningful gene ontology (GO) categories using GO enrichment analysis (Partek. Groups of genes of gene-associated biological and immune processes in PLNs, pancreas and spleen from CB4-infected MDA5$^{+/-}$ mice at day 3pi have been compared to expression of genes in MDA5$^{+/+}$ mice (fold change cut off is 2.0).

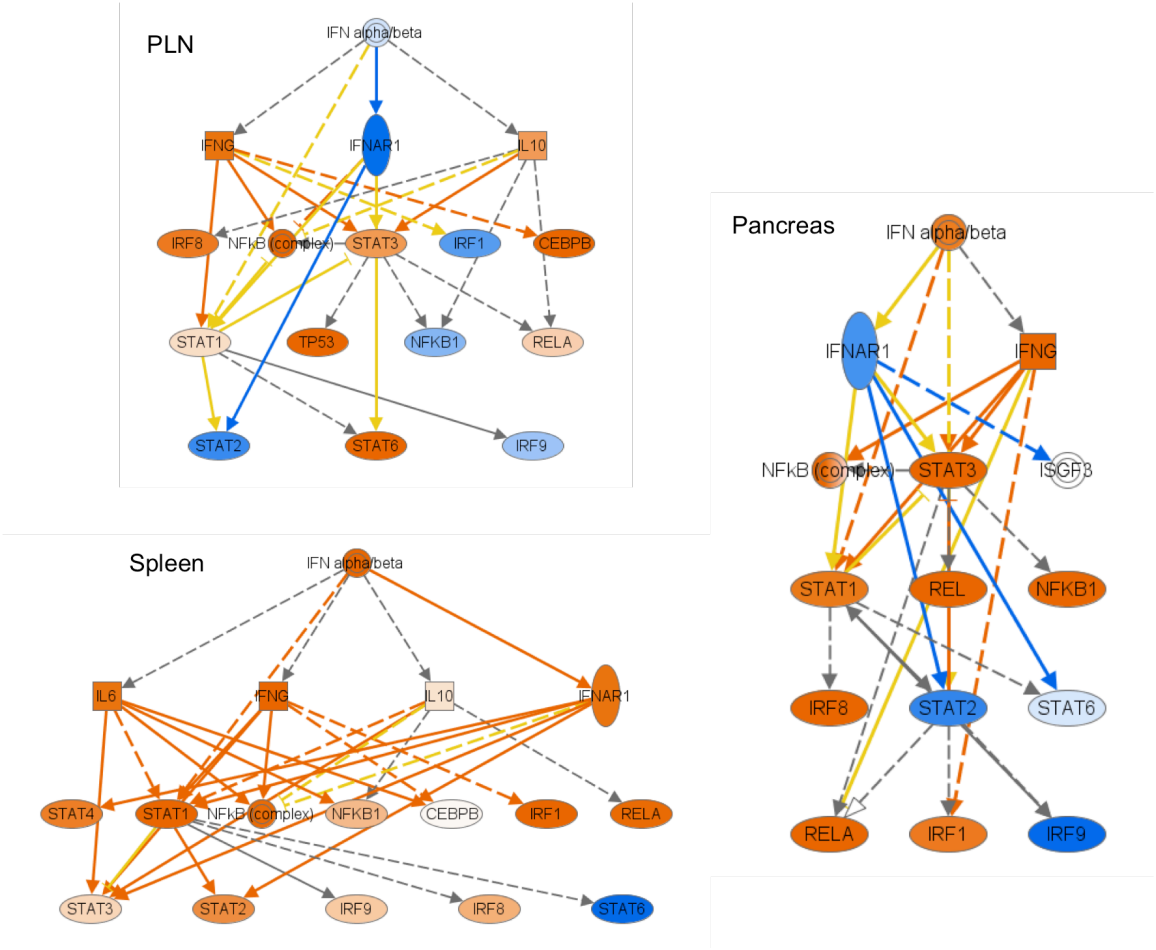


Figure 4.6 Differential expression of genes following CB4 infection in MDA5$^{+/-}$ relative to infected wt mice predict tissue specific differences in IFN-I signaling. Groups of genes expressed in PLNs, pancreas and spleen from CB4-infected MDA5$^{+/-}$ mice at day 3pi have been compared to expression of genes in MDA5$^{+/+}$ mice. Predicted networks are generated by IPA and are based on molecular relationships, interactions, and pathway associations. (Blue) is predicted to be downregulated, whereas (orange) represents genes potentially upregulated. Direct interactions are marked by direct arrows, whereas dotted lines are indirect associations.

The networks predicted by IPA also show that in the PLN, genes that are expressed and involved in IFN α/β expression are indirectly influenced by the expression of IL-10 and IFN-γ (dotted lines) and directly (solid lines) by IFNAR1 expression, whereas in the pancreas just IFN-γ (indirectly) and IFNAR1 (directly) and in the spleen, IL-6 is included indirectly in addition to IL-10 and IFN-γ. In all tissues, IFN α/β expression correlates indirectly with STAT1, and in the pancreas also with STAT3. The difference, though subtle, between gene expression associations in the tissues represented by IPA networks,

further suggests that a reduction in MDA5 alters IFN-I signaling following CB4 infection.

4.4 Discussion

We have previously shown that a reduction in MDA5 expression in NOD mice protects from spontaneous and coxsackievirus B4-induced autoimmune diabetes and induces specific IFN-I and T cell responses following CB4 infection that likely determine disease fate (**see section 2 and 3**). The unique IFN-I signature is characterized by an increase in IFN-β expression in the pancreas and IFN-β production in the serum at day 3 post-infection (pi) that drops significantly by day 7 pi in MDA5$^{+/-}$ mice compared to infect wt mice. Using high throughput 3' RNA sequencing, we have now also described a unique transcriptome signature in CB4-infected MDA5$^{+/-}$ mice relative to infected wt mice at day 3pi.

Gene ontology analysis shows activity expected in the pancreas and spleen tissues following CB4 infection such as higher innate immunity and adaptive responses. There are however, tissue-specific differences in functional grouping of genes that are differentially expressed in MDA5$^{+/-}$ mice relative to infected wt, according to the type of adaptive response (Figure 4.5). In the spleen, the top ranking genes were grouped most prominently in B cell activation function, whereas in the PLN and pancreas, genes grouped in to the T cell activation functional group. This may reflect the ensuing activation of Teff in the PLNs and pancreas directed at clearing viral infection, as day 3pi is typically when CB4 replication in the pancreas peaks. This may also allude to the activation of more Tregs at the site of infection and autoimmunity to control the activation of autoreactive T cells, which could explain why a significant decrease in Teffs is seen in the PLNs of CB4 infected MDA5$^{+/-}$ (Figure 4.1). It is curious though that differential Foxp3 expression was not significant in the PLNs or pancreas, but rather in the spleen. Deeper sequencing and functional studies are required to determine whether the differential expression we have observed for the genes thus far is associated with a reduction or upregulation of these genes in the MDA5$^{+/-}$ relative to the wt. Based on raw and not absolute z-scores for differential expression of genes in the spleen, though

representative of n=1 for each genotype, we can make a preliminary inference that genes associated with anti-viral immunity are downregulated and genes associated with a Treg response are upregulated in MDA5$^{+/-}$ relative to MDA5$^{+/+}$ as a part of a mechanism to control anti-viral immunity leading to the control of autoimmunity in the pancreas and PLN tissues. The downregulation of IL-6 and upregulation of Foxp3 and CD86 are in line with previous observations, where IL-6 has demonstrated suppressive capacity on the expression of Foxp3 and the development of Foxp3+ Tregs [294, 295]. Conversely, in coxsackievirus induced autoimmune myocarditis, IL-6 signalling is required in the early phase of virus infection to avert chronic autoimmune disease[296]. Nonetheless, the differential expression of Foxp3, CD86 and IL-6 genes in the spleen, though preliminary observations, may suggest a mechanism elicited with a reduction in MDA5 signaling to subside disease progression.

It has been shown that autoimmune diabetes induced by CB4 results from a bystander mechanism where previously sequestered antigens are presented to autoreactive T cells and in the context of an inflammatory environment. As such, modifications to the inflammatory milieu have important repercussions on autoreactive responses leading to T1D following CB4 infection. The differential expression of effector T cell activation markers CD44 and CD62L is also significant in the spleen, pointing as well to alterations in the adaptive responses ensued at day 3 pi in MDA5$^{+/-}$ compared to wt mice. The differential expression we observe in the PLNs versus the pancreas and spleen may suggest a mechanism, guided by the differential expression of other inflammatory mediators as a result of a reduction in MDA5, for the eventual accumulation of Tregs in the PLNs that we have previously observed in MDA5$^{+/-}$ at day 7pi.

Innate immune responses, specifically intracellular signaling of viruses, are important to the development of effective antiviral adaptive responses and also to the polarization of an autoreactive response. It is interesting that we observe grouping of genes in the intracellular signaling functional category in the PLN and pancreas and not in the spleen (Figure 4.5). This suggests that a reduction in MDA5 induces tissue-specific differential signaling in response to CB4 infection and relative to infected wt mice. Variance in

intracellular signaling was also observed using IPA and predicting networks of genes differentially expressed in MDA5$^{+/-}$ relative to wt (Figure 4.6), where tissue-specific relations between genes in the IFN-I pathway were observed. Predicted networks generated by IPA are based on molecular relationships, interactions, and pathway associations and as such, can offer a general scope of the molecular changes occurring at a given time in a sample. The variation, though subtle, in the IFN-I pathway networks predicted for the PLNs, pancreas, and spleen suggests that a reduction in MDA5 induces tissue specific IFN-I responses after CB4 infection, where the IFNAR is primarily affected and directly associated with the expression of IFNαβ genes. Importantly, the IFNAR1 is predicted to be downregulated in the PLNs and pancreas and upregulated in the spleen with absolute and raw z-scores for the *ifnar1* gene corroborating this prediction.

Lastly, it was surprising to observe that the gene *xpr1*, encoding the entry receptor for xenotropic endogenous gammaretroviruses, had significant differential expression in the spleen of MDA5$^{+/-}$ relative to wt mice following CB4 infection. This suggested that a reduction in MDA5 and/or CB4 infection stimulated the expression of the *xpr1* gene. Several inbred lab mice including the NOD mouse carry endogenous retrovirus elements, most commonly the xpr1gene that encodes a receptor specific for xenotropic endogenous gammaretroviruses. Endogenous retroelements make up a substantial portion of human genomes and as such, can provide an important source of endogenous nucleic acid that can be sensed by nucleic acid sensors like MDA5 and induce aberrant immune responses leading to autoimmune disease[297]. Interestingly, the insertion of the retroviral HERV-K in the 9th intron of the complement component C4 gene has been demonstrated to contribute to functional protection and not exacerbation of type 1 diabetes[298]. Nonetheless, understanding the role of retroelements and mechanisms such as MDA5 signaling that can influence the sensing of their genomes has important implications in understanding the events leading to and progressing autoimmune disease including T1D.

In comparing the transcriptional profile of MDA5$^{+/-}$ to MDA5$^{+/+}$ following infection with CB4 we have demonstrated unique tissue-specific transcriptional signatures. Our results

suggest that there are distinct immune responses and immune cell interactions conferred by MDA5 signaling following virus infection that are important in determining the outcome of T1D pathogenesis.

Chapter 5: Discussion, future directions and conclusions.

5.1 Discussion

Since the discovery of insulin by Canadians Frederick Banting and Charles Best in 1921[11] insulin has been the primary and most sustainable therapy for the treatment of type 1 diabetes. The autoimmune destruction of β-cells in the pancreas eliminates an individual's ability to produce insulin and regulate blood glucose levels. Most people must take about 1,450 insulin injections a year. Without Banting and Best's discovery of insulin, patients would be completely helpless in treating their disease. And fortunately, over the course of almost a century, science and technology have generated incredible advances in the manufacturing and production of insulin and in personal glucose management with the delivery of insulin through wearable insulin pumps. 'Hands-free' glucose monitoring is now a reality, with the development of a 'Bionic Pancreas' device that uses a subcutaneous removable sensor that automatically monitors glucose levels and outputs insulin or glucagon as required with two automatic pumps[299]. Now, researchers are advancing the field, investigating and devising strategies to circumvent the need for continuous insulin injections and prevent disease altogether. Many therapeutic prospects have been endeavored including surgical islet and immune cell transplant strategies, personalized and regenerative β-cell therapy and immunotherapy injections of regulatory T cells or immunomodulatory cytokines that restore tolerance and help regenerate β-cell function[300-302].

