INNATE IMMUNE SIGNALING DRIVES PATHOGENIC EVENTS LEADING TO AUTOIMMUNE DIABETES

A Dissertation Presented

By

Natasha Qaisar

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DOCTOR OF PHILOSOPHY

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INNATE IMMUNE SIGNALING DRIVES PATHOGENIC EVENTS LEADING TO AUTOIMMUNE DIABETES

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By
Natasha Qaisar

This work was undertaken in the Graduate School of Biomedical Sciences

Under the mentorship of

Jennifer P. Wang, M.D., Thesis Advisor
Egil Lien, Ph.D., Member of Committee
Stuart Levitz, M.D., Member of Committee
Robert Finberg, M.D., Member of Committee
John Mordes, M.D., Member of Committee
Elizabeth Blankenhorn, Ph.D., External Member of Committee
John Harris, M.D., Ph.D., Chair of Committee

Mary Ellen Lane, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

April 26, 2018
DEDICATION

To my Mama and Baba.
ACKNOWLEDGMENTS

This dissertation would not be possible without the contributions of my community of mentors, colleagues, friends and family, whose support has been vital to achieve this milestone and who unequivocally share this victory and joy with me.

Thanks are due firstly to my advisor, Dr. Jennifer Wang, for her overwhelming generosity with time, for her meticulous instruction, for her selfless dedication and for her excellent mentorship, advice and support throughout this journey. I have been fortunate to enjoy immense scientific freedom and good humor under her mentorship but have also received her unique perspective when I desperately needed it. I thank her with all my heart for giving me the opportunity to be her student and to train me as a scientist. Also, I am forever grateful for her flexible and supportive approach to mentoring after I became a mother. I can only aspire to mirror her rigor, passion, kindness and brilliance in my future endeavors.

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ABSTRACT

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by the immune-mediated destruction of insulin-producing beta-cells of pancreatic islets, culminating in critical insulin deficiency. Both genetic and environmental factors likely orchestrate an immune-mediated functional loss of beta cell mass, leading to the clinical manifestation of disease and lifelong dependence on insulin therapy. Additional evidence suggests the role of innate and adaptive immune mechanisms leading to inflammation in beta cells mediated by proinflammatory cytokines and chemokines, activation of beta-cell-reactive T cells, and failure of immune tolerance. Viral infections have been proposed as causal determinants or initiating triggers for T1D but remain unproven. Understanding the relationship between viral infections and the development of T1D is essential for T1D prevention. Importantly, virus-induced innate immune responses, particularly type I interferon (IFN-I, IFN-α/β), have been implicated in the initiation of islet autoimmunity and development of T1D. The goal of my thesis project is to investigate how the IFN-I signaling pathway affects the development of T1D using the LEW.1WR1 rat model of autoimmune diabetes. My hypothesis is that disrupting IFN-I signaling via functional deficiency of the IFN-I interferon receptor (IFNAR) prevents or delays the development of virus-induced diabetes. For this purpose, I generated IFNAR subunit 1(IFNAR1)-deficient LEW.1WR1 rats using CRISPR-Cas9 genome editing and confirmed the functional disruption of IFNAR1. The absence of IFNAR1 results in a significant delay in onset and frequency of diabetes following poly I:C challenge and
reduces the incidence of insulitis after poly I:C treatment. The frequency of diabetes induced by Kilham rat virus (KRV) is also reduced in IFNAR1-deficient LEW.1WR1 rats. Furthermore, I observe a decrease in CD8+ T cells in spleens from KRV-infected IFNAR1-deficient rats relative to that in KRV-infected wild-type rats. While splenic regulatory T cells are depleted in WT rats during KRV-infection, no such decrease is observed in KRV-infected IFNAR1-deficient rats. A comprehensive bulk RNA-seq analysis reveals a decrease of interferon-stimulated genes and inflammatory gene expression in IFNAR1-deficient rats relative to wild-type rats following KRV challenge. Collectively, the results from these studies provided mechanistic insights into the essential role of virus-induced, IFN-I-initiated innate immune responses in the early phase of autoimmune diabetes pathogenesis.
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Chapter II is reprinted from the journal Diabetes (Permission not required)
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APCs</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>APOBEC</td>
<td>apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like</td>
</tr>
<tr>
<td>BACH2</td>
<td>basic leucine zipper transcription factor 2</td>
</tr>
<tr>
<td>BB</td>
<td>biobreeding</td>
</tr>
<tr>
<td>BBDP</td>
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<td>base pair</td>
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<td>cell adhesion molecules</td>
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<td>Database for Annotation, Visualization and Integration Discovery</td>
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<td>i.p.</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>IDIN</td>
<td>interferon regulatory factor 7 driven inflammatory network</td>
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<td>minute virus of mice</td>
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<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
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<td>PFU</td>
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<tr>
<td>PKR</td>
<td>IFN-inducible double-stranded RNA-dependent protein kinase</td>
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<tr>
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RCMV  rat cytomegalovirus
RIG-I  retinoic acid-inducible gene I
RMV1  rat minute virus 1
RNA   ribonucleic acid
rRNA  ribosomal RNA
RSEM  RNA-Seq by Expectation Maximization
RV    rat virus
SDAV  sialodacryoadenitis virus
SFP   specific pathogen-free
sgRNA single guide RNA
SLE   systemic lupus erythematosus
SNPs  single nucleotide polymorphisms
ssDNA single-stranded DNA
STAT1 signal transducer and activator of transcription 1
T1D   Type 1 diabetes
T2D   Type 2 diabetes
TCR   T cell receptor
Teff  T-cell effector
TGF-beta transforming growth factor beta
T_h1  T helper 1
TLR   Toll-like receptor
Treg  regulatory T-cell
TRIMs tripartite motifs
tTreg thymus-derived Treg cells
TYK2  tyrosine kinase 2
UBASH3A ubiquitin-associated and SH3 domain-containing protein
Ubd   diubiquitin
UMass University of Massachusetts Medical School
VAF   viral antibody-free
WT    wild type
ZnT8  zinc transporter 8
Chapter I, which serves as the Introduction to the dissertation, includes new, original text written by Natasha Qaisar that is incorporated with text from the recently published manuscript cited below. The manuscript was written by Natasha Qaisar and edited by Dr. Agata Jurczyk and Dr. Jennifer P. Wang.

CHAPTER I
INTRODUCTION

Type 1 diabetes

Diabetes mellitus comprises a group of metabolic disorders characterized by impaired insulin secretion, insulin function or both. Broadly, diabetes mellitus cases fall into either the immune-associated type 1 diabetes (T1D) (also known as “insulin-dependent diabetes” or “juvenile-onset diabetes”) or obesity-related type 2 diabetes (T2D) (American Diabetes Association 2009). A 2014 comprehensive analysis by a WHO-supported Non-Communicable Disease Risk Factor Collaboration health scientists reported that the global prevalence of diabetes mellitus has alarmingly increased as the number of affected individuals quadrupled from 108 million to 422 million between 1980 and 2014 (N. C. D. Risk Factor Collaboration 2016).

T1D accounts for 5-10% of all diabetes mellitus cases and is historically considered the most prevalent form of diabetes diagnosed in children (Daneman 2006). In the USA, T1D accounts for approximately 80% of pediatric diabetes cases (Redondo, Steck et al. 2017), with peaks in presentations occurring between 5-7 years of age and at or near puberty (Atkinson, Eisenbarth et al. 2014). However, the disease can be diagnosed at any age, with ~30% of patients becoming symptomatic after 18 years of age (Redondo, Steck et al. 2017). Globally, the incidence of T1D has been increasing by 2.8-4.0% per year (Dabelea, Mayer-Davis et al. 2014) with an estimate of doubling in incidence in children every 20 years (Jacobsen and Schatz 2016). While most organ-specific autoimmune
disorders display strong female sex bias, T1D equally affects both sexes in children under 15 years of age but is slightly more prevalent in 15-40-year-old males of European origin (Gale and Gillespie 2001). Globally, Finland has the highest prevalence of T1D among children followed by Sweden (Ludvigsson 2017). In addition, the T1D-associated complications and comorbidities, and the disproportionately higher cost of ~$14.4 billion in the U.S. of managing T1D compared to T2D (Tao, Pietropaolo et al. 2010) necessitates finding effective preventive therapies to reduce the burden of this disease on public health.

Historically and continuing to the present, T1D is considered an autoimmune-mediated, in particular T-cell mediated, disorder although these views have been recently challenged (Skog, Korsgren et al. 2013, Mannering, Pathiraja et al. 2016). Nonetheless, immunological contributions (presence of autoantibodies or genetic predisposition for immune-related genes) resulting from a complex interplay between genetic and environmental factors are believed to cause the progressive loss of insulin-producing beta-cell mass and/or function in the pancreatic islets, leading to persistent hyperglycemia (Atkinson, Eisenbarth et al. 2014). In children and adolescents, the disease clinically presents with the three classic symptoms of polyuria, polydipsia, and rapid weight loss, and approximately one-third present with life-threatening diabetic ketoacidosis (Klingensmith, Tamborlane et al. 2013). These classic symptoms may not be present in adults at the time of disease onset. Clinical diagnosis for T1D is confirmed with random plasma glucose levels of \( \geq 200 \text{ mg/dL} \) [11.1 mmol/L] and a requirement for exogenous insulin (American Diabetes Association 2018).
In the majority of newly diagnosed T1D patients, clinical T1D is also defined by the presence of one or more circulating autoantibodies produced against the following beta-cell autoantigens: insulin, glutamic acid decarboxylase (GAD), insulinoma-associated autoantigen 2 (IA2), and zinc transporter 8 (ZnT8) (Regnell and Lernmark 2017). The development of more than two autoantibodies in at-risk children for T1D is associated with progression to clinical disease over the next 15 years (Ziegler, Rewers et al. 2013). In addition to complications resulting from hyperglycemia and hypoglycemia, T1D patients are at increased risk for developing cardiovascular disease, retinopathy, neuropathy, renal disease, and diabetic foot complications (Smith-Palmer, Bae et al. 2016) as well as additional autoimmune disorders including Hashimoto's thyroiditis and Graves' disease (collectively referred to as autoimmune thyroid diseases), celiac disease, autoimmune gastritis/pernicious anemia, Addison's disease, and vitiligo (Krzewska and Ben-Skowronek 2016, American Diabetes Association 2018).