The insecurity in successfully protecting or treating T1D lies in the complexity of the disease, where little is known about the events leading to the onset of disease. Specific genes and environmental factors, like enterovirus infections, and combinations thereof have been implicated in T1D risk, driving disease susceptibility, yet distinct targets for some golden vaccine or cure remain unclear. Accumulating evidence points to a role for

PRR sensing of viruses and the functional consequences that contribute to the development of autoimmune disease. Until recent years, very little was known of the role of MDA5 in autoimmune diabetes. A common non-synonymous SNP (nsSNP) (rs1990760) and several rare variants in the MDA5 gene *IFIH1* that lead to a reduction in *IFIH1* transcripts and associate with protection from T1D have been identified. The rare alleles of all associated MDA5 polymorphisms consistently protect from T1D, while MDA5 alleles carried by the majority of the population predispose to the disease. This observation suggests strong signaling through MDA5 is an important mediator of autoimmune diabetes[71, 72]. Functional studies have further supported a role for IFN-I signalling and in particular MDA5-mediated IFN-I signalling in the development of autoimmunity emphasizing the important interest in the immunological consequences of MDA5 function. Herein, we further substantiate a position for MDA5 in T1D susceptibility. Specifically, this research has demonstrated a significant role for MDA5 in the development of diabetes in the NOD mouse and following infection with CB4, a virus strongly linked to T1D.

5.1.1 A loss in MDA5 induces a specific IFN-I signature that leads to the polarization of regulatory T cell responses and protection from T1D.

In Chapter 2, we hypothesized that as patient polymorphisms in MDA-5 retain some level of protein function, a reduction in MDA5 expression, as we have translated onto the NOD mouse, alters IFN-I signaling after CB4 infection in a manner that controls and clears virus and mediates the strength, polarization and regulation of T cell responses in favor of T1D protection. We believe there is a direct correlation between the IFN-I signature induced by MDA5 following environmental challenge with the induction of a strong effector T cell (Teff) and a matched regulatory T cell (Treg) response. In line with this, our findings demonstrated that MDA5 sensing acts as an essential regulator of the diabetogenic T cell response.

We observed that partial loss of MDA5 expression created a unique IFN-I signature, where a burst of IFN I is induced early post-infection at day 3, that likely helps to clear viral infection, and returns to a reduced level as the infection is cleared. This unique IFN-I signature was also likely responsible for preventing the triggering of autoimmunity.

MDA5 signaling of IFN-I acts over the course of infection in a tissue specific and kinetic manner with another RNA sensor, TLR3, to develop a unique pathogen-specific signature[122]. As such, it is possible that TLR3 signaling compensates for a lack in MDA5 signaling in order to control virus replication and that this compensation in IFN-I signaling in specific tissues contributed to the unique IFN-I signature we observed in $MDA5^{+/-}$ following CB4 infection. In line with this, we observed tissue-specific TLR3 and MDA5 expression after CB4 infection in $MDA5^{+/-}$ mice and a shift in the IFN-I response in NOD mice heterozygous for TLR3 ($TLR^{+/-}$) from what we observe in $MDA5^{+/-}$ at day 3pi. Additionally, in Chapter 3, we demonstrated that $TLR^{+/-}$ mice infected with CB4 do not retain the ability to polarize Tregs rather than Teffs in the PLNs at day 7 pi as we have observed for $MDA5^{+/-}$. This suggests, together with the tissue-specific expression of TLR3 in the pancreas of $MDA5^{+/-}$ at day 3pi, that TLR3 signaling is important for anti-viral responses to CB4, and rather MDA5 signaling is important for managing autoreactive T cell responses. This work further substantiated that a loss in MDA5 expression induced specific IFN-I signaling that allowed for adaptive T cells responses in favor of protection from T1D. Whether the loss in MDA5 induced tissue specific TLR3 and MDA5 signaling kinetics from day 3 to 7 pi remains unclear and should be further investigated with IFN-I and receptor expression assessed at specific time points.

In the case of the pancreatropic virus EMCV, the loss of one MDA5 allele, though on the T1D resistant C57BL/6 mouse background, resulted in transient hyperglycemia due to direct killing of pancreatic beta cells and did not protect from EMCV- induced diabetes[122]. Our work has demonstrated the importance of MDA5 IFN-I signaling post-infection in diabetes susceptible NOD mice that protects from the establishment of T1D through a change in the polarization of the T cell response to increase regulation of the autoimmune response. The host response is not significantly diminished in its ability to clear virus infection as CB4 does not replicate out of control and directly kill the pancreatic beta cells. Rather CB4 mimics clinical diabetes onset with the presentation of self-antigen through resident CB4 infected cells in the pancreas [118]. Altering MDA5 sensing simply regulates the autoreactive component of the host response to infection.

We have also demonstrated in Chapter 2 $CD11b^+$ $CD11c^+$ cells (containing macrophages and monocytes) from $MDA5^{+/-}$ (and not from $MDA5^{+/+}$ donors) induce Tregs and maintain lower levels of IFN I post-infection suggesting that $CD11b^+$ $CD11c^+$ cells are responsible for driving the unique IFN I signature observed in $MDA5^{+/-}$ mice that protects from T1D. Early intervention of plasmacytoid DC (pDC) function has been recently reported to prevent autoimmune pathologies in an autoimmune lupus model[248]. Further, children at risk for T1D have shown a unique IFN-I transcriptional signature that precedes islet autoimmunity and recent onset of T1D has been strongly associated with infection by RNA viruses like coxsackievirus and an increase in pDCs and IFN-α production, especially following CB4 infection [92, 99, 169, 171, 235]. These studies and our work suggest an important role for natural IFN I- producing cells like pDCs and their contribution, in response to environmental T1D-associated IFN I stimulators like CB4, to an early IFN I signature in the development of autoimmunity.

Our functional and mechanistic studies in Chapter 3 helped identify a unique IFN-I signature limited to MDA5 that does not waver in the presence of sustained IFN-I stimulation and that does not result with reduction in another RNA sensing molecule (TLR3) or T1D-associated receptor (VDR) following CB4 infection. We also demonstrate that disease pathogenesis is linked to the specific IFN-I response induced by the virus as a strain of CB3 failed to modify the IFN-I signature associated with disease. To explore the mechanism responsible for the protective phenotype in $MDA5^{+/-}$ further, we then asked whether IFN-I signaling from APCs was sufficient to induce T cell polarization. $NODIFNAR^{+/+}$ and MDA5 deficient mice ($MDA5^{-/-}$) failed to respond when APCs were transferred from NOD mice deficient in type 1 IFN receptors ($NOD.IFNAR1^{-/-}$). Recipients had lower Tregs and increased Teffs in both the PLNs and spleen. This is contrary to what we observe with APCs adoptively transferred from $MDA5^{+/-}$ that have induced Tregs in $MDA5^{+/+}$ recipients. These observations along with our other results discussed in Chapter 3 suggest that MDA5 IFN-I signaling is essential in regulating the IFN-I signature that mediates T cell responses following virus infection and determines disease fate.

Based on our work from discussed in Chapters 2 and 3 demonstrating a distinct IFN-I expression signature that ultimately leads to adaptive T cell responses in favor of protection from T1D in MDA5$^{+/-}$ following CB4 infection [81], we hypothesized in Chapter 4 that a reduction in MDA5 induces inflammatory changes in the NOD mouse at the transcriptome level and that inflammatory events marked by transcriptional signatures following CB4 infection in MDA5$^{+/-}$ could be traced using high throughput sequencing methods. The transcriptional and cellular landscape of uninfected NOD mice has been examined using micro array and RNAseq methods and with these methods, distinct and chronological phases of spontaneous T1D progression have been described[288]. Our investigation of transcriptional changes in MDA5$^{+/-}$ at day 3 pi also revealed distinct differential expression on a broader scale in genes that grouped to biological and immune response pathways and more specifically, differential expression was observed for genes in the IFN-I and T cell activation pathway. The changes in gene expression in MDA5$^{+/-}$ relative to wt mice were also tissue-specific, with the differential expression for particular genes involved in IFN-I signaling and T cell activation significant in either the PLNs, pancreas, or spleen or combinations thereof. This preliminary transcriptome investigation provided insight into potentially significant expression events in the diabetogenesis of T1D in the NOD mouse following infection with a clinically relevant IFN-I inducer.

Within an autoimmune prone host, amplified inflammatory responses from viral sensing can be detrimental and instigate autoimmune disease. Finding a threshold between pathogen defense and protection from autoimmune disease relies on a balance in viral sensing and peripheral tolerance. As such, this research has aimed to determine how MDA-5 acts as a susceptibility factor in spontaneous T1D and CB4-mediated T1D. Understanding how MDA5 controls anti-viral signaling and consequently T1D pathogenesis is critical in defining disease progression and developing prophylactic and therapeutic measures.

5.2 Future directions

Our first directive was to test the link between protective polymorphisms identified in the human population that result in the reduction of an innate receptor and the susceptibility to T1D following viral infection. We designed experiments in Chapter 2 to test the hypothesis that a reduction in MDA5 expression, as with patients carrying protective *IFIH1* polymorphisms, protects from spontaneous and CB4-induced T1D.

In translating a reduction in MDA5 expression, as seen with protective polymorphisms in the *IFIH1* gene, on to the non-obese diabetic (NOD) mouse we have observed protection from spontaneous and coxsackievirus B4 (CB4)-induced T1D. Our research described in Chapter 2 is the first characterization of a role for MDA5 in the development of diabetes in a clinically relevant model. The generation of $MDA5^{+/-}$ on to the NOD background provided the most suitable model to examine the contribution of MDA5 to the development of spontaneous T1D and in the context of viral-mediated diabetes [119, 120]. Previous work from our lab has shown that infection of 10 week old NOD mice with 400 plaque forming units (pfu) of CB4 (strain Edwards 2) results in a significant increase in T1D compared to an age-matched uninfected control group and demonstrates kinetics of disease development consistent with a mechanism involving an activated memory T cell response[119]. The accelerated induction of T1D in NOD mice provides a clinically relevant model of human disease as it takes into account both genetic and environmental influences.

As such, our research with NOD $MDA5^{+/-}$ could be extended further using other NOD models harboring a reduction in MDA5. The generation of NOD mice expressing mutated copies of MDA5 that correspond to protective risk variants identified in the *IFIH1* gene of patients, could also be used to study the role of MDA5 expression in CB4-mediated T1D. Knock-in (KI) NOD mouse models [303] could be generated, where wild-type MDA5 alleles could be replaced with a mutated version corresponding to the protective nsSNP (rs1990760) in humans[71]. Several rare polymorphisms that confer protection [72] and result in decreased MDA5 function[73] have been identified in the *IFIH1* gene. The study of these polymorphisms in mice will be feasible as the respective

residues for mutagenesis are conserved in humans and mice. Additionally, germline-competent embryonic stem cells derived from NOD mice are readily available, allowing the generation of the KI models on the NOD genetic background and avoiding the need for several lines of backcrossing.

Viruses, including the paramyxoviruses, find specific means to inhibit host immune responses such as the IFN-I responses. In fact, 13 different paramyxoviruses have been demonstrated to use their V protein, encoded by the viral P/V/C gene, to target and inhibit MDA5[144]. The cellular interaction between the viral V protein and MDA5 results in the inhibition of MDA5 and subsequent IFN-I induction. Interestingly, V proteins specifically target the MDA5 helicase domain, interfering with dsRNA binding, MDA5 activation and subsequent oligomerization of MDA5 helical domains[145]. The V protein from parainfluenza virus 5 (PIV5; previously known as SV5) has been well characterized in MDA5 inhibition studies and as such, could be used for further work investigating a reduction in MDA5 signaling.

Besides genetic mutagenesis and the generation of KI models, MDA5 function could be impaired using protein inhibitors and RNA interference. Specifically, MDA5 function could be inhibited with the V protein of parainfluenza virus 5 (PIV5) and RNA interference using MDA5 specific RNAi. Both the V protein from PIV5 and siRNA directed against MDA5 have shown to successfully block MDA5 activity [144, 145]. To assist in the delivery of PIV5 V protein and MDA5 specific siRNA, independent recombinant adenovirus-associated expression vectors could be generated.

Modified double stranded adenovirus-associated vectors (dsAAV8) have been developed to specifically deliver proeins in the pancreatic islet β-cells. The dsAAV vector features a mouse insulin-II promoter that restricts gene transfer to islet β-cells allowing specific delivery of proteins within the islets. *In vivo* delivery of β-cell growth factors glucagon like peptide-1 (GLP-1) and the NK1 fragment of hepatocyte growth factor (HGF/NK1) to pancreatic β-cells has been successfully demonstrated with the dsAAV8 vector in db/db mice [304]The dsAAV8 vector is an ideal tool for studies in the NOD mouse as the

vector does not interfere with pancreatic function, where no alterations in serum amylase or blood glucose levels have been observed and the vector explicitly targets β-cells for transgene expression without causing significant toxicity or immunogenicity[304]. The dsAAV8 platform could be used as an alternative to directly injecting MDA5 inhibiting agents, where issues with tissue bioavailability, turnover and immunogenicity may arise.

As accumulating evidence points to the involvement of viral infections and IFN-I responses in T1D pathogenesis, studies in T1D therapeutic strategies, such as the work we have done using the CB4-induced model, must be designed to consider and replicate both the genetic and viral influence on disease.

Though the specific events leading to autoimmunity in T1D remain unclear, the research presented here advances our understanding of a critical component of innate immune responses that affects adaptive responses and ultimately T1D development. Our demonstration in Chapters 2 and 3 of a unique IFN-I signature that results specifically from a reduction in MDA5 and not TLR3 in NOD mice, following CB4 and not CB3 infection, suggests that MDA5 signaling is essential in regulating the IFN-I signature that mediates T cell responses following virus infection and determines disease fate.

In chapter 3, we explored whether disease pathogenesis and immununological phenotypes such as the unique type 1 interferon signature observed in MDA5$^{+/-}$ were specific to a reduction in MDA5 signaling, if they could sustain under circumstances of increased IFN-I stimulation and whether the unique IFN-I and T cell responses observed in MDA5$^{+/-}$ were specific to CB4 infection. We demonstrate in Chapter 3 that despite challenge from two IFN-I inducers (CB4 and poly i:c), MDA5$^{+/-}$mice and not MDA5$^{+/+}$ mice maintain a regulatory protective phenotype we described in Chapter 1.

To determine whether a unique IFN-I signature can also be produced with interference in signaling at another point of the process, future studies could investigate the immunological consequences and affects on T1D susceptibility with a reduction in other MDA5 signaling pathway molecules. There are several molecules that are integral to the

signaling of IFN-I from MDA5. Those that have been more extensively investigated for their role in IFN-I signaling include the adaptor protein MAVS (IPS-1) and the transcription factors IRF3 and IRF7. MAVS expression has been shown to have a significant role in IFN-β production and controlling virus titers following rotavirus infection[305]. With lipopolysaccharide (LPS) stimulation of TLR4 signaling, IRF3 and NF-κB become activated and IFN-β is the only IFN-I produced [153]. Further, it has recently been demonstrated that following RNA virus infection, signaling from MDA5 and RIG-I leads to the activation of IRF3 and the downstream expression of IFN-β and the downregulation of Smad signaling in the TGF-β signaling pathway that consequently inhibits TGF-β responses including TGF-β-induced Treg cell differentiation. A loss in IRF3 expression rescued TGF-β signaling and allowed TGF-β induction of Tregs even following virus infection[235]. This suggests that absolving IFN-I signaling with the loss of an MDA5 signaling pathway molecule, IRF3, can alter T cell polarity in favor of a regulatory T cell response albeit at the sacrifice of antiviral responses.

The kinetics and cell-specific expression of IRF3 and IRF7, where IRF3 is constitutively produced across many cell types and IRF7 is modestly expressed mainly in immune cells and specifically pDCs, may also play a role in altering the IFN-signature after viral infection. Both transcription factors offer induction of IFN-I, however they differ in the type and timing of IFN-I produced. Activation of IRF3 causes IFN-β and IFN- α1 (IFN-α4 in mice) to be produced, establishing a low level stimulation of the IFNAR and priming of IFN-I responses, whereas after virus infection, IRF3 gets degraded and activation of IRF7 rapidly induces the production of many IFN-α genes within pDCs that can then activate virus-specific adaptive responses[306]. Intriguingly, IFN-α subtypes and IFN-β have distinct IFNAR binding affinity and have demonstrated individual immunomodulatory functions under different stimulatory conditions. The specificities in the type and kinetics of IFN-I responses that are mediated by IRF3 and IRF7 and produced after viral infection emphasize their essential and non-redundant role in shaping the IFN-I signature. The unique IFN-I signature we observe with a reduction in MDA5 and following CB4 infection could therefore, also result from interruption in the activation of downstream molecules, and merits further investigation. Future studies that

address the potential role of MDA5 signaling pathway molecules in CB4-mediated T1D could extend our understanding and offer new insight in to the contribution of the IFN-I signature in viral-mediated T1D and could offer new targets for the design of T1D therapeutic strategies.

Despite epidemiological, clinical and animal studies that have strongly linked CB4 to T1D to in patients, CB4 may only be responsible for disease pathogenesis in a subset of new onset cases. Other viruses such as the enterovirus coxsackievirus B 1 (CB1) and rotaviruses have more recently been implicated [87]. Since MDA5 recognizes dsRNA from enteroviruses and rotaviruses it would be worth pursuing in other studies, the immunological consequences of these other T1D-associated viruses in the context of a reduction in MDA5.

In chapter 4 we characterized the type 1 interferon transcriptional landscape associated with a reduction in MDA5 following CB4 infection. With RNAseq technology, we established a transcriptome phenotype at day 3 pi in MDA5$^{+/-}$ PLNs, spleen and pancreas that helped identify potential changes in IFN-I and T cell activation associated genes that supported our molecular and cell data. As our initial RNA samples were modestly degraded following CB4 infection and due to costs, the number of samples analyzed for the sequencing were few. Further work using supplemental purification methods to maintain the integrity and purity of the RNA and the use of more replicates in sequencing will enhance the reliability of the transcriptome analysis. It has been shown that in RNA-seq differential studies, sequencing less reads and performing more biological replication, increases accuracy in subsequent analysis[307]. Deeper sequencing and functional studies with qPCR are required to determine whether the differential expression we have observed for the genes thus far is associated with a reduction or upregulation of these genes in the MDA5$^{+/-}$ relative to the wt.

Deep sequencing and the use of bioinformatics and computational tools like R programming will enable the identification of potential SNPs and specific transcriptional

changes that are induced following CB4 infection and with a reduction in MDA5. As costs of sequencing limited the number of samples we were able to analyze, we did not compare the infected mice to uninfected mice. This is an important control as it will help distinguish transcriptional signatures that are a result of CB4 infection and which signatures are transcribed as a result of a reduction in MDA5. Although the transcriptome involved in the spontaneous diabetogensis of uninfected NOD mice has been previously described, comparing transcriptional profiles observed following CB4 infection in NOD to uninfected age-matched controls residing in the same vivarium facility would eliminate other potential factors like the gut microbiome and other potential exposures to environmental factors that are specific to the housing facility and could influence immune responses following coxsackievirus challenge[308, 309]. As such, it would be interesting to extend studies of the role of MDA5 in diabetogenesis and coxsackievirus signaling to high throughput sequencing and functional analysis of the gut microbiome in MDA5$^{+/-}$ and wt mice.

Lastly, it was surprising to observe that the gene xpr1, encoding the entry receptor for xenotropic endogenous gammaretroviruses, had significant differential expression in the spleen of MDA5$^{+/-}$ relative to wt mice following CB4 infection. Endogenous retroelements make up a substantial portion of human genomes and as such, can provide an important source of endogenous nucleic acids that can be sensed by nucleic acid sensors like MDA5 and induce aberrant immune responses leading to autoimmune disease[297]. Low-level IFN-I signaling caused by the stimulation of RNA sensors like MDA5 induced by the emergence of endogenous retrovirus elements could be an underlying inflammatory factor that increases susceptibility to autoimmunity [91] and as such, merits the investigation of a potential role of endogenous retroviruses in the context of MDA5 signaling and T1D.

The information gained from this research has strengthened our understanding of the immunological consequences of viral infection and the induction of T1D in the context of reduced viral sensing. Further, this work has enhanced our understanding of the immunological requirements for the induction of autoimmunity following viral infection.

The results from this research can likely extend mechanistically to other autoimmune diseases for which viral infections are strongly tied to susceptibility and provides a foundation for a more rational approach in the design of therapeutics for the prevention and treatment of autoimmunity.

5.3 Conclusions

We have shown here that a reduction in MDA5 creates a unique IFN-I signature and leads to the development of a regulatory rather than an effector T cell response following infection with a T1D-inducing RNA virus in a T1D susceptible model. We have further demonstrated that a loss in MDA5 retains a unique IFN-I signature and regulatory T cell response despite IFN-I stimulation from viral and synthetic RNA mimetic inducers and that this unique phenotype is specific to the virus infection and is not maintained with a loss in another RNA sensor, TLR3, or T1D-associated factor, VDR. Our identification of a specific IFN-I signature as a result of reduced (not eliminated) MDA5 sensing of CB4 substantiates the importance of IFN-I signaling in the development of T1D. Using RNAseq we have ascribed for the first time, the transcriptional effects of a reduction in MDA5 on NOD mouse IFN-I responses after CB4 infection.

Identifying susceptibility factors and their functional consequences on disease development has opened the door for further studies that pursue these susceptibility factors as targets for T1D therapeutic intervention. As such, the research presented here provides evidence to support a role for MDA5 in T1D susceptibility and as a potential target for T1D therapeutic intervention.

Bibliography

1. Pandiri, A.R., *Overview of exocrine pancreatic pathobiology.* Toxicol Pathol, 2014. **42**(1): p. 207-16.
2. Kim, A., et al., *Islet architecture: A comparative study.* Islets, 2009. **1**(2): p. 129-36.
3. Rodriguez-Calvo, T., et al., *Increased immune cell infiltration of the exocrine pancreas: a possible contribution to the pathogenesis of type 1 diabetes.* Diabetes, 2014. **63**(11): p. 3880-90.
4. El-Gohary, Y., et al., *Three-dimensional analysis of the islet vasculature.* Anat Rec (Hoboken), 2012. **295**(9): p. 1473-81.
5. Hellman, B., *The frequency distribution of the number and volume of the islets Langerhans in man. I. Studies on non-diabetic adults.* Acta Soc Med Ups, 1959. **64**: p. 432-60.
6. Cabrera, O., et al., *The unique cytoarchitecture of human pancreatic islets has implications for islet cell function.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2334-9.
7. Bosco, D., et al., *Unique arrangement of alpha- and beta-cells in human islets of Langerhans.* Diabetes, 2010. **59**(5): p. 1202-10.
8. Rodriguez-Diaz, R., et al., *Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans.* Nat Med, 2011. **17**(7): p. 888-92.
9. Leibiger, I.B., B. Leibiger, and P.O. Berggren, *Insulin signaling in the pancreatic beta-cell.* Annu Rev Nutr, 2008. **28**: p. 233-51.
10. Rorsman, P. and M. Braun, *Regulation of insulin secretion in human pancreatic islets.* Annu Rev Physiol, 2013. **75**: p. 155-79.
11. Banting, F.G. and C.H. Best, *The internal secretion of the pancreas*. University of Toronto studies Physiological series,. 1922, Toronto: The University Library: pub. by the librarian. 16 p.
12. Dean, P.M. and E.K. Matthews, *Electrical activity in pancreatic islet cells.* Nature, 1968. **219**(5152): p. 389-90.
13. Menting, J.G., et al., *How insulin engages its primary binding site on the insulin receptor.* Nature, 2013. **493**(7431): p. 241-5.
14. Aronoff, S.e.a., *Glucose metabolism and regulation: beyond insulin and glucagon.* Diabetes Spectrum, 2004. **17**: p. 183-190.
15. Unger, R.H., *Glucagon physiology and pathophysiology.* N Engl J Med, 1971. **285**(8): p. 443-9.
16. Cho, Y.M., Y. Fujita, and T.J. Kieffer, *Glucagon-like peptide-1: glucose homeostasis and beyond.* Annu Rev Physiol, 2014. **76**: p. 535-59.
17. Hayes, M.R., et al., *Incretins and amylin: neuroendocrine communication between the gut, pancreas, and brain in control of food intake and blood glucose.* Annu Rev Nutr, 2014. **34**: p. 237-60.
18. Kowluru, A., *Deoxysphingolipids: beta-cell, beware of these new kids on the block.* Diabetes, 2014. **63**(4): p. 1191-3.