**Genetic risk factors in type 1 diabetes**

T1D has a strong genetic component with ~50% risk of T1D conferred by the highly polymorphic human leukocyte antigen (HLA) on chromosome 6. The gene products of some genes in the HLA region are involved in immune response, in particular class I and II major histocompatibility complex (MHC) proteins that are involved in antigen presentation to CD8⁺ and CD4⁺ T cells, respectively (Noble and Valdes 2011, Pociot and Lernmark 2016). In general, the HLA-class II genes (DR, DQ, and DP) confer the greatest T1D risk, where the highest T1D predisposing haplotypes are DR4-
DQA1*03:01-DQB1*03:02 (DR4-DQ8 haplotype) and DRB1*03:01-DQA1*05:01-
DQB1*02:01 (DR3-DRQ2 haplotype), with the presence of DR4-DQ8/DR3-DQ2
genotype associated with overall greatest risk for T1D (Noble and Valdes 2011,
Redondo, Steck et al. 2017). In contrast, protective alleles in HLA-class II genes provide
disease resistance (Erlich, Valdes et al. 2008). HLA-class I genes (A, B, C) also impact
T1D risk independently of class II genes (Erlich, Valdes et al. 2008). The remaining
genetic risks are from several non-HLA loci including the insulin gene (INS), cytotoxic
T-lymphocyte-associated protein 4 (CTLA-4), protein tyrosine phosphatase, non-receptor
type 22 (PTPN22), and interleukin 2 receptor α (IL2RA, CD25)(Noble and Valdes 2011).
Since 2006, the advent of genome-wide association studies (GWAS) has dramatically
increased the number of identified T1D risk loci and more than 50 genetic loci affect
disease susceptibility (Pociot 2017). As of March 2017, the ImmunoBase
(www.immunobase.org) lists 57 loci linked with T1D. Some examples of T1D-risk
associated genes discovered through GWAS include interferon-induced with helicase C
domain 1 (IFIH1) (Smyth, Cooper et al. 2006), protein tyrosine phosphatase, non-
receptor type 2 (PTPN2) (Todd, Walker et al. 2007), the basic leucine zipper transcription
factor 2 (BACH2) (Cooper, Smyth et al. 2008), and ubiquitin-associated and SH3
domain-containing protein A (UBASH3A) (Concannon, Onengut-Gumuscu et al. 2008).
As expected, most of the genes associated with T1D are involved in immune
mechanisms, suggesting that genetic risk may impact responses to environmental factors
such as viral infection.
Environmental risk factors for type 1 diabetes

Although T1D has a strong genetic component, the differences in the concordance rate between monozygotic twins (Insel, Dunne et al. 2015), an increasing incidence of T1D, and geographical and seasonal variance suggest the contribution of non-genetic factors such as environment or lifestyle in its pathogenesis (Principi, Berioli et al. 2017). An increased number of cases are diagnosed in autumn and winter (Moltchanova, Schreier et al. 2009) and spring births are associated with an increased T1D risk (Kahn, Morgan et al. 2009). Despite having similar predisposing HLA genotypes, the incidence of T1D in Finland is six times higher compared to the neighboring Russian Karelia (Kondrashova, Reunanen et al. 2005), implicating the role of environmental factors in T1D. Studies on migrant populations report an increased likelihood of migrants acquiring same risk for T1D as the population in the new area of residence (Oilinki, Otonkoski et al. 2012, Soderstrom, Aman et al. 2012). Additionally, not all islet-autoantibody-positive individuals progress to clinical T1D as evident in fulminant T1D, a subtype of T1D prevalent in Japan that is characterized by a rapid onset of insulin-deficiency and severe hyperglycemia without islet-related autoantibodies.

Several environmental factors have been proposed to influence the epidemiology of T1D. Candidate environmental triggers for T1D include infant and adolescent dietary factors, toxins and chemical contaminants, composition of the gut microbiome, prenatal and postnatal stressors, cesarean delivery, vitamin D deficiency, and viral infections (Rewers and Ludvigsson 2016). Overall, viruses have received particular attention as putative causal determinants based on the evidence from extensive epidemiologic and

**Viral triggers initiating type 1 diabetes**

Enteroviruses are a prime candidate environmental trigger of T1D. Coxsackie B virus (CVB) is a suspected causal factor for T1D based on seroepidemiological, histological, and experimental data (Op de Beeck and Eizirik 2016). Epidemiological studies support a role for viral infections in T1D development; specifically, geographic variation in incidence, seasonality of disease presentation, and an increased incidence of T1D follow enterovirus epidemics (Yeung, Rawlinson et al. 2011). Both enterovirus immunoglobulin M antibodies and viral ribonucleic acid (RNA) are frequently detected in blood of individuals with recent-onset T1D compared with healthy controls (Frisk and Tuvemo 2004, Elfaitouri, Berg et al. 2007, Sarmiento, Cabrera-Rode et al. 2007, Schulte, Bakkers et al. 2010). In the Diabetes and Autoimmunity Study in the Young, children positive for islet autoantibodies and enteroviral RNA in blood reportedly progress to diabetes faster compared to children with islet autoantibodies alone (Stene, Oikarinen et al. 2010). Staining of postmortem pancreatic specimens from T1D patients reveals viral proteins (Richardson, Willcox et al. 2009, Richardson, Leete et al. 2013). Enteroviral RNA detection precedes islet autoimmunity by several months in genetically susceptible T1D children from a prospective longitudinal study (Diabetes Prediction and Prevention Project) (Honkanen, Oikarinen et al. 2017). In prospective studies, such as the Environmental Triggers of T1D Study, enteroviruses are frequently detected in the blood
of T1D patients at the islet autoantibody seroconversion stage compared with healthy controls (Cinek, Stene et al. 2014). Pancreatic beta cells from T1D donors contain CVB4 particles, viral capsid protein, and reduced levels of insulin (Dotta, Censini et al. 2007). The presence of sustained, low-grade CVB in biopsies from islets of freshly isolated pancreatic tissue from living recent-onset T1D patients is reported by the Diabetes Virus Detection (DiViD) study (Krogvold, Edwin et al. 2015). An increased frequency of coxsackie and adenovirus receptor (CAR) expression is observed in the pancreatic islets of both T1D and autoantibody-positive non-diabetic donors compared with non-diabetic controls, suggesting that enhanced CAR expression promotes virus spread to islets during T1D pathogenesis (Hodik, Anagandula et al. 2016). Children with incompetent antibody responses against CVB capsid proteins develop early insulin-targeting autoimmunity with impaired ability to clear CVB infections in early childhood (Ashton, Eugster et al. 2016). Moreover, in the Environmental Determinants of Diabetes in the Young study, recent respiratory infections in young children are associated with increased risk of islet autoimmunity (Lonnrot, Lynch et al. 2017). Similarly, early childhood infections are linked to islet autoimmunity and progression to T1D in children having HLA-conferred T1D risk (Mustonen, Siljander et al. 2017).

Despite abundant data suggesting associations between enteroviral infection and T1D, proof of causality is lacking. Specific mechanisms by which viruses trigger T1D are unknown, but viral infections may modulate immune responses in susceptible individuals to promote T1D.
The type I interferon signaling pathway

The innate immune system consists of pattern-recognition receptors (PRRs) which detect conserved pathogen-derived structural motifs known as pathogen-associated molecular patterns (PAMPs) (Niedzwiedzka-Rystwej, Ratajczak et al. 2017). PRR-activated signaling following recognition of viral PAMPs establishes an effective immune response driven by interferon (IFN)-I (α, β), IFN-II (γ), and IFN-III (λ1–4), and induces IFN-stimulated genes (ISGs) which encode mediators for establishing an antiviral and inflammatory state in the host (Niedzwiedzka-Rystwej, Ratajczak et al. 2017). The multigene family of human IFN-I consists of IFN-α (13 variants), IFN-β, and several less-defined members (IFN-κ, ε, and υ) which signal through the cognate IFN-α/β receptor (IFNAR) comprised of the IFNAR1 and IFNAR2 subunits (McNab, Mayer-Barber et al. 2015). Blood leukocytes and fibroblasts as well as plasmacytoid dendritic cells (DCs) secrete IFN-I (McNab, Mayer-Barber et al. 2015). The differential tissue expression and PRR preference of IFN-I and unique binding affinities to IFNAR result in diverse antiviral, anti-proliferative, and immunomodulatory outcomes (McNab, Mayer-Barber et al. 2015). Canonical binding of IFN-I to IFNAR activates the phosphorylation of tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) which phosphorylate the cytoplasmic effectors signal transducer and activator of transcription 1 (STAT1) and STAT2, leading to dimerization and interaction with IFN regulatory factor (IRF) 9 to form the ISG factor 3 complex (Niedzwiedzka-Rystwej, Ratajczak et al. 2017). Upon nuclear translocation, the ISG factor 3 complex binds to IFN-stimulated response elements in ISG promoters to coordinate the transcriptional induction of hundreds of
ISGs (Niedzwiedzka-Rystwej, Ratajczak et al. 2017). IFIH1, DExD/H-box helicase 58 (DDX58), and Toll-like receptor 3 are also instantly upregulated as ISGs for enhanced viral detection and IFN signaling. ISG protein products, including myxovirus resistance 1, ISG15, protein kinase R, 2,5-oligoadenylate synthetase, and many others, primarily act to restrict viral entry, replication, and release; they may also function to regulate the production of various cytokines and chemokines to orchestrate innate and adaptive responses in various biological and pathological settings. This pattern of upregulated ISGs following IFN-I stimulation, termed the IFN gene signature, is detectable by transcriptome analysis (Banchereau, Cepika et al. 2017).

The IFN-activated inflammatory response is further amplified by recruitment of innate immune cells including macrophages, monocytes, natural killer (NK) cells, and DCs that secrete inflammatory molecules and establish an effector immunological response by antigen presentation to lymphocytes (Niedzwiedzka-Rystwej, Ratajczak et al. 2017). Innate immune responses are likely activated through viral-activated PRRs during beta-cell autoimmunity. Endosomal Toll-like receptors are expressed in islets and their activation by viral replication intermediates results in production of IFN-I(Tai, Wong et al. 2016). The IFIH1-encoded melanoma differentiation-associated protein 5 (MDA5) and DDX58-encoded retinoic acid-inducible gene I are cytosolic sensors. Both sensors contain a helicase domain that binds RNA ligands derived during viral replication (Morse and Horwitz 2017) and a caspase activation and recruitment domain required for mitochondrial antiviral signaling protein (MAVS) downstream signaling (Morse and Horwitz 2017). MAVS signaling leads to the phosphorylation and nuclear translocation
of IRF3 or IRF7 and activation of the nuclear factor-κB pathway. This is followed by transcriptional induction of IFN-I and IFN-III and activation of ISGs, proinflammatory cytokines, and antigen presentation for antiviral immunity (Morse and Horwitz 2017). MDA5 senses double-stranded RNA, polyinosinic:polycytidylic acid (poly I:C), and CVB (Kato, Takeuchi et al. 2006, Wang, Cerny et al. 2010). Compelling data on IFIH1 suggest its critical role for the regulation of innate immune responses in T1D, discussed below.

**Type I interferon pathways and type 1 diabetes susceptibility**

More than 50 genetic loci are implicated in T1D development. The strongest genetic determinant of T1D, HLA class II genes, confer 50% genetic predisposition for disease development. Non-HLA loci are also linked to T1D (Pociot and Lernmark 2016). Novel candidate genes, identified by GWAS, have several naturally occurring single-nucleotide polymorphisms (SNPs) associated with T1D susceptibility (Pociot and Lernmark 2016). These variants are thought to influence T1D development through modulation of innate and adaptive immunity, inflammatory responses, apoptosis, endocrine function, and responses to environmental cues (Santin and Eizirik 2013). Both protective and risk variants of *IFIH1* have been identified in T1D and other autoimmune diseases (Smyth, Cooper et al. 2006, Nejentsev, Walker et al. 2009, Kato and Fujita 2014). A common risk variant of *IFIH1* is a nonsynonymous SNP at rs1990760 associated with multiple autoimmune disorders including T1D (Cen, Wang et al. 2013). This variant causes the substitution A946T
within MDA5 and is present at an allelic frequency of ~57% in European populations (Gorman, Hundhausen et al. 2017). Homozygous T946/T946 increases the risk of T1D by 35% compared to the nonpredisposing A946 allele (Todd 2014). Peripheral blood mononuclear cells from healthy donors homozygous for the risk variant allele IFIHI A946T and cell lines expressing it display elevated basal and poly I:C-triggered IFN-I production (Gorman, Hundhausen et al. 2017). T1D-protective variants of IFIHI are predicted to be complete or partial loss-of-function mutations, leading to reduced IFN-α/β response. Two such variants, E627* and I923V, are associated with reduced IFN-I levels in poly I:C-stimulated peripheral blood mononuclear cells of T1D patients (Chistiakov, Voronova et al. 2010). SNPs identified in TYK2 are also associated with multiple autoinflammatory and autoimmune disorders including T1D (Wallace, Smyth et al. 2010, Tao, Zou et al. 2011). The nonsynonymous SNP rs2304256 may confer protection against T1D by reducing TYK2 function (Tao, Zou et al. 2011). This SNP causes a missense mutation in the JAK-homology 4 region, a region critical for interactions between TYK2 and IFNAR1. Knocking down TYK2 in human beta cells resulted in diminished levels of poly I:C-induced IFN-α production and IFN-α-induced MHC class I expression (Marroqui, Dos Santos et al. 2015). A TYK2 promoter variant has been associated with an enhanced risk for T1D (Nagafuchi, Kamada-Hibio et al. 2015).

The molecular mechanisms by which loss-of-function or gain-of-function loci specific to innate immune responses influence T1D development continue to be
defined. Given that ~80% of T1D susceptibility genes (Santin and Eizirik 2013), including innate immune genes, are expressed in pancreatic islet beta cells, beta cells may play a direct role in mediating the early events related to T1D pathogenesis. Therefore, it is crucial to understand how beta cells are influenced by the innate immune response leading to their destruction.

The role of type I interferon and the interferon gene signature in type 1 diabetes

IFN signaling is implicated in the initiation of islet autoimmunity and development of T1D (Crow 2010, Newby and Mathews 2017). Enhanced IFN-α was first reported by Foulis et al. (Foulis, Farquharson et al. 1987) in pancreata removed at necropsy from recent-onset T1D diabetes. Subsequent reports show that treatment of hepatitis C virus (Fabris, Floreani et al. 2003) or hairy cell leukemia with IFN-α therapy (Guerci, Guerci et al. 1994) is associated with T1D development. Pancreatic islets isolated postmortem or from biopsies from T1D patients reveal the presence of IFN-I (Huang, Yuan et al. 1995, Dotta, Censini et al. 2007), cytokines (Roep, Kleijwegt et al. 2010, Waugh, Snell-Bergeon et al. 2017), or ISGs (Lundberg, Krogvold et al. 2016). Elevated levels of IFN-α have been detected in sera of T1D patients (Chehadeh, Weill et al. 2000, Mavragani, Niewold et al. 2013). Lundberg et al. (Lundberg, Krogvold et al. 2016) report increased expression of ISGs in insulitic islets from pancreatic biopsies of patients with recent-onset T1D participating in the DiViD study.

Independent work identifies a strong IFN gene signature in peripheral blood
samples from genetically predisposed children prior to development of autoantibodies from two longitudinal birth cohorts, the BABYDIET cohort (Ferreira, Guo et al. 2014) and Diabetes Prediction and Prevention Project (Kallionpaa, Elo et al. 2014). In the BABYDIET cohort, Ferreira et al. (Ferreira, Guo et al. 2014) temporally associate an IFN signature with recent episodes of upper respiratory tract infections and note that the signature is strongest prior to seroconversion and begins to decline after detection of autoantibodies. Most recently, Meyer et al. (Meyer, Woodward et al. 2016) report the presence of self-reacting neutralizing antibodies against IFN-α in autoimmune regulator-deficient patients that conferred protection against T1D development, whereas those patients lacking anti-IFN-α antibodies progressed to T1D.

Studies conducted more than three decades ago have shown the over expression of HLA-class I and IFN-α in human pancreatic tissues (Foulis, Farquharson et al. 1987, Rodriguez-Calvo, Suwandi et al. 2015, Richardson, Rodriguez-Calvo et al. 2016). More recent data from the Network for Pancreatic Organ Donors with Diabetes, DiViD, and archival collection of postmortem samples demonstrate hyperexpression of HLA class I RNA and protein in insulin-containing beta cells from T1D patients (Richardson, Rodriguez-Calvo et al. 2016). Hyperexpressed HLA class I is strongly associated with increased expression of STAT1 in all these cohorts (Richardson, Rodriguez-Calvo et al. 2016). Prior to the symptomatic phase of T1D, beta cells undergo critical pathological changes (Richardson, Morgan et al. 2014). Two recent studies independently analyzed effects of IFN-α on endoplasmic reticulum (ER) stress in beta cells. One report shows that IFN-α treatment of either human islets or the human beta-cell line EndoC-βH1
upregulates ER stress markers and slows the conversion of proinsulin to insulin (Lombardi and Tomer 2017). Another demonstrates the crucial contribution of IFN-α in the early phases of diabetes as a common mediator of HLA class I hyperexpression, excessive inflammation, and elevated expression of ER stress markers (Marroqui, Dos Santos et al. 2017).

The growing evidence from recent studies supports IFN-α-mediated immune effects, including hyperexpressed HLA class I, in the early pathogenic events in T1D. The presence of the IFN-I signature in the early events leading to T1D and its absence in protected individuals strongly implicates viruses in T1D.

**Interferon-III: an additional companion to type I interferon in type 1 diabetes pathogenesis**

The host antiviral innate immune defense also includes IFN-III (IFNλ1 –4). IFN-I and IFN-III induce similar sets of genes following infection. Whereas IFN-I acts globally by targeting almost all nucleated cells, IFN-III utilizes a distinct receptor complex and acts primarily on mucosal epithelial cells (Wack, Terczynska-Dyla et al. 2015). Human primary islets produce IFN-III following infection with CVB3 and treatment of islets with IFN-III results in upregulation of ISGs (Lind, Richardson et al. 2013). Domsgen et al. (Domsgen, Lind et al. 2016) report a role for the common T1D-associated rs1990760 SNP in *IFIH1* in regulating IFN-III-related responses in islets following CVB3 infection. Human islets carrying the protective *IFIH1* genotype T946A have significantly elevated IFN-III and ISG responses to CVB infection compared with islets carrying the risk-
conferring genotype A946T. Novel findings from this study emphasize the need to focus on the immunomodulatory role of IFN-III with respect to host immune response to virus infections in T1D pathogenesis.

Dysregulated interferon: making a case for type 1 diabetes interferonopathy

Type I interferonopathies are a group of rare Mendelian disorders of abnormal upregulated IFN-I that lead to autoinflammation and/or autoimmunity (Rodero and Crow 2016). The inappropriate overproduction of IFN-I is a central common phenotypic feature that originates from molecular defects in genes of functionally diverse biological pathways affecting IFN-I regulation (Rodero and Crow 2016). The upregulated IFN-I and IFN gene signatures are associated with sporadic auto-inflammatory and autoimmune diseases including systemic lupus erythematosus (SLE) (Banchereau, Cepika et al. 2017, Psarras, Emery et al. 2017). Multiple autoimmune disorders including T1D and SLE exhibit significant genetic overlap (Richard-Miceli and Criswell 2012). Indeed, SLE has been described as an interferonopathy with an ‘overlapping IFN gene signature with T1D’ (Jean-Baptiste, Xia et al. 2017).

Although definitive proof for an IFN-related clinical phenotype in T1D pathogenesis is lacking, a conceptual model in which IFN signaling acts as a common central player in the early stages of T1D is highly conceivable. Viruses such as CVB could establish a persistent infection in beta cells in genetically predisposed individuals, creating a diabetogenic environment characterized by continuous production of IFN-I to sustain local inflammation of the pancreatic islets, eventually leading to islet
autoimmunity.

**Rodent models of type 1 diabetes**

Since the early discovery and testing of secreted insulin in dogs almost a century ago, animal models of T1D have immensely contributed to the understanding and characterization of the pathophysiologic mechanisms of the disease and the development of novel therapeutic agents and treatments. Researchers are heavily reliant on animal models because the human population is outbred and is constantly exposed to chemical and microbial agents, making it difficult to study, test and develop novel therapeutic agents (Mordes, Bortell et al. 2004). In particular, rodent models are indispensable for studying T1D due to their easy accessibility for procuring target tissues and planning clinical interventions. In addition, they provide an excellent system for testing genetic and environmental factors relevant to T1D.

An extensively studied rodent model of T1D is the non-obese diabetic (NOD) mouse. NOD mice spontaneously develop autoimmune diabetes (Makino, Kunimoto et al. 1980) and shares several genetic and etiopathogenic features with human T1D such as susceptibility genes (in particular MHC-loci), the presence of autoantibodies, and T-cell-mediated insulitis (Wicker, Todd et al. 1995, Thayer, Wilson et al. 2010); with influence from the environment, incidence of disease in NOD mice can be influenced by the response to viral infections (Oldstone 1988, Serreze, Ottendorfer et al. 2000). However, differences exist in the cellular architecture and composition of the islets, the innate and adaptive immune systems, and histopathological features of insulitis in NOD mice compared to human T1D (Brehm, Powers et al. 2012, In't Veld 2014). Such differences
have likely impeded the successful translation of otherwise promising therapies developed in NOD mice to humans (Reed and Herold 2015). Collectively, the limitations in correlating results between NOD-based studies and human T1D necessitate the need for an alternative rodent model for studying T1D—the rat model of autoimmune diabetes.

**Rat models of type 1 diabetes, in particular the LEW.1WR1 rat model**

The laboratory rat, *Rattus norvegicus*, has served as an important model for investigating human diseases and for the identification, validation, and testing of potential drugs for improving human health for over 150 years (Shimoyama, Smith et al. 2017). Before the advent of the NOD mice, several rat models of T1D have provided essential foundations for understanding the genetics and immune processes underlying T1D and serve as an invaluable resource for testing and developing therapies (Mordes, Bortell et al. 2004).