19. Grill, V., U. Adamson, and E. Cerasi, *Immediate and time-dependent effects of glucose on insulin release from rat pancreatic tissue. Evidence for different mechanisms of action.* J Clin Invest, 1978. **61**(4): p. 1034-43.
20. Grodsky, G.M., *A threshold distribution hypothesis for packet storage of insulin and its mathematical modeling.* J Clin Invest, 1972. **51**(8): p. 2047-59.
21. Gerich, J.E., M.A. Charles, and G.M. Grodsky, *Regulation of pancreatic insulin and glucagon secretion.* Annu Rev Physiol, 1976. **38**: p. 353-88.
22. Halperin, F., et al., *Insulin augmentation of glucose-stimulated insulin secretion is impaired in insulin-resistant humans.* Diabetes, 2012. **61**(2): p. 301-9.
23. Bhattacharya, P., et al., *A novel pancreatic beta-cell targeting bispecific-antibody (BsAb) can prevent the development of type 1 diabetes in NOD mice.* Clin Immunol, 2014. **153**(1): p. 187-98.
24. Komatsu, M., et al., *Glucose-stimulated insulin secretion: A newer perspective.* J Diabetes Investig, 2013. **4**(6): p. 511-6.
25. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2014. **37 Suppl 1**: p. S81-90.
26. Westwell-Roper, C. and J.A. Ehses, *Is there a role for the adaptive immune system in pancreatic beta cell failure in type 2 diabetes?* Diabetologia, 2014. **57**(3): p. 447-50.
27. Ndisang, J.F., S. Rastogi, and A. Vannacci, *Insulin resistance, type 1 and type 2 diabetes, and related complications: current status and future perspective.* J Diabetes Res, 2014. **2014**: p. 276475.
28. Herold, K.C., et al., *Type 1 diabetes: translating mechanistic observations into effective clinical outcomes.* Nat Rev Immunol, 2013. **13**(4): p. 243-56.
29. JDRF, P.G.f., *Type 1 Diabetes, 2010.* JDRF, 2010.
30. Chiang, J.L., et al., *Type 1 diabetes through the life span: a position statement of the American Diabetes Association.* Diabetes Care, 2014. **37**(7): p. 2034-54.
31. Eisenbarth, G.S., *Type I diabetes mellitus. A chronic autoimmune disease.* N Engl J Med, 1986. **314**(21): p. 1360-8.
32. Roep, B.O. and T.I. Tree, *Immune modulation in humans: implications for type 1 diabetes mellitus.* Nat Rev Endocrinol, 2014. **10**(4): p. 229-42.
33. Anderson, M.S. and J.A. Bluestone, *The NOD mouse: a model of immune dysregulation.* Annu Rev Immunol, 2005. **23**: p. 447-85.
34. Jorns, A., et al., *Islet infiltration, cytokine expression and beta cell death in the NOD mouse, BB rat, Komeda rat, LEW.1AR1-iddm rat and humans with type 1 diabetes.* Diabetologia, 2014. **57**(3): p. 512-21.
35. Yang, Y. and P. Santamaria, *Lessons on autoimmune diabetes from animal models.* Clin Sci (Lond), 2006. **110**(6): p. 627-39.
36. Roep, B.O., et al., *Islet inflammation and CXCL10 in recent-onset type 1 diabetes.* Clin Exp Immunol, 2010. **159**(3): p. 338-43.
37. Stewart, T.A., et al., *Induction of type I diabetes by interferon-alpha in transgenic mice.* Science, 1993. **260**(5116): p. 1942-6.
38. Willcox, A., et al., *Analysis of islet inflammation in human type 1 diabetes.* Clin Exp Immunol, 2009. **155**(2): p. 173-81.
39. Roep, B.O. and M. Peakman, *Antigen targets of type 1 diabetes autoimmunity.* Cold Spring Harb Perspect Med, 2012. **2**(4): p. a007781.

40. Culina, S., V. Brezar, and R. Mallone, *Insulin and type 1 diabetes: immune connections.* Eur J Endocrinol, 2013. **168**(2): p. R19-31.
41. Brezar, V., et al., *Beyond the hormone: insulin as an autoimmune target in type 1 diabetes.* Endocr Rev, 2011. **32**(5): p. 623-69.
42. Arvan, P., et al., *Islet autoantigens: structure, function, localization, and regulation.* Cold Spring Harb Perspect Med, 2012. **2**(8).
43. Anderson, M.S., et al., *Projection of an immunological self shadow within the thymus by the aire protein.* Science, 2002. **298**(5597): p. 1395-401.
44. Bonner, S.M., et al., *Sequence variation in promoter of Ica1 gene, which encodes protein implicated in type 1 diabetes, causes transcription factor autoimmune regulator (AIRE) to increase its binding and down-regulate expression.* J Biol Chem, 2012. **287**(21): p. 17882-93.
45. Gardner, J.M., et al., *Deletional tolerance mediated by extrathymic Aire-expressing cells.* Science, 2008. **321**(5890): p. 843-7.
46. Pugliese, A., *The insulin gene in type 1 diabetes.* IUBMB Life, 2005. **57**(7): p. 463-8.
47. Daniel, M. and D. Gamble, *Diabetes and Canada's aboriginal peoples: the need for primary prevention.* Int J Nurs Stud, 1995. **32**(3): p. 243-59.
48. Muir, A. and D. Schatz, *Prevention of insulin-dependent diabetes-1995.* Trends Endocrinol Metab, 1995. **6**(9-10): p. 312-7.
49. Achenbach, P., et al., *Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes.* J Clin Invest, 2004. **114**(4): p. 589-97.
50. Toma, A., et al., *Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients.* Proc Natl Acad Sci U S A, 2005. **102**(30): p. 10581-6.
51. Soleimanpour, S.A. and D.A. Stoffers, *The pancreatic beta cell and type 1 diabetes: innocent bystander or active participant?* Trends Endocrinol Metab, 2013. **24**(7): p. 324-31.
52. Kramer, C.K., et al., *Prospective associations of vitamin D status with beta-cell function, insulin sensitivity, and glycemia: the impact of parathyroid hormone status.* Diabetes, 2014. **63**(11): p. 3868-79.
53. O'Sullivan-Murphy, B. and F. Urano, *ER stress as a trigger for beta-cell dysfunction and autoimmunity in type 1 diabetes.* Diabetes, 2012. **61**(4): p. 780-1.
54. Urano, F., *Diabetes: Targeting endoplasmic reticulum to combat juvenile diabetes.* Nat Rev Endocrinol, 2014. **10**(3): p. 129-30.
55. Pociot, F., et al., *Genetics of type 1 diabetes: what's next?* Diabetes, 2010. **59**(7): p. 1561-71.
56. Barrett, J.C., et al., *Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes.* Nat Genet, 2009. **41**(6): p. 703-7.
57. Harjutsalo, V., T. Podar, and J. Tuomilehto, *Cumulative incidence of type 1 diabetes in 10,168 siblings of Finnish young-onset type 1 diabetic patients.* Diabetes, 2005. **54**(2): p. 563-9.
58. Steck, A.K. and M.J. Rewers, *Genetics of type 1 diabetes.* Clin Chem, 2011. **57**(2): p. 176-85.

59. Foulis, A.K., M.A. Farquharson, and R. Hardman, *Aberrant expression of class II major histocompatibility complex molecules by B cells and hyperexpression of class I major histocompatibility complex molecules by insulin containing islets in type 1 (insulin-dependent) diabetes mellitus.* Diabetologia, 1987. **30**(5): p. 333-43.
60. Foulis, A.K., M.A. Farquharson, and A. Meager, *Immunoreactive alpha-interferon in insulin-secreting beta cells in type 1 diabetes mellitus.* Lancet, 1987. **2**(8573): p. 1423-7.
61. Ghosh, S., et al., *Polygenic control of autoimmune diabetes in nonobese diabetic mice.* Nat Genet, 1993. **4**(4): p. 404-9.
62. Todd, J.A., et al., *Dissection of the pathophysiology of type 1 diabetes by genetic analysis.* Autoimmunity, 1993. **15 Suppl**: p. 16-7.
63. Deruytter, N., O. Boulard, and H.J. Garchon, *Mapping non-class II H2-linked loci for type 1 diabetes in nonobese diabetic mice.* Diabetes, 2004. **53**(12): p. 3323-7.
64. Wherrett, D.K., S.M. Singer, and H.O. McDevitt, *Reduction in diabetes incidence in an I-Ag7 transgenic nonobese diabetic mouse line.* Diabetes, 1997. **46**(12): p. 1970-4.
65. Marron, M.P., et al., *Functional evidence for the mediation of diabetogenic T cell responses by HLA-A2.1 MHC class I molecules through transgenic expression in NOD mice.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13753-8.
66. Noble, J.A. and H.A. Erlich, *Genetics of type 1 diabetes.* Cold Spring Harb Perspect Med, 2012. **2**(1): p. a007732.
67. Noble, J.A., et al., *HLA class I and genetic susceptibility to type 1 diabetes: results from the Type 1 Diabetes Genetics Consortium.* Diabetes, 2010. **59**(11): p. 2972-9.
68. Smyth, D.J., et al., *Shared and distinct genetic variants in type 1 diabetes and celiac disease.* N Engl J Med, 2008. **359**(26): p. 2767-77.
69. Wandstrat, A. and E. Wakeland, *The genetics of complex autoimmune diseases: non-MHC susceptibility genes.* Nat Immunol, 2001. **2**(9): p. 802-9.
70. Santin, I. and D.L. Eizirik, *Candidate genes for type 1 diabetes modulate pancreatic islet inflammation and beta-cell apoptosis.* Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 71-81.
71. Smyth, D.J., et al., *A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region.* Nat Genet, 2006. **38**(6): p. 617-9.
72. Nejentsev, S., et al., *Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes.* Science, 2009. **324**(5925): p. 387-9.
73. Shigemoto, T., et al., *Identification of loss of function mutations in human genes encoding RIG-I and MDA5: implications for resistance to type I diabetes.* J Biol Chem, 2009. **284**(20): p. 13348-54.
74. Downes, K., et al., *Reduced expression of IFIH1 is protective for type 1 diabetes.* PLoS One, 2010. **5**(9).
75. Ivashkiv, L.B. and L.T. Donlin, *Regulation of type I interferon responses.* Nat Rev Immunol, 2014. **14**(1): p. 36-49.

76. Molineros, J.E., et al., *Admixture mapping in lupus identifies multiple functional variants within IFIH1 associated with apoptosis, inflammation, and autoantibody production.* PLoS Genet, 2013. **9**(2): p. e1003222.
77. Robinson, T., et al., *Autoimmune disease risk variant of IFIH1 is associated with increased sensitivity to IFN-alpha and serologic autoimmunity in lupus patients.* J Immunol, 2011. **187**(3): p. 1298-303.
78. Todd, J.A., *Constitutive antiviral immunity at the expense of autoimmunity.* Immunity, 2014. **40**(2): p. 167-9.
79. Ferreira, R.C., et al., *Association of IFIH1 and other autoimmunity risk alleles with selective IgA deficiency.* Nat Genet, 2010. **42**(9): p. 777-80.
80. Rice, G.I., et al., *Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling.* Nat Genet, 2014. **46**(5): p. 503-9.
81. Lincez, P.J., I. Shanina, and M.S. Horwitz, *Reduced expression of the MDA5 gene IFIH1 prevents autoimmune diabetes.* Diabetes, 2015.
82. Howson, J.M., et al., *Analysis of 19 genes for association with type I diabetes in the Type I Diabetes Genetics Consortium families.* Genes Immun, 2009. **10 Suppl 1**: p. S74-84.
83. Eerligh, P., et al., *Functional genetic polymorphisms in cytokines and metabolic genes as additional genetic markers for susceptibility to develop type 1 diabetes.* Genes Immun, 2004. **5**(1): p. 36-40.
84. Nokoff, N.J., M. Rewers, and M. Cree Green, *The interplay of autoimmunity and insulin resistance in type 1 diabetes.* Discov Med, 2012. **13**(69): p. 115-22.
85. Bluestone, J.A., K. Herold, and G. Eisenbarth, *Genetics, pathogenesis and clinical interventions in type 1 diabetes.* Nature, 2010. **464**(7293): p. 1293-300.
86. Richer, M.J. and M.S. Horwitz, *Coxsackievirus infection as an environmental factor in the etiology of type 1 diabetes.* Autoimmun Rev, 2009. **8**(7): p. 611-5.
87. Kondrashova, A. and H. Hyoty, *Role of viruses and other microbes in the pathogenesis of type 1 diabetes.* Int Rev Immunol, 2014. **33**(4): p. 284-95.
88. Schneider, D.A. and M.G. von Herrath, *Viruses and Type 1 diabetes: a dynamic labile equilibrium.* Diabetes Manag (Lond), 2013. **3**(3): p. 217-223.
89. Craig, M.E., et al., *Viruses and type 1 diabetes: a new look at an old story.* Pediatr Diabetes, 2013. **14**(3): p. 149-58.
90. Jankosky, C., et al., *Viruses and vitamin D in the etiology of type 1 diabetes mellitus and multiple sclerosis.* Virus Res, 2012. **163**(2): p. 424-30.
91. Richardson, S.J. and M.S. Horwitz, *Is type 1 diabetes "going viral"?* Diabetes, 2014. **63**(7): p. 2203-5.
92. Yeung, W.C., W.D. Rawlinson, and M.E. Craig, *Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies.* BMJ, 2011. **342**: p. d35.
93. *Diabetes: Enterovirus infection associated with T1DM risk in children.* Nat Rev Endocrinol, 2015. **11**(1): p. 4.
94. Hober, D. and P. Sauter, *Pathogenesis of type 1 diabetes mellitus: interplay between enterovirus and host.* Nat Rev Endocrinol, 2010. **6**(5): p. 279-89.