Similar to humans and mice, autoimmune diabetes in rats is strongly associated with certain MHC genes, in particular class II MHC. The rat MHC is designated as *RT1* and haplotypes in rats are called *A, B/D* and *C*, respectively, where *RT1 A* and *C* are two class I loci and the *RT1 B/D* (designated as Iddm1) are the two class II loci which are in linkage disequilibrium (Gunther and Walter 2001, Mordes, Bortell et al. 2004). The rat *RT1-B* and *RT1-D* loci are homologous to human HLA-DQ and -DR, respectively (Cort, Habib et al. 2014). The repertoire of rat models of T1D is comprehensive with a variety of spontaneous and inducible models of rats being available as discussed below.
1. **Spontaneous rat models of type 1 diabetes:** Rats can develop diabetes “spontaneously” similar to NOD mice under viral antibody-free (VAF) conditions (Mordes, Bortell et al. 2004). The major spontaneous models of rat T1D (Shafrir 2007) include the biobreeding (BB) rats (Mordes, Bortell et al. 2004), Long–Evans Tokushima lean (LETL) (Kawano, Hirashima et al. 1991), Komeda diabetes-prone (KDP) (Yokoi, Komeda et al. 2002), and the LEW.1AR1/Ztm-iddm (Lenzen, Tiedge et al. 2001). The Introduction here will focus on the Diabetes Prone Biobreeding (BBDP/BBdp) rats.

   Diabetes-Prone Biobreeding (BB) rats are the oldest known, extensively studied rat model of T1D. Spontaneous hyperglycemia and ketoacidosis were first observed in the rat colony of non-obese outbred Wistar rats in the Biobreeding Laboratories at Canada in 1974 (Nakhooda, Like et al. 1977). These BB rats were the founders for two major colonies: one inbred colony of spontaneously diabetic rats, named “diabetes-prone BB rat (BBDP)/Wor” (hereafter referred to as “BBDP”), was established at Biomere (formerly known as Biomedical Research Models) in Worcester, Massachusetts (www.biomere.com). A second colony, “BBdp” rats, was an outbred rat colony and remained in Canada (Shafrir 2007).

   Studies on different rat strains have demonstrated that the *RT1 B/D* region is an essential locus for genetic predisposition to T1D in rats and at least one class II *RT1 B/Du* allele is required for the expression of diabetes (Colle 1990, Fuks, Ono et al. 1990). BBDP rats develop diabetes between 50 and 90 days of age after autoimmune destruction of beta cells, and insulitic islets are observed in 100% of diabetic rats, with both sexes equally affected (Like, Guberski et al. 1991). Insulitis in BBDP rats is usually observed
2-3 weeks before overt diabetes, characterized by lymphocytic infiltration of the islets and the presence of detectable serum anti-islet antibodies (Dyrberg, Nakhoooda et al. 1982, Castano and Eisenbarth 1990). Infiltration by macrophages and dendritic cells precedes invasion of NK cells, T cells, and B cells; macrophage infiltration is a prerequisite for the lymphocytic invasion in prediabetic BBDP rats (Hanenberg, Kolb-Bachofen et al. 1989). Furthermore, eosinophils and the eosinophil-recruiting chemokine eotaxin may also be involved in the insulitis process in BBDP rats (Hessner, Wang et al. 2004). Interestingly, the characteristic persistent mononuclear cell accumulation around the islets, termed “peri-insulitis” as observed in NOD mice is typically absent in diabetic rats. Instead, the morphology of insulitis in rats, including BB rats, more closely mimics that for human insulitis than in NOD mice (Kolb, Worz-Pagenstert et al. 1996, Zipris 1996).

A unique feature of all BBDP rats is severe peripheral T cell lymphopenia due to a mutation in the *Gimap5* gene, characterized by apoptosis of peripheral T cells soon after their migration from the thymus (Bortell and Yang 2012). It was found that a frameshift mutation in *Gimap5*, a member of the GTPase of the immunity-associated gene family (gimaps; formerly known as IANs; previous other name lyp/iddm1 gene) (Hornum, Romer et al. 2002, MacMurray, Moralejo et al. 2002), is required for T1D onset in these rats (Awata, Guberski et al. 1995). The deficiency of GIMAP5 protein causes mitochondrial dysfunction in T cells and T cell-specific apoptosis (Pandarpurkar, Wilson-Fritch et al. 2003). Interestingly, polymorphisms in human *GIMAP5* have been linked with increased risk of T1D (Shin, Janer et al. 2007), SLE (Hellquist, Zucchelli et
al. 2007) and asthma (Heinonen, Laine et al. 2015). It was further demonstrated that apoptosis in T cells of Gimap5−/− BBDP rats is mediated through elevated ER stress via upregulated C/EBP homologous protein (CHOP) apoptotic pathway, as diminishing CHOP protein in Gimap5−/− BBDP rat T cells inhibited T cell apoptosis (Pino, O'Sullivan-Murphy et al. 2009). Potential autoantigens in BBDP rats include insulin, GAD, and sulfatide but the primary autoantigen(s) for islet autoimmunity in rats remain largely uncharacterized (Shafrir 2007). Prevention of diabetes in BBDP rats involves administration of insulin, intrathymic islets transplantation, thymectomy, CD8+ T cell depletion, or immunosuppression of the immune system using cyclosporine (Shafrir 2007, Bortell and Yang 2012).

2. Inducible rat models of T1D: As discussed in previous sections, the role of viral infection in the development of T1D in humans is not fully or definitively characterized. As such, the availability of reliable inbred animal models is critical for designing studies aimed at investigating the role of virus and other environmental factors in the development of T1D, or paradoxically, in protection as well as to overcome the challenges associated with studying T1D in humans due to the existing genetic complexity and random exposure to microbes and chemicals in human populations (Mordes, Bortell et al. 2004). For such studies, NOD mice offer limited utility given that viral infections often prevent or reduce the incidence of diabetes as discussed previously (Atkinson and Leiter 1999). In contrast, a predisposition to “induced” autoimmune diabetes with virus or other environmental perturbants is prevalent in rats (Mordes,
Some inducible models of rats include the biobreeding resistant rat (BBDR) (Like, Guberski et al. 1991), LEW.1WR1 (Mordes, Guberski et al. 2005), and the LEW.1AR1 (Wederkind, Weiss et al. 2005). The remainder of Chapter I places focus on the LEW.1WR1 rat model, from which the central hypotheses of this dissertation have been developed.

The LEW.1WR1 rat

Diabetes in LEW.1WR1 rat – Spontaneous: The diabetes-resistant (DR)/Wor sub-line of the BB rat, designated BBDR, was derived from DP progenitors by inbreeding for more than 50 generations in order to select for resistance against diabetes. Under VAF conditions, 0% of BBDR rats develop diabetes in contrast to BBDP rats. BBDR rats share the RT1<sup>a</sup> MHC haplotype of the BBDP rats but are not lymphopenic due to the presence of WT *Gimap5* allele, in contrast to BBDP rats (Ellerman, Richards et al. 1996, Bortell and Yang 2012). However, the BBDR rat is susceptible to diabetes following treatment with immune perturbants such as poly I:C stimulation, viral infections, or regulatory T cell (Treg) depletion (Zipris 2010). The incidence of spontaneous diabetes in a colony of MHC-congenic LEW.1WR1 rats maintained at Biomere led to the emergence of another rat model of T1D, the LEW.1WR1/Wor/Brm (hereafter referred to as LEW.1WR1). These rats were acquired from the Hanover Institute in Hanover, Germany in 1989, initially maintained at the University of Massachusetts Medical School (Worcester, MA) and thereafter at BRM. Following their acquisition, the rats remained diabetes-free until
2000. Beginning in 2000, spontaneous diabetes appeared with a low frequency (~2.5%) at a median age of 59 days (Mordes, Guberski et al. 2005).

Mordes et al. demonstrated that diabetes in LEW.1WR1 rats with the MHC haplotype RT1.A\textsuperscript{a}B/D\textsuperscript{a}C\textsuperscript{a} is characterized by hyperglycemia, glucosuria, ketonuria, and polyuria, and that these symptoms can be ameliorated with exogenous insulin therapy. Both sexes are equally affected. Histopathological analysis of the islets from the diabetic animals shows “end-stage” insulitis, characterized by the absence of beta cells, and intact alpha and delta cells. In agreement with the islet pathology of BBDP and BBDR rats, the islets in diabetic LEW.1WR1 rat are also devoid of “peri-insulitis” lesions, a peculiar feature of NOD mice but not of human and rat T1D (Mordes, Guberski et al. 2005). It is known that the development of T1D increases the risk of other autoimmune diseases including Hashimoto’s thyroiditis and Graves’ disease (collectively referred to as autoimmune thyroid diseases), celiac disease, autoimmune gastritis/pernicious anemia, Addison’s disease, and vitiligo (Krzewska and Ben-Skowronek 2016). In line with this data, the LEW.1WR1 rats also develop collagen-induced arthritis similar to BBDR, although they do not develop lymphocytic thyroiditis spontaneously (Mordes, Guberski et al. 2005).

Immunophenotypic analysis of LEW.1WR1 rats have shown that unlike BBDP rats, these rats are not lymphopenic, having comparable percentages of T cells including CD4\textsuperscript{+}, CD8\textsuperscript{+}, and ART2\textsuperscript{+} cells (now Tregs) to those analyzed in the ancestral LEW rats and the BBDR rat in the lymphoid organs including spleen, cervical lymph nodes, mesenteric lymph nodes, and pancreatic lymph nodes (Mordes, Guberski et al. 2005).
Diabetes in LEW.1WR1 rat—Induced

i. Diabetes induction by Kilham rat virus infection: The genus Paroviruses (from *Parvus*—Latin for “small”) belongs to the family *Parvoviridae* that includes small non-enveloped viruses having a linear, single-stranded (ss)DNA of ~5 kb, encapsidated within a simple icosahedral protein coat. Due to absence of accessory proteins and a duplex transcription template, paroviruses depend on actively-dividing mitotic (S-phase) host cells to utilize DNA synthesis machinery of the host for generating complementary-sense DNA strand to initiate replication cycle (Cotmore and Tattersall 2007). Paroviruses have evolved several mechanisms to evade the host’s antiviral mechanisms including antiviral type I IFN responses (Mattei, Cotmore et al. 2013).

Kilham rat virus (KRV), synonymous with rat virus (RV) and parovirus R-1, belongs to “rat virus group” that also include rat minute virus 1 (RMV1) and H-1 parovirus. Together with the mouse paroviruses such as the prototype minute virus of mice (MVM), these subgroups of paroviruses form a broadly related but serologically diverse cluster of “rodent paroviruses.” KRV was discovered by Kilham and Olivier in 1959 (named RV at that time) by isolation from rats with experimental tumors and is remarkably resistant to heat and desiccation (National Research Council (US) 1991).