95. Hober, D. and F. Sane, *Enteroviral pathogenesis of type 1 diabetes.* Discov Med, 2010. **10**(51): p. 151-60.
96. Elfving, M., et al., *Maternal enterovirus infection during pregnancy as a risk factor in offspring diagnosed with type 1 diabetes between 15 and 30 years of age.* Exp Diabetes Res, 2008. **2008**: p. 271958.
97. Viskari, H., et al., *Maternal enterovirus infection as a risk factor for type 1 diabetes in the exposed offspring.* Diabetes Care, 2012. **35**(6): p. 1328-32.
98. Oikarinen, M., et al., *Analysis of pancreas tissue in a child positive for islet cell antibodies.* Diabetologia, 2008. **51**(10): p. 1796-802.
99. Dotta, F., et al., *Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients.* Proc Natl Acad Sci U S A, 2007. **104**(12): p. 5115-20.
100. Jubelt, B. and H.L. Lipton, *Enterovirus/picornavirus infections.* Handb Clin Neurol, 2014. **123**: p. 379-416.
101. Minor, P.D.a.M., Peter, *Enteroviruses*, in *Principles and Practice of Clinical Virology*, A.J. Zuckerman, Banatvala, J.E., Pattison, J.R., Griffiths, P.D., and Schoub, B.D., Editor. 2004, John Wiley & Sons Ltd. p. 467-489.
102. Esfandiarei, M. and B.M. McManus, *Molecular biology and pathogenesis of viral myocarditis.* Annu Rev Pathol, 2008. **3**: p. 127-55.
103. Huber, S.A., C.J. Gauntt, and P. Sakkinen, *Enteroviruses and myocarditis: viral pathogenesis through replication, cytokine induction, and immunopathogenicity.* Adv Virus Res, 1998. **51**: p. 35-80.
104. Gamble, D.R., et al., *Viral antibodies in diabetes mellitus.* Br Med J, 1969. **3**(5671): p. 627-30.
105. King, M.L., et al., *Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus.* Lancet, 1983. **1**(8339): p. 1397-9.
106. Banatvala, J.E., et al., *Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria, and Australia.* Lancet, 1985. **1**(8443): p. 1409-12.
107. Frisk, G., et al., *Coxsackie B virus IgM in children at onset of type 1 (insulin-dependent) diabetes mellitus: evidence for IgM induction by a recent or current infection.* Diabetologia, 1992. **35**(3): p. 249-53.
108. Clements, G.B., D.N. Galbraith, and K.W. Taylor, *Coxsackie B virus infection and onset of childhood diabetes.* Lancet, 1995. **346**(8969): p. 221-3.
109. Helfand, R.F., et al., *Serologic evidence of an association between enteroviruses and the onset of type 1 diabetes mellitus. Pittsburgh Diabetes Research Group.* J Infect Dis, 1995. **172**(5): p. 1206-11.
110. Andreoletti, L., et al., *Detection of coxsackie B virus RNA sequences in whole blood samples from adult patients at the onset of type I diabetes mellitus.* J Med Virol, 1997. **52**(2): p. 121-7.
111. Lonnrot, M., et al., *Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study.* Diabetes, 2000. **49**(8): p. 1314-8.

112. Sadeharju, K., et al., *Enterovirus antibody levels during the first two years of life in prediabetic autoantibody-positive children.* Diabetologia, 2001. **44**(7): p. 818-23.
113. Sadeharju, K., et al., *Enterovirus infections as a risk factor for type I diabetes: virus analyses in a dietary intervention trial.* Clin Exp Immunol, 2003. **132**(2): p. 271-7.
114. Sarmiento, L., et al., *Occurrence of enterovirus RNA in serum of children with newly diagnosed type 1 diabetes and islet cell autoantibody-positive subjects in a population with a low incidence of type 1 diabetes.* Autoimmunity, 2007. **40**(7): p. 540-5.
115. Schulte, B.M., et al., *Detection of enterovirus RNA in peripheral blood mononuclear cells of type 1 diabetic patients beyond the stage of acute infection.* Viral Immunol. **23**(1): p. 99-104.
116. Huang, X., et al., *Interferon expression in the pancreases of patients with type I diabetes.* Diabetes, 1995. **44**(6): p. 658-64.
117. Yoon, J.W., et al., *Isolation of a virus from the pancreas of a child with diabetic ketoacidosis.* N Engl J Med, 1979. **300**(21): p. 1173-9.
118. Horwitz, M.S., et al., *Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry.* Nat Med, 1998. **4**(7): p. 781-5.
119. Richer, M.J., et al., *Regulatory T-cells protect from type 1 diabetes after induction by coxsackievirus infection in the context of transforming growth factor-beta.* Diabetes, 2008. **57**(5): p. 1302-11.
120. Serreze, D.V., et al., *Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets.* Diabetes, 2000. **49**(5): p. 708-11.
121. Reetoo, K.N., et al., *Quantitative analysis of viral RNA kinetics in coxsackievirus B3-induced murine myocarditis: biphasic pattern of clearance following acute infection, with persistence of residual viral RNA throughout and beyond the inflammatory phase of disease.* J Gen Virol, 2000. **81**(Pt 11): p. 2755-62.
122. McCartney, S.A., et al., *RNA sensor-induced type I IFN prevents diabetes caused by a beta cell-tropic virus in mice.* J Clin Invest, 2011. **121**(4): p. 1497-507.
123. Bason, C., et al., *In type 1 diabetes a subset of anti-coxsackievirus B4 antibodies recognize autoantigens and induce apoptosis of pancreatic beta cells.* PLoS One, 2013. **8**(2): p. e57729.
124. Schneider, D.A. and M.G. von Herrath, *Potential viral pathogenic mechanism in human type 1 diabetes.* Diabetologia, 2014. **57**(10): p. 2009-18.
125. Morgan, N.G. and S.J. Richardson, *Enteroviruses as causative agents in type 1 diabetes: loose ends or lost cause?* Trends Endocrinol Metab, 2014. **25**(12): p. 611-619.
126. Richer, M.J. and M.S. Horwitz, *Viral infections in the pathogenesis of autoimmune diseases: focus on type 1 diabetes.* Front Biosci, 2008. **13**: p. 4241-57.
127. Richer, M.J. and M.S. Horwitz, *The Innate Immune Response: An Important Partner in Shaping Coxsackievirus-Mediated Autoimmunity.* Journal of Innate Immunity, 2009. **1**(5): p. 421-434.

128. Kawai, T. and S. Akira, *Toll-like receptor and RIG-I-like receptor signaling.* Ann N Y Acad Sci, 2008. **1143**: p. 1-20.
129. Takeuchi, O. and S. Akira, *MDA5/RIG-I and virus recognition.* Curr Opin Immunol, 2008. **20**(1): p. 17-22.
130. Meylan, E. and J. Tschopp, *Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses.* Mol Cell, 2006. **22**(5): p. 561-9.
131. Swiecki, M., et al., *TLR7/9 versus TLR3/MDA5 signaling during virus infections and diabetes.* J Leukoc Biol, 2011. **90**(4): p. 691-701.
132. Kato, H., et al., *Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses.* Nature, 2006. **441**(7089): p. 101-5.
133. Triantafilou, K., et al., *Visualisation of direct interaction of MDA5 and the dsRNA replicative intermediate form of positive strand RNA viruses.* J Cell Sci, 2012. **125**(Pt 20): p. 4761-9.
134. Feng, Q., et al., *MDA5 detects the double-stranded RNA replicative form in picornavirus-infected cells.* Cell Rep, 2012. **2**(5): p. 1187-96.
135. Wu, B., et al., *Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5.* Cell, 2013. **152**(1-2): p. 276-89.
136. Wies, E., et al., *Dephosphorylation of the RNA sensors RIG-I and MDA5 by the phosphatase PP1 is essential for innate immune signaling.* Immunity, 2013. **38**(3): p. 437-49.
137. Wang, J.P., et al., *MDA5 and MAVS mediate type I interferon responses to coxsackie B virus.* J Virol, 2010. **84**(1): p. 254-60.
138. Longhi, M.P., et al., *Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant.* J Exp Med, 2009. **206**(7): p. 1589-602.
139. Trumpfheller, C., et al., *The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2574-9.
140. McCartney, S., et al., *Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells.* J Exp Med, 2009. **206**(13): p. 2967-76.
141. Wang, Y., et al., *Cutting edge: polyinosinic:polycytidylic acid boosts the generation of memory CD8 T cells through melanoma differentiation-associated protein 5 expressed in stromal cells.* J Immunol, 2010. **184**(6): p. 2751-5.
142. Bruns, A.M., et al., *The innate immune sensor LGP2 activates antiviral signaling by regulating MDA5-RNA interaction and filament assembly.* Mol Cell, 2014. **55**(5): p. 771-81.
143. Rodriguez, K.R., A.M. Bruns, and C.M. Horvath, *MDA5 and LGP2: accomplices and antagonists of antiviral signal transduction.* J Virol, 2014. **88**(15): p. 8194-200.
144. Andrejeva, J., et al., *The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter.* Proc Natl Acad Sci U S A, 2004. **101**(49): p. 17264-9.
145. Childs, K.S., et al., *Mechanism of mda-5 Inhibition by paramyxovirus V proteins.* J Virol, 2009. **83**(3): p. 1465-73.