The common natural hosts for KRV infection are the laboratory and wild rats (*Rattus norvegicus*) but experimental infection in other species has been reported. It is also a frequent contaminant of a number of cultured cells lines and transplantable tumors (Besselsen, Besch-Williford et al. 1995). No case of human infection with KRV has been
reported, but an acute infection with human parvovirus B19 has been associated with T1D, rheumatoid arthritis, and Graves’ disease (Munakata, Kodera et al. 2005). As an autonomously replicating parvovirus, KRV infects S-phase cells and replicates in the nucleus of dividing cells and thus, has a propensity for rapidly proliferating and growing tissues such as during fetal development and early life. Besides fetal and neonatal hosts, KRV has a natural tendency to infect adult tissues with actively dividing cell populations such as cells of bone marrow, lymphoid organs, and gut epithelium (Jun and Yoon 2003, Cotmore and Tattersall 2007).

The first evidence for the role of virus-induced autoimmune diabetes came from the analysis of unexplained outbreaks of spontaneous diabetes in a BBDR rat breeding colony maintained under less stringent specific pathogen-free (SFP) conditions, which temporally coincided with changes in the viral serological profiles of sentinel rats. Serological testing was positive for KRV as well as other viruses including Sendai virus, sialodacryoadenitis virus (SDAV), and H1 virus (Thomas, Woda et al. 1991). To test the hypothesis that KRV infection contributed to lymphocytic insulitis and diabetes in spontaneously diabetic BBDR rats, a plaque-purified pancreatic isolate from a KRV-seropositive diabetic BBDR rat (named KRV-UMASS) was inoculated into BBDR rats. KRV-UMASS induced autoimmune diabetes in 31% of naïve BBDR rats under VAF conditions. In contrast, SDAV-treated BBDR rats remained diabetes-free and insulitis-free, supporting the specificity of KRV as a diabetes inducer (Guberski, Thomas et al. 1991). Interestingly KRV antigens were detectable in hepatocytes and splenocytes (including lymphocytes and megakaryocytes), but were not identified in the beta cells of
KRV-infected BBDR rats, leading to the hypothesis that KRV-induced diabetes may not involve direct beta cell lysis (Guberski, Thomas et al. 1991).

Studies conducted by Ellerman et al. on MHC congenic and inbred rat strains demonstrated that KRV selectively induces insulitis and T1D in rat strains having class I A\textsuperscript{u} and class II B/D\textsuperscript{u} gene products shared by BBDR and LEW.1WR1 rats, although contributions from RT1\textsuperscript{u} alone to diabetes susceptibility were not sufficient (Ellerman, Richards et al. 1996). Combined treatment with poly I:C and KRV, and depletion of RT6.1\textsuperscript{+} regulatory T cells followed by KRV infection increased the frequency of diabetes to >90\% in BBDR and LEW.1WR1 rats. Furthermore, KRV-induced T1D is T-cell mediated as monoclonal antibodies targeted against TCR\textsuperscript{+}, CD5\textsuperscript{+}, and CD8\textsuperscript{+} T cells protected against KRV-induced diabetes in BBDR and LEW.1WR1 (Ellerman, Richards et al. 1996). In addition, adoptive transfer of concavalin A (ConA; a polyclonal mitogen that activates all T cells)-activated splenocytes from KRV-infected BBDR rats conferred diabetes into RT1\textsuperscript{u}-compatible recipient rats, which were not naturally susceptible to direct KRV-induced diabetes. These results demonstrated that KRV infection of susceptible rats involve the activation of diabetogenic class II\textsuperscript{u}-restricted T cells (Ellerman, Richards et al. 1996).

Tirabassi et al. tested the diabetogenic potential of other viruses including rat cytomegalovirus (RCMV), H-1, vaccinia, and CVB4 viruses in LEW.1WR1 rats (Tirabassi, Guberski et al. 2010). They reported that RCMV alone or in combination with KRV induces diabetes in 60\% or 100\% of treated rats, respectively. Diabetes failed to be induced with H-1 virus (0\%) or CVB4 (0\%) and only 7\% of rats developed diabetes
following vaccinia virus challenge. The failure to induce diabetes with the parvovirus H-1, a close homolog of KRV sharing ~98% identity at the genomic level, in the LEW.WR1 rats is a consistent observation with the data from BBDR rats (Tirabassi, Guberski et al. 2010). Work from the Mordes and Blankenhorn groups established Iddm14 as a dominant diabetes susceptibility locus in the LEW.1WR1 rat following viral infection (Mordes, Leif et al. 2002, Blankenhorn, Rodemich et al. 2005, Blankenhorn, Cort et al. 2009). Beyond requirements for RT1.B/D\* and Iddm14, another locus, Iddm37 was found to significantly modify the incidence of diabetes in response to poly I:C+KRV challenge (Mordes, Leif et al. 2002, Blankenhorn, Rodemich et al. 2005, Blankenhorn, Cort et al. 2009).

**ii. Diabetes induction by poly I:C:** Poly I:C is a synthetic double-stranded RNA which is a potent stimulator of immune system and is commonly used as experimental surrogate for viral infection, primarily due to its ability to activate type I IFN responses. Historically, and at present, poly I:C has been used to induce diabetes in rats, including spontaneously diabetic rat models such as BBDP (Ewel, Sobel et al. 1992). Work by Ellerman & Like revealed that treatment with poly I:C induces insulitis and diabetes in LEW.1WR1 rats and histopathological analysis of islets revealed lymphocytic insulitis and selective beta cells destruction (Ellerman and Like 2000). Analysis of the inflammatory cells infiltrating islets showed the presence of monocyte/macrophage lineage cells and CD8\(^+\) T cells. Adoptive transfer of Con-A activated splenocytes from poly I:C treated LEW.1WR1 rats to Class II\(^\alpha\)-compatible recipient rats induced diabetes,
suggesting that poly I:C generates diabetes-transferring spleen cells (Ellerman and Like 2000). In addition to poly I:C, treatment of LEW.1WR1 rats with the Toll-like receptor 4 agonist LPS failed to induce diabetes in rats (Mordes, Guberski et al. 2005).

iii. Diabetes induction by Treg depletion: Earlier studies on BB rats recognized the importance of putative Tregs, initially defined as CD4⁺ART2⁺ in rats (Mordes, Gallina et al. 1987), in the prevention of diabetes (Rossini, Mordes et al. 1983, Greiner, Handler et al. 1986, Burstein, Mordes et al. 1989). ART2 (formerly designated RT6) is a rat maturational T cell alloantigen with nicotinamide adenine dinucleotide (NAD) glycohydrolase activity. It has immunoregulatory properties including Treg function and identification and exists in both cell surface and soluble forms (Bortell, Kanaitsuka et al. 1999, Bortel, Waite et al. 2001). Specifically, these studies demonstrated that BBDP rats lacked peripheral ART2⁺ T cells (Greiner, Handler et al. 1986) and adoptive transfer of normal histocompatible T cells to BBDP rats partially remediated the peripheral T cell lymphopenia and prevented diabetes, the protective mechanism for this effect was attributed to donor-derived CD4⁺RT6⁺ cells (Rossini, Mordes et al. 1983, Rossini, Faustman et al. 1984, Mordes, Gallina et al. 1987, Burstein, Mordes et al. 1989). In contrast, BBDR treated with anti-ART2⁺ monoclonal antibody (mAb) to deplete CD4⁺ART2⁺ Tregs became diabetic when housed in conventional, non-VAF facility by depleting CD4⁺ART2⁺Treg cells using anti-ART2 mAb (Greiner, Handler et al. 1986). Interestingly, anti-ART2⁺-depleted BBDR rats rarely progress to diabetes in VAF
conditions, indicating the requirement for additional treatment such as poly I:C to induce diabetes (Guberski, Thomas et al. 1991, Thomas, Woda et al. 1991).

Consistent with reports from BBDR rats, Mordes et al. demonstrated that combined treatment with both poly I:C and Treg depletion with anti-ART2.1 mAb induced diabetes in 96% of LEW.1WR1 rats within 40 days of starting treatment, comparable to 100% diabetes penetrance observed with poly I:C treatment alone. Strikingly, Treg depletion alone induced diabetes in 46% of the anti-ART2.1 mAb-treated rats (Mordes, Guberski et al. 2005). In contrast, this treatment fails to induce diabetes in BBDR rats housed in SPF barrier facilities (Like 1990).

In summary, the LEW.1WR1 and BBDR rat strains are the only genetically unaltered rat models receptive to viral-induced beta-cell autoimmunity, offering an excellent system to investigate virus-induced innate immune responses occurring before the onset of aggressive autoimmune attack against beta cells. As LEW.1WR1 rats follow a predictable time course of insulitis and overt diabetes, it is possible to mechanistically dissect the innate immune responses, specifically type I IFN-induced signaling, to determine which pathways induce and orchestrate critical events during the early phase of the T1D development.

**Hypothesis and objectives**

The central hypothesis of this dissertation is that type I IFN signaling mediated though IFNAR plays a critical role in the development of autoimmune diabetes in the LEW.1WR1 rats. Furthermore, IFN signaling modulate the adaptive immune system by
tipping the balance in favor of effector T cells such that, when unrestrained, they promote islet autoimmunity, eventually leading to autoimmune diabetes in LEW.1WR1 rats.

Specifically, in the following chapters of this dissertation, we tested our hypotheses by pursuing three goals:

1. **The generation of an IFNAR1 knockout LEW.1WR1 rat to establish the essential role of type I IFN signaling in the development of autoimmune diabetes.** We used CRISPR-Cas9 gene editing to specifically disrupt the IFNAR1 signaling and monitor the development of diabetes following poly I:C or KRV-induced diabetes.

2. **The analysis of immunomodulatory effects of IFN signaling on the effector and regulatory T cell populations in the pathogenesis of virus-induced diabetes.** With the specific goal to investigate the protective effects mediated through functional disruption of IFNAR signaling, we analyzed T cell populations to provide a mechanistic link between innate and adaptive immune systems via IFN signaling.

3. **Dissecting the IFNAR-mediated transcriptional alterations during antiviral innate immune response in rats.** We performed a comprehensive RNA-seq study to characterize IFN and ISGs and inflammatory networks induced at the transcriptional level in the spleen following KRV infection during the early immune response in rats.
Conclusion to Chapter I

Designing curative and preventive therapies for T1D requires a comprehensive understanding of the relationship between viral infections and the development of T1D. A complex interaction between genetic and environmental factors is commonly believed to instigate an autoimmune attack selectively against the beta cells. Virus-induced innate immune responses, specifically IFN-I and the IFN gene signature, orchestrate early events of beta-cell dysfunction preceding islet autoimmunity. Rat models have historically been helpful in improving our understanding of the interaction between genetics and environmental factors, in particular viruses, and the resulting immunological responses emanating from such complex interactions in the development of T1D. This dissertation focuses on investigating the role of critical antiviral innate immune IFN pathways mediated through the IFNAR in the development of T1D in LEW.1WR1 rats. Additional interests of the dissertation include the analysis of the immunomodulatory role of IFN signaling in the adaptive immune response and the transcriptional expression of IFN and inflammatory gene networks.
Preface to Chapter II

Natasha Qaisar designed and performed the experiments, analyzed data, and wrote and critically reviewed the manuscript. Suvana Lin and Glennice Ryan designed and performed experiments, analyzed data, and critically reviewed the manuscript. Chaoxing Yang and Sarah R. Oikemus performed experiments, analyzed data, and critically reviewed the manuscript. Michael H. Brodsky, Rita Bortell, and John P. Mordes designed the experiments and critically reviewed the manuscript. Jennifer P. Wang designed experiments and wrote and critically reviewed the manuscript.

The work presented in Chapter II has been published in Diabetes (Qaisar, Lin et al. 2017).
CHAPTER II
A CRITICAL ROLE FOR THE TYPE I INTERFERON RECEPTOR IN VIRUS-INDUCED AUTOIMMUNE DIABETES IN RATS

2.1. Abstract

The pathogenesis of human type 1 diabetes, characterized by immune-mediated damage of insulin-producing β-cells of pancreatic islets, may involve viral infection. Essential components of the innate immune antiviral response, including type I interferon (IFN) and IFN receptor–mediated signaling pathways, are candidates for determining susceptibility to human type 1 diabetes. Numerous aspects of human type 1 diabetes pathogenesis are recapitulated in the LEW.1WR1 rat model. Diabetes can be induced in LEW.1WR1 weanling rats challenged with virus or with the viral mimetic polyinosinic:polycytidylic acid (poly I:C). We hypothesized that disrupting the cognate type I IFN receptor (type I IFN α/β receptor [IFNAR]) to interrupt IFN signaling would prevent or delay the development of virus-induced diabetes. We generated IFNAR1 subunit-deficient LEW.1WR1 rats using CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-associated protein 9) genome editing and confirmed functional disruption of the Ifnar1 gene. IFNAR1 deficiency significantly delayed the onset and frequency of diabetes and greatly reduced the intensity of insulitis after poly I:C treatment. The occurrence of Kilham rat virus-induced diabetes was also diminished in IFNAR1-deficient animals. These findings firmly establish that alterations in innate immunity influence the course of autoimmune diabetes and support the use of targeted strategies to limit or prevent the development of type 1 diabetes.
2.2. Introduction

Type 1 diabetes (T1D) is a T-cell-mediated autoimmune disease that destroys insulin-producing pancreatic β-cells (Atkinson, Eisenbarth et al. 2014). It is heritable but non-Mendelian, and genetic susceptibility loci are insufficient for predicting diabetes onset; most people with risk alleles never become diabetic (Polychronakos and Li 2011). Interaction of genes with environmental factors has been invoked as a determinant of disease (Akerblom, Vaarala et al. 2002, Hawa, Beyan et al. 2002). Viral infection, particularly with enterovirus, is believed to be a key environmental modulator of T1D, and its possible role in pathogenesis has been reviewed in detail (Kondrashova and Hyoty 2014, Drescher, von Herrath et al. 2015). The mechanisms that underlie viral triggering of T1D remain unclear; β-cell infection, bystander activation, antigenic spreading, and molecular mimicry have been proposed. Alternatively, viruses could prevent T1D through immunoregulation or induction of protective immunity (Boettler and von Herrath 2011).

To gain better insight into the mechanism of virus-induced diabetes, we used a rat model of the disease. Rats are the only naturally occurring virus-induced T1D model that closely resembles that of human T1D in terms of histopathology, pathogenesis, lack of sex bias, and MHC class II association (Mordes, Zipris et al. 2013). Type 1-like autoimmune diabetes, both spontaneous and inducible, is relatively common among inbred rat strains, which, like humans, express a high risk class II MHC haplotype; in rats, this is designated RT1B/Du. Among susceptible rat strains, the LEW.1WR1 strain
has been particularly useful. About 2.5% of LEW.1WR1 rats develop T1D spontaneously, typically during their early reproductive years; both sexes are affected, and islets show insulitis (Mordes, Guberski et al. 2005). Various perturbations of the immune system, however, can efficiently trigger autoimmune diabetes in up to 100% of animals. Perturbants include regulatory T-cell (Treg) depletion, innate immune activation with thrice-weekly doses of polyinosinic:polycytidylic acid (poly I:C), and infection with Kilham rat virus (KRV) or rat cytomegalovirus. LEW.1WR1 rats also develop diabetes at an increased rate (18%) after Coxsackie B serotype 4 (CVB4) infection, but only if pretreated with a low dose of poly I:C daily for 3 days before viral challenge (Tirabassi, Guberski et al. 2010). Of note, weanling but not adult LEW.1WR1 rats are prone to diabetes after any viral challenge, making these animals a faithful model of the generally juvenile aspect of T1D.
After poly I:C challenge or during viral infection, a cascade of cytokines, including type I interferon (IFN) (i.e., IFN-α/β), could contribute to the induction of diabetes in LEW.1WR1 rats (Fig. 2.1). Furthermore, genome-wide association studies (GWASs) have established associations between the risk for human TID and polymorphisms in genes that mediate type I IFN responses, including IFIH1 (Smyth, Cooper et al. 2006, Nejentsev, Walker et al. 2009), Ebi2 (Heinig, Petretto et al. 2010), and Tyk2 (Mein, Esposito et al. 1998, Shimoda, Kato et al. 2000, Izumi, Mine et al. 2015). Thus, we aimed to define the role of type I IFN on the development of autoimmune diabetes in LEW.1WR1 rats by disrupting the IFNAR1 subunit of the type I IFN receptor complex, a key component of IFN signaling. The generation of knockout rats has previously been challenging, but advances in zinc finger nuclease gene targeting (Geurts, Cost et al. 2009) and, more recently, CRISPR-Cas9 technology (Guan, Shao et

Figure 2.1. Type I IFN is induced by poly I:C in sera and pancreata in WT LEW.1WR1 rats. Poly I:C was administered to weanling WT LEW.1WR1 rats at a dose of 1 µg/g body weight by intraperitoneal (i.p.) injection. Samples were collected at 4 and 24 hours. Each point represents a sample from an independent animal and the horizontal bar shows the mean value. **, \( P = 0.0035 \) for serum at 4 hours; ***, \( P = 0.0064 \) for pancreas at 4 hours (unpaired t test). Error bars represent the S.D. Type I IFN values were determined by conventional bioassay as described (Seeds and Miller 2011).
al. 2014) have made this feasible. We generated Ifnar1+/ LEW.1WR1 rats using CRISPR-Cas9 gene editing and challenged weanling wild type (WT) and Ifnar1+/- rats with either poly I:C or KRV and assessed for the development of diabetes. We found that IFNAR1 deficiency protects against diabetes.

2.3. Materials and Methods

**Animals.** LEW.1WR1 rats (RT1B/Du) were from Biomere (Worcester, MA). They develop spontaneous diabetes at a rate of ~2.5% (Mordes, Guberski et al. 2005), but treatment with poly I:C (Mordes, Guberski et al. 2005) or infection with viruses from several families (Tirabassi, Guberski et al. 2010) increases the frequency of diabetes to 30–100%. Animals were housed in viral antibody-free conditions, confirmed monthly to be serologically free of rat pathogens (Mordes, Leif et al. 2002), and maintained in accordance with institutional and national guidelines (Institute of Laboratory Animal Resources (U.S.) 1996).

**Generation of Ifnar1+/- Rats.** An Ifnar1 target region in exon 4, encoding the IFN-binding domain, was disrupted in the genome of the LEW.1WR1 rat using the CRISPR-Cas9 method. The IFNAR1ex4_guide RNA (gRNA) 2 target site (AGGAGAGATGTAGACTA|GTATGG) includes an overlapping SpeI restriction site. The IFNAR1ex4_gRNA3 target site (TCAATTACACGATACGG|ATCTGG) includes an overlapping XhoII restriction site. Note that for both target sites, the cleavage site is
indicated with a vertical line and the protospacer adjacent motif (PAM) sequence is underlined. To confirm high activity of the single guide RNA (sgRNA)/Cas9 nucleases before embryo injection, the guide sequences were cloned into plasmid pX330 (Plasmid #42230; Addgene, Cambridge, MA) (Cong, Ran et al. 2013), using the following primers:

IFNAR1ex4_gRNA2_F 5’-gtggaaggacgaaacaccgAGGAGAGATGTAGACTAGTA-3’
IFNAR1ex4_gRNA3_F 5’-gtggaaggacgaaacaccgTCAATTACACGATAACGGATC-3’
IFNAR1ex4_gRNA2_R 5’-ctatttctagctctaaaacTACTAGTCTACATCTCTCCT-3’
IFNAR1ex4_gRNA3_R 5’-ctatttctagctctaaaacGATCCGTATCGTGTAATTGA-3’

The full target sites, including PAM sequence, were cloned into the nuclease reporter plasmid M427 (provided by M. Porteus, Stanford University) (Wilson, Chateau et al. 2013), using the following primers:

IFNAR1ex4_gRNA2_M427F
5’-gaattcgacgacggcccagGAGAGATGTAGACTAGTTG-3’
IFNAR1ex4_gRNA3_M427F
5’-gaattcgacgacggccCTCAATTACACGATAACGGATCTG-3’
IFNAR1ex4_gRNA2_M427R
5’-aaaattgtgccctctgcCCATAGACATCTCCTCTCTCCT-3’
IFNAR1ex4_gRNA3_M427R
5’-aaaattgtgccctctgcCCAGATCCGTATCGTGTAATTG-3’

The M427 reporter plasmid expresses green fluorescent protein after cotransfection with a nuclease that cleaves the target site. Nuclease activity was
confirmed by examining green fluorescent protein-positive cells after cotransfection into 293T cells with the corresponding nuclease plasmid or with a negative control.