146. Nagano, Y. and Y. Kojima, *[Inhibition of vaccinia infection by a liquid factor in tissues infected by homologous virus].* C R Seances Soc Biol Fil, 1958. **152**(11): p. 1627-9.
147. Isaacs, A., J. Lindenmann, and R.C. Valentine, *Virus interference. II. Some properties of interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 268-73.
148. Isaacs, A. and J. Lindenmann, *Virus interference. I. The interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 258-67.
149. Wang, B.X. and E.N. Fish, *The yin and yang of viruses and interferons.* Trends Immunol, 2012. **33**(4): p. 190-7.
150. Begitt, A., et al., *STAT1-cooperative DNA binding distinguishes type 1 from type 2 interferon signaling.* Nat Immunol, 2014. **15**(2): p. 168-76.
151. Gessani, S., et al., *Bacterial lipopolysaccharide and gamma interferon induce transcription of beta interferon mRNA and interferon secretion in murine macrophages.* J Virol, 1989. **63**(6): p. 2785-9.
152. Hamilton, J.A., et al., *Endogenous IFN-alpha beta suppresses colony-stimulating factor (CSF)-1-stimulated macrophage DNA synthesis and mediates inhibitory effects of lipopolysaccharide and TNF-alpha.* J Immunol, 1996. **156**(7): p. 2553-7.
153. Takayanagi, H., S. Kim, and T. Taniguchi, *Signaling crosstalk between RANKL and interferons in osteoclast differentiation.* Arthritis Res, 2002. **4 Suppl 3**: p. S227-32.
154. de Weerd, N.A., et al., *Structural basis of a unique interferon-beta signaling axis mediated via the receptor IFNAR1.* Nat Immunol, 2013. **14**(9): p. 901-7.
155. Crow, M.K., *Type I interferon in organ-targeted autoimmune and inflammatory diseases.* Arthritis Res Ther, 2010. **12 Suppl 1**: p. S5.
156. Crow, M.K., M. Olferiev, and K.A. Kirou, *Targeting of type I interferon in systemic autoimmune diseases.* Transl Res, 2014.
157. Theofilopoulos, A.N., et al., *Type I interferons (alpha/beta) in immunity and autoimmunity.* Annu Rev Immunol, 2005. **23**: p. 307-36.
158. Sadler, A.J. and B.R. Williams, *Interferon-inducible antiviral effectors.* Nat Rev Immunol, 2008. **8**(7): p. 559-68.
159. Malireddi, R.K. and T.D. Kanneganti, *Role of type I interferons in inflammasome activation, cell death, and disease during microbial infection.* Front Cell Infect Microbiol, 2013. **3**: p. 77.
160. Bogdan, C., J. Mattner, and U. Schleicher, *The role of type I interferons in non-viral infections.* Immunol Rev, 2004. **202**: p. 33-48.
161. Gough, D.J., et al., *Constitutive type I interferon modulates homeostatic balance through tonic signaling.* Immunity, 2012. **36**(2): p. 166-74.
162. Rai, E. and E.K. Wakeland, *Genetic predisposition to autoimmunity--what have we learned?* Semin Immunol, 2011. **23**(2): p. 67-83.
163. Maurano, M.T., et al., *Systematic localization of common disease-associated variation in regulatory DNA.* Science, 2012. **337**(6099): p. 1190-5.
164. Hall, J.C. and A. Rosen, *Type I interferons: crucial participants in disease amplification in autoimmunity.* Nat Rev Rheumatol, 2010. **6**(1): p. 40-9.

165. Tomasello, E., et al., *Harnessing Mechanistic Knowledge on Beneficial Versus Deleterious IFN-I Effects to Design Innovative Immunotherapies Targeting Cytokine Activity to Specific Cell Types.* Front Immunol, 2014. **5**: p. 526.
166. Funabiki, M., et al., *Autoimmune disorders associated with gain of function of the intracellular sensor MDA5.* Immunity, 2014. **40**(2): p. 199-212.
167. Trinchieri, G., *Type I interferon: friend or foe?* J Exp Med, 2010. **207**(10): p. 2053-63.
168. Delgado-Vega, A.M., M.E. Alarcon-Riquelme, and S.V. Kozyrev, *Genetic associations in type I interferon related pathways with autoimmunity.* Arthritis Res Ther, 2010. **12 Suppl 1**: p. S2.
169. Ferreira, R.C., et al., *A type I interferon transcriptional signature precedes autoimmunity in children genetically at-risk of type 1 diabetes.* Diabetes, 2014.
170. Kallionpaa, H., et al., *Innate immune activity is detected prior to seroconversion in children with HLA-conferred type 1 diabetes susceptibility.* Diabetes, 2014. **63**(7): p. 2402-14.
171. Dotta, F. and G. Sebastiani, *Enteroviral infections and development of type 1 diabetes: The Brothers Karamazov within the CVBs.* Diabetes, 2014. **63**(2): p. 384-6.
172. Lind, K., M.H. Huhn, and M. Flodstrom-Tullberg, *Immunology in the clinic review series; focus on type 1 diabetes and viruses: the innate immune response to enteroviruses and its possible role in regulating type 1 diabetes.* Clin Exp Immunol, 2012. **168**(1): p. 30-8.
173. Reynier, F., et al., *Specific gene expression signature associated with development of autoimmune type-I diabetes using whole-blood microarray analysis.* Genes Immun, 2010. **11**(3): p. 269-78.
174. Li, Q., et al., *Interferon-alpha initiates type 1 diabetes in nonobese diabetic mice.* Proc Natl Acad Sci U S A, 2008. **105**(34): p. 12439-44.
175. Eizirik, D.L., et al., *The human pancreatic islet transcriptome: expression of candidate genes for type 1 diabetes and the impact of pro-inflammatory cytokines.* PLoS Genet, 2012. **8**(3): p. e1002552.
176. Dogusan, Z., et al., *Double-stranded RNA induces pancreatic beta-cell apoptosis by activation of the toll-like receptor 3 and interferon regulatory factor 3 pathways.* Diabetes, 2008. **57**(5): p. 1236-45.
177. Garcia, M., et al., *Regulation and function of the cytosolic viral RNA sensor RIG-I in pancreatic beta cells.* Biochim Biophys Acta, 2009. **1793**(11): p. 1768-75.
178. Rasschaert, J., et al., *Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-gamma-induced apoptosis in primary pancreatic beta-cells.* J Biol Chem, 2005. **280**(40): p. 33984-91.
179. Ylipaasto, P., et al., *Enterovirus-induced gene expression profile is critical for human pancreatic islet destruction.* Diabetologia, 2012. **55**(12): p. 3273-83.
180. Pichlmair, A., et al., *Viral immune modulators perturb the human molecular network by common and unique strategies.* Nature, 2012. **487**(7408): p. 486-90.
181. Eizirik, D.L., M.L. Colli, and F. Ortis, *The role of inflammation in insulitis and beta-cell loss in type 1 diabetes.* Nat Rev Endocrinol, 2009. **5**(4): p. 219-26.

182. Richer, M.J. and M.S. Horwitz, *The innate immune response: an important partner in shaping coxsackievirus-mediated autoimmunity.* J Innate Immun, 2009. **1**(5): p. 421-34.
183. Ganguly, D., et al., *The role of dendritic cells in autoimmunity.* Nat Rev Immunol, 2013. **13**(8): p. 566-77.
184. Creusot, R.J., et al., *It's time to bring dendritic cell therapy to type 1 diabetes.* Diabetes, 2014. **63**(1): p. 20-30.
185. Jun, H.S., et al., *The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice.* J Exp Med, 1999. **189**(2): p. 347-58.
186. Dahlen, E., G. Hedlund, and K. Dawe, *Low CD86 expression in the nonobese diabetic mouse results in the impairment of both T cell activation and CTLA-4 up-regulation.* J Immunol, 2000. **164**(5): p. 2444-56.
187. Richer, M.J., et al., *Immunomodulation of antigen presenting cells promotes natural regulatory T cells that prevent autoimmune diabetes in NOD mice.* PLoS One, 2012. **7**(2): p. e31153.
188. Karimi, M.H., et al., *Tolerance Induction by CD40 Blocking through Specific Antibody in Dendritic Cells.* Iran J Allergy Asthma Immunol, 2010. **9**(3): p. 141-7.
189. Peters, A.L., L.L. Stunz, and G.A. Bishop, *CD40 and autoimmunity: the dark side of a great activator.* Semin Immunol, 2009. **21**(5): p. 293-300.
190. Sun, W., et al., *Blockade of CD40 pathway enhances the induction of immune tolerance by immature dendritic cells genetically modified to express cytotoxic T lymphocyte antigen 4 immunoglobulin.* Transplantation, 2003. **76**(9): p. 1351-9.
191. Vaitaitis, G.M., et al., *A CD40-targeted peptide controls and reverses type 1 diabetes in NOD mice.* Diabetologia, 2014. **57**(11): p. 2366-73.
192. Bluestone, J.A. and Q. Tang, *How do CD4+CD25+ regulatory T cells control autoimmunity?* Curr Opin Immunol, 2005. **17**(6): p. 638-42.
193. Sakaguchi, S. and F. Powrie, *Emerging challenges in regulatory T cell function and biology.* Science, 2007. **317**(5838): p. 627-9.
194. Smigiel, K.S., et al., *Regulatory T-cell homeostasis: steady-state maintenance and modulation during inflammation.* Immunol Rev, 2014. **259**(1): p. 40-59.
195. Zhang, Y., E. Bandala-Sanchez, and L.C. Harrison, *Revisiting regulatory T cells in type 1 diabetes.* Curr Opin Endocrinol Diabetes Obes, 2012. **19**(4): p. 271-8.
196. Jeker, L.T., H. Bour-Jordan, and J.A. Bluestone, *Breakdown in peripheral tolerance in type 1 diabetes in mice and humans.* Cold Spring Harb Perspect Med, 2012. **2**(3): p. a007807.
197. Campbell, D.J. and M.A. Koch, *Phenotypical and functional specialization of FOXP3+ regulatory T cells.* Nat Rev Immunol, 2011. **11**(2): p. 119-30.
198. Akimova, T., et al., *Helios expression is a marker of T cell activation and proliferation.* PLoS One, 2011. **6**(8): p. e24226.
199. McClymont, S.A., et al., *Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes.* J Immunol, 2011. **186**(7): p. 3918-26.
200. Gottschalk, R.A., E. Corse, and J.P. Allison, *Expression of Helios in peripherally induced Foxp3+ regulatory T cells.* J Immunol, 2012. **188**(3): p. 976-80.

201. Yadav, M., et al., *Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo.* J Exp Med, 2012. **209**(10): p. 1713-22, S1-19.
202. Sarris, M., et al., *Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition.* Immunity, 2008. **28**(3): p. 402-13.
203. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
204. Khattri, R., et al., *An essential role for Scurfin in CD4+CD25+ T regulatory cells.* Nat Immunol, 2003. **4**(4): p. 337-42.
205. Wildin, R.S., et al., *X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy.* Nat Genet, 2001. **27**(1): p. 18-20.
206. Chaudhry, A. and A.Y. Rudensky, *Control of inflammation by integration of environmental cues by regulatory T cells.* J Clin Invest, 2013. **123**(3): p. 939-44.
207. von Boehmer, H., *Mechanisms of suppression by suppressor T cells.* Nat Immunol, 2005. **6**(4): p. 338-44.
208. Anz, D., et al., *Immunostimulatory RNA blocks suppression by regulatory T cells.* J Immunol, 2010. **184**(2): p. 939-46.
209. Lehner, T., *Special regulatory T cell review: The resurgence of the concept of contrasuppression in immunoregulation.* Immunology, 2008. **123**(1): p. 40-4.
210. Kabelitz, D., D. Wesch, and H.H. Oberg, *Regulation of regulatory T cells: role of dendritic cells and toll-like receptors.* Crit Rev Immunol, 2006. **26**(4): p. 291-306.
211. Swain, S.L., K.K. McKinstry, and T.M. Strutt, *Expanding roles for CD4(+) T cells in immunity to viruses.* Nat Rev Immunol, 2012. **12**(2): p. 136-48.
212. Mayer, C.T., L. Berod, and T. Sparwasser, *Layers of dendritic cell-mediated T cell tolerance, their regulation and the prevention of autoimmunity.* Front Immunol, 2012. **3**: p. 183.
213. Bin Dhuban, K., et al., *Functional dynamics of Foxp3(+) regulatory T cells in mice and humans.* Immunol Rev, 2014. **259**(1): p. 140-58.
214. Fu, W., et al., *A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells.* Nat Immunol, 2012. **13**(10): p. 972-80.
215. Tree, T.I., et al., *Naturally arising human CD4 T-cells that recognize islet autoantigens and secrete interleukin-10 regulate proinflammatory T-cell responses via linked suppression.* Diabetes, 2010. **59**(6): p. 1451-60.
216. Maizels, R.M. and K.A. Smith, *Regulatory T cells in infection.* Adv Immunol, 2011. **112**: p. 73-136.
217. Tang, Q., et al., *Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction.* Immunity, 2008. **28**(5): p. 687-97.
218. Tritt, M., et al., *Functional waning of naturally occurring CD4+ regulatory T-cells contributes to the onset of autoimmune diabetes.* Diabetes, 2008. **57**(1): p. 113-23.

219. Zhou, X., et al., *Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo.* Nat Immunol, 2009. **10**(9): p. 1000-7.
220. Thomas, D., P. Zaccone, and A. Cooke, *The role of regulatory T cell defects in type I diabetes and the potential of these cells for therapy.* Rev Diabet Stud, 2005. **2**(1): p. 9-18.
221. Feuerer, M., et al., *Enhanced thymic selection of FoxP3+ regulatory T cells in the NOD mouse model of autoimmune diabetes.* Proc Natl Acad Sci U S A, 2007. **104**(46): p. 18181-6.
222. Lindley, S., et al., *Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes.* Diabetes, 2005. **54**(1): p. 92-9.
223. Putnam, A.L., et al., *CD4+CD25high regulatory T cells in human autoimmune diabetes.* J Autoimmun, 2005. **24**(1): p. 55-62.
224. Glisic, S. and P. Jailwala, *Interaction between Treg apoptosis pathways, Treg function and HLA risk evolves during type 1 diabetes pathogenesis.* PLoS One, 2012. **7**(4): p. e36040.
225. Ferraro, A., et al., *Interindividual variation in human T regulatory cells.* Proc Natl Acad Sci U S A, 2014. **111**(12): p. E1111-20.
226. Tree, T.I., B.O. Roep, and M. Peakman, *A mini meta-analysis of studies on CD4+CD25+ T cells in human type 1 diabetes: report of the Immunology of Diabetes Society T Cell Workshop.* Ann N Y Acad Sci, 2006. **1079**: p. 9-18.
227. Leung, S., et al., *The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease.* Cell Mol Immunol, 2010. **7**(3): p. 182-9.
228. Goudy, K.S., et al., *Inducible adeno-associated virus-mediated IL-2 gene therapy prevents autoimmune diabetes.* J Immunol, 2011. **186**(6): p. 3779-86.
229. Grinberg-Bleyer, Y., et al., *IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells.* J Exp Med, 2010. **207**(9): p. 1871-8.
230. Long, S.A., J.H. Buckner, and C.J. Greenbaum, *IL-2 therapy in type 1 diabetes: "Trials" and tribulations.* Clin Immunol, 2013. **149**(3): p. 324-31.
231. Hartemann, A., et al., *Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial.* Lancet Diabetes Endocrinol, 2013. **1**(4): p. 295-305.
232. Rosenzwajg, M., et al., *Interleukin 2 in the pathogenesis and therapy of type 1 diabetes.* Curr Diab Rep, 2014. **14**(12): p. 553.
233. Peng, Y., et al., *TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes.* Proc Natl Acad Sci U S A, 2004. **101**(13): p. 4572-7.
234. Ishigame, H., et al., *Excessive Th1 responses due to the absence of TGF-beta signaling cause autoimmune diabetes and dysregulated Treg cell homeostasis.* Proc Natl Acad Sci U S A, 2013. **110**(17): p. 6961-6.
235. Xu, P., et al., *Innate Antiviral Host Defense Attenuates TGF-beta Function through IRF3-Mediated Suppression of Smad Signaling.* Mol Cell, 2014. **56**(6): p. 723-37.

236. Srivastava, S., et al., *Type I interferons directly inhibit regulatory T cells to allow optimal antiviral T cell responses during acute LCMV infection.* J Exp Med, 2014. **211**(5): p. 961-74.
237. You, S., et al., *Immunoregulatory pathways controlling progression of autoimmunity in NOD mice.* Ann N Y Acad Sci, 2008. **1150**: p. 300-10.
238. Marek-Trzonkowska, N., et al., *Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets - Results of one year follow-up.* Clin Immunol, 2014. **153**(1): p. 23-30.
239. Bluestone, J.A. and Q. Tang, *Therapeutic vaccination using CD4+CD25+ antigen-specific regulatory T cells.* Proc Natl Acad Sci U S A, 2004. **101 Suppl 2**: p. 14622-6.
240. Tang, Q., et al., *In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes.* J Exp Med, 2004. **199**(11): p. 1455-65.
241. von Boehmer, H. and C. Daniel, *Therapeutic opportunities for manipulating T(Reg) cells in autoimmunity and cancer.* Nat Rev Drug Discov, 2013. **12**(1): p. 51-63.
242. Singer, B.D., L.S. King, and F.R. D'Alessio, *Regulatory T cells as immunotherapy.* Front Immunol, 2014. **5**: p. 46.
243. Smilek, D.E., M.R. Ehlers, and G.T. Nepom, *Restoring the balance: immunotherapeutic combinations for autoimmune disease.* Dis Model Mech, 2014. **7**(5): p. 503-13.
244. Reikine, S., J.B. Nguyen, and Y. Modis, *Pattern Recognition and Signaling Mechanisms of RIG-I and MDA5.* Front Immunol, 2014. **5**: p. 342.
245. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.
246. Gonzalez-Navajas, J.M., et al., *Immunomodulatory functions of type I interferons.* Nat Rev Immunol, 2012. **12**(2): p. 125-35.
247. Guerder, S., et al., *Dendritic cells in tolerance and autoimmune diabetes.* Curr Opin Immunol, 2013. **25**(6): p. 670-5.
248. Rowland, S.L., et al., *Early, transient depletion of plasmacytoid dendritic cells ameliorates autoimmunity in a lupus model.* J Exp Med, 2014. **211**(10): p. 1977-91.
249. Xia, C.Q., et al., *Increased IFN-alpha-producing plasmacytoid dendritic cells (pDCs) in human Th1-mediated type 1 diabetes: pDCs augment Th1 responses through IFN-alpha production.* J Immunol, 2014. **193**(3): p. 1024-34.
250. Ferreira, R.C., et al., *A type I interferon transcriptional signature precedes autoimmunity in children genetically at risk for type 1 diabetes.* Diabetes, 2014. **63**(7): p. 2538-50.
251. Krogvold, L., et al., *Detection of a low-grade enteroviral infection in the islets of Langerhans of living patients newly diagnosed with type 1 diabetes.* Diabetes, 2014.
252. Richer, M.J., et al., *Toll-like receptor 3 signaling on macrophages is required for survival following coxsackievirus B4 infection.* PLoS One, 2009. **4**(1): p. e4127.
253. Toubi, E. and Y. Shoenfeld, *The role of CD40-CD154 interactions in autoimmunity and the benefit of disrupting this pathway.* Autoimmunity, 2004. **37**(6-7): p. 457-64.

254. Taylor, P.A., et al., *Tolerance induction of alloreactive T cells via ex vivo blockade of the CD40:CD40L costimulatory pathway results in the generation of a potent immune regulatory cell.* Blood, 2002. **99**(12): p. 4601-9.
255. Baker, R.L., et al., *T cells interact with T cells via CD40-CD154 to promote autoimmunity in type 1 diabetes.* Eur J Immunol, 2012. **42**(3): p. 672-80.
256. Wagner, D.H., Jr., et al., *Expression of CD40 identifies a unique pathogenic T cell population in type 1 diabetes.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3782-7.
257. Baker, R.L., D.H. Wagner, Jr., and K. Haskins, *CD40 on NOD CD4 T cells contributes to their activation and pathogenicity.* J Autoimmun, 2008. **31**(4): p. 385-92.
258. Salomon, B., et al., *B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes.* Immunity, 2000. **12**(4): p. 431-40.
259. Chambers, E.S. and C.M. Hawrylowicz, *The Impact of Vitamin D on Regulatory T Cells.* Curr Allergy Asthma Rep, 2010.
260. Takiishi, T., et al., *Vitamin D and diabetes.* Endocrinol Metab Clin North Am, 2010. **39**(2): p. 419-46, table of contents.
261. Thacher, T.D. and B.L. Clarke, *Vitamin d insufficiency.* Mayo Clin Proc, 2011. **86**(1): p. 50-60.
262. Szodoray, P., et al., *The complex role of vitamin D in autoimmune diseases.* Scand J Immunol, 2008. **68**(3): p. 261-9.
263. Plum, L.A. and H.F. Deluca, *Vitamin D, disease and therapeutic opportunities.* Nat Rev Drug Discov, 2010. **9**(12): p. 941-55.
264. Chen, J., D. Bruce, and M.T. Cantorna, *Vitamin D receptor expression controls proliferation of naive CD8+ T cells and development of CD8 mediated gastrointestinal inflammation.* BMC Immunol, 2014. **15**: p. 6.
265. Cantorna, M.T., et al., *Vitamin D, immune regulation, the microbiota, and inflammatory bowel disease.* Exp Biol Med (Maywood), 2014.
266. Cantorna, M.T. and A. Waddell, *The vitamin D receptor turns off chronically activated T cells.* Ann N Y Acad Sci, 2014. **1317**: p. 70-5.
267. Bergelson, J.M., et al., *Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5.* Science, 1997. **275**(5304): p. 1320-3.
268. Horwitz, M.S., et al., *Pancreatic expression of interferon-gamma protects mice from lethal coxsackievirus B3 infection and subsequent myocarditis.* Nat Med, 2000. **6**(6): p. 693-7.
269. Horwitz, M.S., et al., *Requirements for viral-mediated autoimmune diabetes: beta-cell damage and immune infiltration.* J Autoimmun, 2001. **16**(3): p. 211-7.
270. Roep, B.O., *beta-Cells, autoimmunity, and the innate immune system: "un menage a trois"?* Diabetes, 2013. **62**(6): p. 1821-2.
271. Wen, L., et al., *The effect of innate immunity on autoimmune diabetes and the expression of Toll-like receptors on pancreatic islets.* J Immunol, 2004. **172**(5): p. 3173-80.
272. Serreze, D.V., K. Hamaguchi, and E.H. Leiter, *Immunostimulation circumvents diabetes in NOD/Lt mice.* J Autoimmun, 1989. **2**(6): p. 759-76.

273. Sobel, D.O., et al., *Low dose poly I:C prevents diabetes in the diabetes prone BB rat.* J Autoimmun, 1998. **11**(4): p. 343-52.
274. Ambrosini, Y.M., et al., *The multi-hit hypothesis of primary biliary cirrhosis: polyinosinic-polycytidylic acid (poly I:C) and murine autoimmune cholangitis.* Clin Exp Immunol, 2011. **166**(1): p. 110-20.
275. Chehadeh, W., et al., *Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells.* J Virol, 2000. **74**(21): p. 10153-64.
276. Flodstrom, M., et al., *Target cell defense prevents the development of diabetes after viral infection.* Nat Immunol, 2002. **3**(4): p. 373-82.
277. Taylor, K., Hyöty, H., Toniolo, A., Zuckerman, A. (eds), *Diabetes and Viruses*, 2013, Springer. p. 373.
278. Laitinen, O.H., et al., *Coxsackievirus B1 is associated with induction of beta-cell autoimmunity that portends type 1 diabetes.* Diabetes, 2014. **63**(2): p. 446-55.
279. Richer, M.J., et al., *Toll-like receptor 4-induced cytokine production circumvents protection conferred by TGF-beta in coxsackievirus-mediated autoimmune myocarditis.* Clin Immunol, 2006. **121**(3): p. 339-49.
280. Horwitz, M.S., et al., *Transforming growth factor-beta inhibits coxsackievirus-mediated autoimmune myocarditis.* Viral Immunol, 2006. **19**(4): p. 722-33.
281. Boettler, T. and M. von Herrath, *Protection against or triggering of Type 1 diabetes? Different roles for viral infections.* Expert Rev Clin Immunol, 2011. **7**(1): p. 45-53.
282. Kemball, C.C., et al., *Coxsackievirus B3 inhibits antigen presentation in vivo, exerting a profound and selective effect on the MHC class I pathway.* PLoS Pathog, 2009. **5**(10): p. e1000618.
283. Mukherjee, A., et al., *The coxsackievirus B 3C protease cleaves MAVS and TRIF to attenuate host type I interferon and apoptotic signaling.* PLoS Pathog, 2011. **7**(3): p. e1001311.
284. Casanova, J.L., L. Abel, and L. Quintana-Murci, *Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics.* Annu Rev Immunol, 2011. **29**: p. 447-91.
285. Wong, F.S., et al., *The role of Toll-like receptors 3 and 9 in the development of autoimmune diabetes in NOD mice.* Ann N Y Acad Sci, 2008. **1150**: p. 146-8.
286. Munz, C., et al., *Antiviral immune responses: triggers of or triggered by autoimmunity?* Nat Rev Immunol, 2009. **9**(4): p. 246-58.
287. Banchereau, R., A.M. Cepika, and V. Pascual, *Systems approaches to human autoimmune diseases.* Curr Opin Immunol, 2013. **25**(5): p. 598-605.
288. Carrero, J.A., et al., *Defining the transcriptional and cellular landscape of type 1 diabetes in the NOD mouse.* PLoS One, 2013. **8**(3): p. e59701.
289. Tattermusch, S., et al., *Systems biology approaches reveal a specific interferon-inducible signature in HTLV-1 associated myelopathy.* PLoS Pathog, 2012. **8**(1): p. e1002480.
290. Wang, Y., et al., *Genome-wide dynamic transcriptional profiling in Clostridium beijerinckii NCIMB 8052 using single-nucleotide resolution RNA-Seq.* BMC Genomics, 2012. **13**: p. 102.