Capped and tailed Cas9 mRNA was prepared using the mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Austin, TX), and gRNA was prepared using the HiScribe T7 High Yield RNA Synthesis Kit (New England BioLabs, Ipswich, MA) as previously described (Wang, Yang et al. 2013). Linear DNA templates for Cas9 mRNA synthesis and sgRNA synthesis through T7 RNA polymerase were prepared by PCR using the pX330 sgRNA plasmids and the following oligonucleotides:

Cas9T7_F

5'-TAATACGACTCACTATAGGGAGAATGGACTATAAGGACCACGCAC-3’

Cas9T7_R

5’-GCGAGCTCTAGGAATTCTTAC-3’

IFNAR1ex4_gRNA2_vT7F

5’-ttaatgcactctataggAGGAGAGATGTAGACTAGTA-3’

IFNAR1ex4_gRNA3_vT7F

5’-ttaatgcactctataggTCAATTACACGATACGGATC-3’

gRNA_RsEq

5’-AAAAAAAGcaccgactcggtgccac-3’

Two independent in vitro-transcribed sgRNAs (50 ng) were coinjected with Cas9 mRNA (50 ng) in 0.5-day-old LEW.1WR1 single-cell embryos by intracytoplasmic microinjection to create site-specific DNA double-strand breaks, thereby stimulating targeted gene disruptions. After injection, LEW.1WR1 embryos were transferred into
pseudopregnant Sprague Dawley female rats. Embryonic injections and transfers were performed at the University of Massachusetts (UMass) Medical School Transgenic Animal Modeling Core facility. Genomic DNA was isolated from tail samples. The genotypes of individual pups (i.e., presence of insertions/deletions [indels]) were determined by PCR, restriction enzyme digests, and sequencing (Macrogen, Rockville, MD).

Of nine rats born, five founder animals that contained monoallelic or biallelic mutations in Ifnar1 were identified by nuclease screening and used for further breeding (Fig. 2.2B) to establish two distinct homozygous lines, designated IFNAR1^A81 and IFNAR1^A81+4, that were based on the indels present. Total RNA was extracted from heart and brain samples of encephalomyocarditis virus (EMCV)-infected rats 2 days postinfection using TRIzol reagent (Sigma-Aldrich, St. Louis, MO). One microgram of RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. For RT-PCR analysis, primers targeting a 574-base pair (bp) region between exons 2 and 5 of the Ifnar1 gene (IFNAR1-F [5’-CCGTAGCCTCAGGTGAAGAC-3’] and IFNAR1-R [5’-GCTGTGTCTCTGAAGCGATG-3’]) were designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast). As a control, primers targeting a 543-bp region between exons 1 and 5 were designed for the Ifnar2 gene (IFNAR2-F [5’-AAGCCCAGAACAGGGGAAAC-3’] and IFNAR2-R [5’-CCAACCACCTCGTCAGTCACA-3’]). The reference cDNA sequences used for rat Ifnar1 and Ifnar2 are NM_001105893.1 and XM_006248107.2, respectively. A 50-µL
PCR master mix comprising HotStartTaq Master Mix (QIAGEN), nuclease-free water, forward and reverse primers (0.2 µmol/L each), and cDNA (2 µL per reaction) was prepared. For PCR cycling conditions, initial denaturation at 95 °C for 15 min was followed by 40 cycles of 95 °C for 40 s, 59 °C for 45 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Amplified products were run on a 1.5% agarose gel and visualized with ethidium bromide under ultraviolet illumination after electrophoresis.

**Cell Culture and In Vitro Stimulation.** Spleens were isolated from rats of either sex and immediately washed with phosphate-buffered saline (PBS, Corning, Manassas, VA) and then minced and passed through a 40-µm sterile nylon mesh with a 3-mL rubber syringe plunger. Cells were collected by centrifugation at 1,500 rpm for 3 min. The supernatant was discarded, and cells were resuspended in red blood cell lysis buffer (Sigma, St. Louis, MO) for 7 min at room temperature. After removal of erythrocytes, the splenocytes were washed once with PBS, centrifuged and resuspended in appropriate volumes of RPMI medium supplemented with 10% fetal bovine serum (FBS), counted, and seeded in 96-well plates at a density of 1 x 10^6 cells/well. Cultured splenocytes were stimulated with 1,000 units/mL of recombinant rat IFN-β or IFN-α (PBL Assay Science, Piscataway, NJ) for 18 h, then cell lysates were harvested for total RNA preparation using the RNeasy Plus Mini Kit (QIAGEN).

**Real-Time and RT-PCR Analysis.** cDNA was synthesized from 100 ng of total RNA using the QuantiTect Reverse Transcription Kit according to the manufacturer’s protocol.
Gene expression was quantified by quantitative RT-PCR (RT-qPCR) with the QuantiTect Primer Assay (Rn_Isg15_1_SG, Rn_Oas1a_1_SG) for interferon-stimulated gene (ISG) 15 and OAS1a. Expression levels were normalized to GusB (Rn_GusB_1_SG QuantiTect Primer Assay). The QuantiFast SYBR Green PCR Kit (QIAGEN) was used for real-time PCR amplification according to the manufacturer’s protocol on a Mastercycler ep realplex (Eppendorf, Hauppauge, NY).

To determine splenic KRV transcript levels, total RNA was extracted from spleens of KRV-infected WT and IFNAR1\textsuperscript{-/-} rats. cDNA was synthesized by using 1 µg of total RNA followed by RT-qPCR as described above. We used previously published primers (Hara, Alkanani et al. 2014) with the following sequences: forward primer 5’-GGAAACGCTTACTCCGATGA-3’ and reverse primer 5’-AACCGATGTCCTTCCCATTT-3’. The expression levels of viral transcripts were normalized to GusB.

All real-time PCR reactions were run in duplicate, including a no-template control reaction. Fold changes in gene expression of test and control samples were determined by using the $2^{-\Delta\Delta Ct}$ method.

**Diabetes Induction Protocols.** Studies of induced diabetes were performed in WT LEW.1WR1 and Ifnar1\textsuperscript{-/-} LEW.1WR1 rats (backcrossed $\geq$ F5) using two different perturbants known to trigger autoimmunity in WT animals and one not previously tested. High-molecular-weight poly I:C (InvivoGen, San Diego, CA) was administered to rats of either sex at 21–25 days of age by using a dose of 1 µg/g body weight by intraperitoneal
(i.p.) injection three times weekly for 3 weeks as previously described (Mordes, Guberski et al. 2005). Rats were monitored for a total of 39 days after the first injection with poly I:C, defined as day 0. Spleens, pancreata, pancreatic lymph nodes (PLNs), and sera were collected at day 4 from age-matched animals injected on days 0 and 2 with either saline or poly I:C in one experiment.

In a second set of experiments, weanling WT and Ifnar1−/− rats 21–25 days of age were infected with a single i.p. dose of KRV-UMass strain (1 x 10⁷ plaque forming units [PFU]) on day 0 and monitored for a total of 39 days for diabetes. KRV was prepared as previously described (Zipris, Hillebrands et al. 2003). Spleens, pancreata, PLNs, and sera were collected from age-matched uninfected (i.e., injected with culture media) or KRV-infected animals at day 5 post inoculation in one experiment.

In a third set of experiments, weanling WT LEW.1WR1 rats were infected with a single dose of 1 x 10⁷ PFU of EMCV (ATCC strain VR-129 propagated in BHK-21 cells) by i.p. injection on day 0 and monitored for diabetes for a total of 40 days. EMCV has not been previously tested in the LEW.1WR1 rat model. In one experiment, adult WT and Ifnar1−/− LEW.1WR1 rats were infected with 1 x 10⁷ PFU of EMCV i.p. and monitored for 14 days. Rats were euthanized if they exhibited gross signs of illness (e.g., ruffling, hunching). Some adult WT and Ifnar1−/− rats were euthanized 48 h after infection with 1 x 10⁷ PFUs of EMCV, and serum and select organs were harvested and stored at –80 °C until used for quantifying viral titers.
Blood glucose concentrations were measured at least three times weekly with a glucometer (Breeze2; Bayer, Carlsbad, CA). Rats were diagnosed as diabetic when the blood glucose concentration exceeded 250 mg/dL on 2 consecutive days.

**Plaque Assays.** EMCV was measured in rat serum and organs based on previously published methods (Guy, Chilmonczyk et al. 2009).

**Cytokine and Insulin Assays.** A ProcartaPlex kit (Affymetrix, Santa Clara, CA) was used according to the manufacturer’s instructions to measure cytokines and chemokines (CCL2, interleukin-1β [IL-1β], CCL5, CXCL10) in rat samples. Insulin was measured in serum samples by using an ultrasensitive insulin ELISA kit (ALPCO, Salem, NH).

**Histopathology.** After the diagnosis of diabetes or at the conclusion of an experiment, rats were euthanized, and pancreata were removed and fixed in 10% buffered formalin. Paraffin-embedded sections of pancreas were sectioned and prepared for light microscopy in the UMass Medical School Morphology Core laboratory (www.umassmed.edu/morphology/protocols). Sections stained with hematoxylin-eosin (H-E) were scored for insulitis as previously described (Mordes, Guberski et al. 2005) by an experienced reader (J.P.M.) who was not aware of the animal’s glycemic status. Intensity of insulitis was scored as follows: 0, no inflammatory mononuclear cell (MNC) infiltration; 1+, small numbers of infiltrating MNCs with preservation of islet architecture; 2+, moderate infiltrating MNCs with preservation of architecture; 3+, many
MNCs, with most islets affected and distortion of islet architecture; 4+, florid infiltration and distorted islet architecture or end-stage islets with or without residual inflammation. Histology images in Fig. 2.4 were adjusted for clarity by setting the white point for each image using Adobe Photoshop CS6.

**Statistics.** Statistical procedures were carried out with either GraphPad Prism version 6 (GraphPad Software, La Jolla, CA) or SPSS version 19 (IBM Corporation, Armonk, NY) software. Survival and disease-free survival were analyzed using Kaplan-Meier methodology; equality of survival distributions was tested by the log-rank statistic (Bewick, Cheek et al. 2004). Parametric data are given as arithmetic means ± 1 SD or ± SE as indicated in the figure legends and Table 2.1. Fisher exact test was used to analyze 2 x 2 tables, and the $\chi^2$ test was used for larger tables. For comparisons of three or more means, we used one-way and two-way ANOVAs and either Bonferroni correction or the least significant differences procedure for posteriori contrasts (Bewick, Cheek et al. 2004). $P < 0.05$ was considered statistically significant.
2.4. Results