291. Pocock, S.J., *The simplest statistical test: how to check for a difference between treatments.* BMJ, 2006. **332**(7552): p. 1256-8.
292. Adiconis, X., et al., *Comparative analysis of RNA sequencing methods for degraded or low-input samples.* Nat Methods, 2013. **10**(7): p. 623-9.
293. Kozak, C.A., *Origins of the Endogenous and Infectious Laboratory Mouse Gammaretroviruses.* Viruses, 2014. **7**(1): p. 1-26.
294. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
295. Pasare, C. and R. Medzhitov, *Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells.* Science, 2003. **299**(5609): p. 1033-6.
296. Poffenberger, M.C. and M.S. Horwitz, *IL-6 during viral-induced chronic autoimmune myocarditis.* Ann N Y Acad Sci, 2009. **1173**: p. 318-25.
297. Stetson, D.B., *Endogenous retroelements and autoimmune disease.* Curr Opin Immunol, 2012. **24**(6): p. 692-7.
298. Mason, M.J., et al., *Low HERV-K(C4) copy number is associated with type 1 diabetes.* Diabetes, 2014. **63**(5): p. 1789-95.
299. Russell, S.J., et al., *Outpatient glycemic control with a bionic pancreas in type 1 diabetes.* N Engl J Med, 2014. **371**(4): p. 313-25.
300. Pugliese, A., et al., *The Juvenile Diabetes Research Foundation Network for Pancreatic Organ Donors with Diabetes (nPOD) Program: goals, operational model and emerging findings.* Pediatr Diabetes, 2014. **15**(1): p. 1-9.
301. Pugliese, A., et al., *New insight on human type 1 diabetes biology: nPOD and nPOD-transplantation.* Curr Diab Rep, 2014. **14**(10): p. 530.
302. Ehlers, M.R. and G.T. Nepom, *Immune-directed therapy for type 1 diabetes at the clinical level: the Immune Tolerance Network (ITN) experience.* Rev Diabet Stud, 2012. **9**(4): p. 359-71.
303. Manis, J.P., *Knock out, knock in, knock down--genetically manipulated mice and the Nobel Prize.* N Engl J Med, 2007. **357**(24): p. 2426-9.
304. Gaddy, D.F., et al., *In vivo expression of HGF/NK1 and GLP-1 From dsAAV vectors enhances pancreatic ss-cell proliferation and improves pathology in the db/db mouse model of diabetes.* Diabetes, 2010. **59**(12): p. 3108-16.
305. Broquet, A.H., et al., *RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus-infected intestinal epithelium.* J Immunol, 2011. **186**(3): p. 1618-26.
306. Reder, A.T. and X. Feng, *Aberrant Type I Interferon Regulation in Autoimmunity: Opposite Directions in MS and SLE, Shaped by Evolution and Body Ecology.* Front Immunol, 2013. **4**: p. 281.
307. Liu, Y., J. Zhou, and K.P. White, *RNA-seq differential expression studies: more sequence or more replication?* Bioinformatics, 2014. **30**(3): p. 301-4.
308. Wolf, K.J., et al., *Consumption of acidic water alters the gut microbiome and decreases the risk of diabetes in NOD mice.* J Histochem Cytochem, 2014. **62**(4): p. 237-50.
309. Campbell, J.H., et al., *Host genetic and environmental effects on mouse intestinal microbiota.* ISME J, 2012. **6**(11): p. 2033-44.

Appendix A: Knowledge translation and science communication

My fascination in scientific research was initially sparked by my grandfather's stories of exciting travels on icebreaker ships through the Arctic and across the Atlantic Ocean to run and analyze experiments. Growing up watching David Suzuki on the Nature of Things and reading amazing discoveries in the monthly delivered National Geographic fueled my interest in scientific discovery and the art of scientific journalism. From my childhood through my academic career I have maintained a strong interest in all facets of science by following online Science magazines and the Science and Health sections of newspapers and news stations like the BBC. Now as a researcher I have realized and embraced the benefits in being aware and understanding different fields of science. Various methods of science communication (print and electronic media, video clips etc.) have facilitated my understanding of new discoveries from across different fields and have allowed me to integrate a variety of scientific thought in to my own experimental design and analysis.

Science communication is not only important for progress in the scientific community, but also for the intellectual progress and participation of the general public. As a citizen of the world you are affected by discoveries in science, nature, and health fields at personal, governmental, and societal levels, which is why it is important that proper communication of scientific discoveries is achieved. Science journalists and writers become a megaphone for researchers that can pitch and advocate scientific findings to both the scientific community and general public at a level staying true to the facts and still, uncomplicated by troublesome scientific jargon. I am drawn to science communication for the opportunity to research and learn new scientific discoveries and share these discoveries with my peers and the public, as well as the potential to immerse myself in the art of combining the written word with new media technology.

With advancements in digital technology, scientific magazines, newspapers, and their journalists can communicate and teach exciting, but complicated results to the public using video, images and interactive media.

At this stage in my scientific career, at the end of my Doctorate in Microbiology and Immunology, I am comfortably adept in searching, interpreting and documenting scientific literature. For five years, under the supervision of Dr. Horwitz, I have received outstanding training in virus-induced autoimmunity research. Other projects that I have pursued in my graduate studies include the design of a knock-in transgenic mouse model and the use of vitamin D as a therapeutic for type 1 diabetes. I have also written an encyclopedia entry, a book chapter, grant proposals, reviewed papers, written abstracts, designed posters and presentations for conferences.

Prior to my graduate studies I had the opportunity to intern in four different research environments, where I worked with clinical oncologists, analytical chemists, protein biochemists, antibody engineers, virologists and immunologists. Working with this range of scientific expertise afforded not only extensive technical training, but also exposure to diverse behaviours in scientific thought.
In 2012 I completed the acclaimed Banff Science Communications Program in Banff, Alberta. Participating in the Banff program afforded the opportunity to interact and learn from renowned science communication professionals- Jay Ingram, John Rennie, Maggie Koerth-Baker, Mary-Anne Moser, Henry Kowalski and Robert Davidson- who greatly helped refine my skills as a scientific communicator. At the Banff Centre I wrote and edited a journalistic news report about a scientific discovery and for another team project, I designed a website with a graphic designer and scripted, edited and recorded a podcast and video to promote a newly created science communication company.

This past fall, I finished filming the web series The Lab that I co-created and co-wrote along with another alumnus of the Banff Science Communication program. I scripted the 6 episodes of Season 1, drawing on humor from real life molecular biology lab experience.

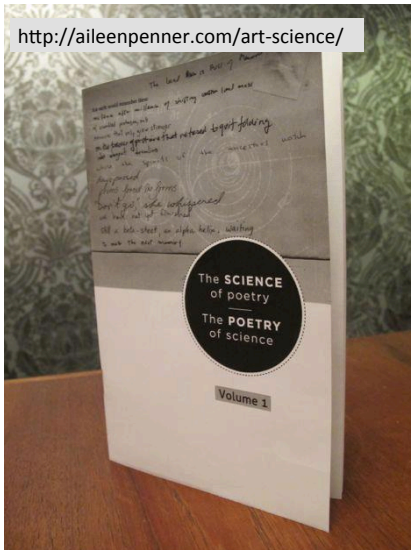
I have enriched my portfolio further with writing blog posts for my own site pamlincez.wordpress.com as well as guest blog for various online magazines and non-profit organizations including the Society of Canadian Women in Science and Technology and the Canadian Science Writer's Association. I have also worked with poets and other scientists in a Science and Poetry workshop to write and present a Science Poem.

At my university I have written Department newsletter updates, participated in Day of Immunology and Let's Talk Science programs teaching elementary kids basic and exciting experiments in Microbiology and Immunology and have assisted teaching three different undergraduate courses in the Faculty of Science including a Science Communications course.

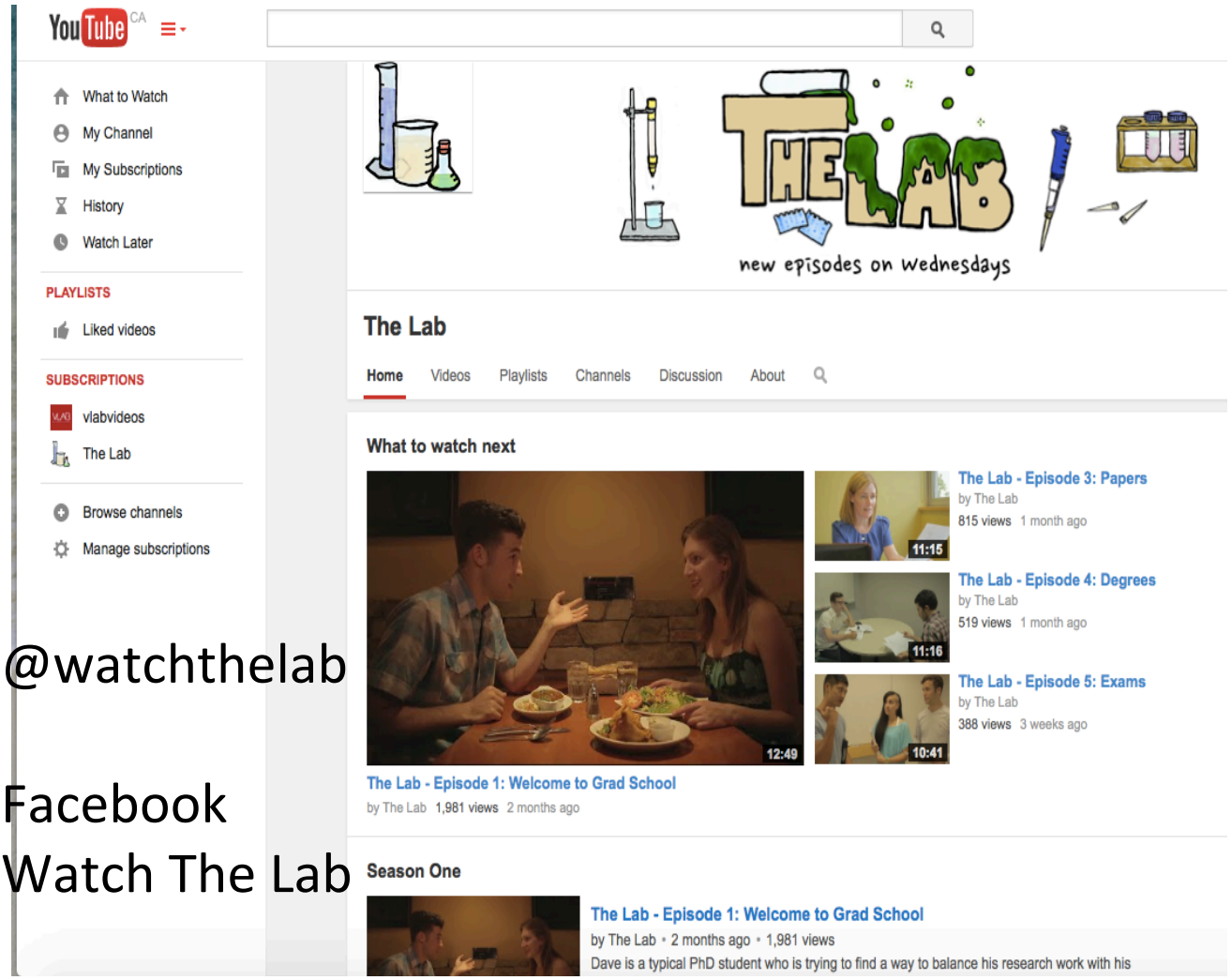
Enriching my science communication skills with all available mediums as a science communicator has been and will continue to be a rewarding and enjoyable process.

At the Banff Centre I had the opportunity to devise a new science communication company. The mock company Maggie Pie can be found here : http://www.banffscience.ca/projects/2012-group-projects/MaggiePie/index.html

Following the Banff program I had the opportunity to work in a workshop with poets and write science poetry. My poem Frustration can be found at aileenpenner.com/art-science/



In the fall of 2014, I finished filming a web series called the The Lab for which I am a co-creator and co-author. I contributed writing to 6 full episodes that can be viewed on YouTube. The comedy show depicts the many interesting adventures and interactions a grad student experiencing as they work in a lab.



In my ‘spare’ time I contribute to my own blog and guest blog for the Canadian Science Writer’s Association, Sustainable Balance and The Society for Canadian Women in Science and Technology. Examples and links to my work can be found on my website at pamlincez.wordpress.com.