2.4.1. Targeting Ifnar1 in LEW.1WR1 Rats Using CRISPR-Cas9.

We induced mutations in rat Ifnar1 using a CRISPR-Cas9 strategy. Two sgRNAs were designed to target exon 4 of rat Ifnar1, which, together with exon 5, encodes an IFN-binding domain (Fig. 2.2A). The sgRNAs were coinjected with Cas9 mRNA into single-cell LEW.1WR1 rat embryos. We assayed Ifnar1 somatic mutations in F0 pups and identified either deletions spanning the region between the two sgRNA/Cas9 target sites (Fig. 2.2B, lower bands) or small indels at the individual target sites (data not shown). Two lines with germline mutations due to error prone nonhomologous end joining repair were established and designated IFNAR1\textsuperscript{D81} and IFNAR1\textsuperscript{D81+4}, and confirmed by sequencing rat genomic DNA from F0 founder, F1 heterozygous, and F2 homozygous animals. Sequences of IFNAR1\textsuperscript{D81} and IFNAR1\textsuperscript{D81+4} F2 homozygous animals are shown in Fig. 2.2C. PCR on cDNA using primers spanning exons 2–5 of Ifnar1 yielded the appropriate-sized product in WT rats but truncated products in IFNAR1\textsuperscript{D81} and IFNAR1\textsuperscript{D81+4} rats (Fig. 2.2D, left panel). Sequence analysis of Ifnar1 PCR products confirmed that the amplicons contained the predicted mutations (data not shown). In contrast, PCR analysis of Ifnar2 cDNA, which was not targeted, revealed the expected products for WT and both IFNAR1 rat lines for a region spanning exons 1–5 (Fig. 2.2D, right panel).
Figure 2.2. Generation of IFNAR1-deficient LEW.1WR1 rats using a CRISPR-Cas9 strategy. A: Schematic of the predicted protein domain structure of rat IFNAR1 (top panel). Exons 4 and 5 encode the first interferon-binding domain of IFNAR1 (bottom panel). Arrowheads indicate the two sgRNA target sites (gray boxes) for editing exon 4. B: PCR products for nine F0 rats using primers targeting sequences residing in introns that flank exons 4–5. The WT PCR product size is 559 bp (arrow). The smaller PCR products amplified in rats 5–7 reflect deletions between the two CRISPR-Cas9 target sites. Rats 3–7 all contained indels and/or larger deletions in the targeted region, confirmed by sequencing the PCR products. C: Sequence analysis of PCR products amplified from the genomic DNA of two distinct F2 homozygous lines (IFNAR1^{Δ81+4} and IFNAR1^{Δ81}) reveal deletion mutations mediated by nonhomologous end joining at the targeted Ifnar1 exon 4. The two sgRNAs designed for targeting exon 4, each containing a 20 bp target sequence, are shown in bold, and the adjacent PAM sequences are boxed. Dots indicate base deletions and underlined nucleotides indicate base insertions. Each vertical line indicates a CRISPR-Cas9 cleavage site. D: PCR products amplified from cDNA generated from brain (B) and heart (H) from WT and homozygous rats for a region of Ifnar1 spanning exons 2–5 (left panel). A truncated PCR product (arrow) is detected for Ifnar1 mRNA in IFNAR1^{Δ81+4} and IFNAR1^{Δ81} rats. As a control, cDNA was amplified from a region of Ifnar2 spanning exons 1–5 (arrow) and is identical for WT, IFNAR1^{Δ81+4}, and IFNAR1^{Δ81} rats (right panel). NTC, no template control.
2.4.2. Homozygous Ifnar1-Deficient Rat Lines Are Phenotypically IFNAR1 Deficient.

We tested several commercially available antibodies against rat IFNAR1 but were unable to validate their target specificity. Therefore, we confirmed the IFNAR1 deficiency phenotype of our mutant rats using two approaches. First, we isolated splenocytes from WT IFNAR1$^{Δ81+4}$ rats and IFNAR1$^{Δ81}$ rats and challenged them in vitro with either recombinant rat IFN-β or IFN-α. At 18 h postchallenge with IFN-β or IFN-α, robust induction of lsg15 was present with WT but not with IFNAR1$^{Δ81+4}$ and IFNAR1$^{Δ81}$ splenocytes (Fig. 2.3A). A second ISG, Oas1a, was similarly induced by IFN-β in WT but not IFNAR1-deficient rat splenocytes. The inability to respond to recombinant type I IFNs indicates that the cognate type I IFN receptor was lacking in the Ifnar1-targeted rats.

Second, we examined rat survival after challenge with EMCV, which has been shown to induce type I IFN by engaging the IFI11-encoded melanoma differentiation-associated protein 5 (MDA5) (Gitlin, Barchet et al. 2006, Kato, Takeuchi et al. 2006). Ifnar1$^{-/-}$ mice are highly susceptible to EMCV infection compared with control mice (Hwang, Hertzog et al. 1995, Kato, Takeuchi et al. 2006). In addition, viral titers in Ifnar1$^{-/-}$ mice are much higher than in controls after challenge with viruses such as vesicular stomatitis virus and Semliki Forest virus (Muller, Steinhoff et al. 1994, Hwang, Hertzog et al. 1995). Thus, we expected that rats lacking IFNAR1 would have a heightened susceptibility to EMCV. After EMCV challenge, all WT adult rats survived $>14$ days postinfection without exhibiting signs of disease. In contrast, 100% of
Figure 2.3. *Ifnar1*-targeted rats are phenotypically IFNAR1 deficient. A: *Isg15* is induced in WT but not IFNAR1Δ81+4 or IFNAR1Δ81 LEW.1WR1 rat splenocytes after an 18-h challenge with either recombinant IFN-β or IFN-α (four independent wells per condition). Similarly, *Oas1a* message is induced in WT but not IFNAR1Δ81+4 or IFNAR1Δ81 LEW.1WR1 rat splenocytes after challenge with recombinant IFN-β. For IFN-β, ***P < 0.001 for WT vs. IFNAR1Δ81+4 (unpaired t-test) and ***P < 0.001 for WT vs. IFNAR1Δ81 (unpaired t-test); for IFN-α, **P < 0.01 for WT vs. IFNAR1Δ81+4 (unpaired t-test) and **P < 0.01 for WT vs. IFNAR1Δ81 (unpaired t-test). Error bars represent the SD. B: IFNAR1Δ81+4 and IFNAR1Δ81 rats exhibit decreased survival after EMCV challenge. **P = 0.0013 for WT vs. IFNAR1Δ81+4 (log-rank test); **P = 0.0070 for WT vs. IFNAR1Δ81 (log-rank test). C: IFNAR1Δ81+4 rats have increased viral titers in serum and heart 48 h post-EMCV challenge. Dotted line indicates the limit of detection of the assay. Each point represents a sample from an independently infected animal, and the horizontal bar shows the mean value. **P = 0.0079 for WT vs. IFNAR1Δ81+4 serum (Fisher exact test); **P = 0.0079 for WT vs. IFNAR1Δ81 heart (Fisher exact test). D: IFNAR1Δ81 rats have elevated viral titers compared with WT rats following EMCV challenge. **P = 0.0079 for WT vs. IFNAR1Δ81 serum (Fisher exact test); **P = 0.0079 for WT vs. IFNAR1Δ81 heart (Fisher exact test). Error bars represent the SD.
IFNAR1<sup>Δ81+</sup> rats and 80% of IFNAR1<sup>Δ81</sup> rats died by 4–5 days postinfection (<i>P</i> = 0.0013 and 0.0070, respectively) (Fig. 2.3B). Serum and heart from IFNAR1-targeted rats also showed high viral titers compared with WT rats 48 h after inoculation with EMCV (Fig. 2.3C and D), indicating that the type I IFN response is impaired in these rats. The consistent findings between the two IFNAR1-targeted lines suggest that off-target effects from the CRISPR-Cas9 editing are unlikely. From this point on, we considered the two lines equivalent and henceforth term these <i>Ifnar1</i><sup>−/−</sup> rats.

In mice, only certain strains of EMCV are diabetogenic (Yoon, McClintock et al. 1980), whereas EMCV pathogenesis in rats has been only partly characterized (Psalla, Psychas et al. 2006). Therefore, we challenged weanling WT LEW.1WR1 rats (<i>n</i> = 7) with 1 x 10<sup>7</sup> PFU EMCV and monitored for diabetes for 40 days. None of the rats became diabetic over the course of the experiment (data not shown).

### 2.4.3. Weanling <i>Ifnar1</i><sup>−/−</sup> LEW.1WR1 Rats Are Protected From Poly I:C–Induced Autoimmune Diabetes.

Previous studies established that spontaneous diabetes in LEW.1WR1 rats occurs with a cumulative frequency of ~2.5%, but administration of poly I:C to weanling rats leads to diabetes in 100% of rats (Mordes, Guberski et al. 2005). We challenged WT and <i>Ifnar1</i><sup>−/−</sup> rats with poly I:C and monitored them for diabetes. Poly I:C administration resulted in diabetes in 13 of 15 (87%) WT rats by 23 days after the first dose (Fig. 2.4A). In contrast, only 2 of 11 (18%) <i>Ifnar1</i><sup>−/−</sup> rats became diabetic and not until day 28 at the earliest. This difference was highly significant (<i>P</i> < 0.0001). Of note, the difference between WT and
Figure 2.4. IFNAR1 deficiency protects rats from poly I:C-induced diabetes.
Animals 21–25 days old of either sex were injected with poly I:C as described in the Methods section. A: The frequency of poly I:C-induced diabetes was reduced in Ifnar1<sup>−/−</sup> rats compared with WT rats. ***P < 0.0001 (log-rank test). Blood glucose values for individual WT and Ifnar1<sup>−/−</sup> rats are plotted vs. time. B: Representative images of H-E-stained pancreatic samples from normoglycemic WT and Ifnar1<sup>−/−</sup> rats as well as from diabetic WT and Ifnar1<sup>−/−</sup> rats are shown. An image from a saline-treated, normoglycemic WT rat without insulitis is included for reference. Islets from normoglycemic poly I:C-treated Ifnar1<sup>−/−</sup> rats have normal architecture and are free of insulitis at day 40. One normoglycemic WT rat had evidence of moderate insulitis (2+), as shown. Islets from diabetic WT and Ifnar1<sup>−/−</sup> rats are architecturally distorted and display intense or severe insulitis. Representative images of pancreatic samples immunostained for insulin reveal abundant insulin-staining cells in normoglycemic Ifnar1<sup>−/−</sup> rats similar to that in the saline treated control WT animals. Diabetic WT and Ifnar1<sup>−/−</sup> rats have reduced numbers of insulin-positive cells. Islets from the saline-treated WT rats and poly I:C-treated Ifnar1<sup>−/−</sup> normoglycemic rats show normal peripheral staining of glucagon-positive cells. Although glucagon-positive cells are preserved in the diabetic WT and diabetic Ifnar1<sup>−/−</sup> rats, the islets are architecturally distorted. C: Ifnar1<sup>−/−</sup> rats have decreased splenic CXCL10 and CCL5 compared with WT rats at 4 days after poly I:C challenge. Each point represents a sample from an independent animal, and the horizontal bar shows the mean value. No differences in total splenic IL-1β or CCL2 in WT and Ifnar1<sup>−/−</sup> rats are observed. **P = 0.0074 for CXCL10; **P = 0.0063 for CCL5 in WT vs. Ifnar1<sup>−/−</sup> poly I:C–challenged rats (unpaired t test). Error bars represent the SD.
Ifnar1<sup>−/−</sup> rats was statistically significant regardless of whether the rats were of IFNAR1<sup>Δ81+4</sup> or IFNAR1<sup>Δ81</sup> lineage (Fig. 2.5), again indicating that protection from diabetes is not simply because of an off-target effect.

For poly I:C experiments, animals were euthanized at the time of diabetes or at the end of the study, and serial sections of pancreas were stained for H-E, insulin, and glucagon. Histopathological analysis revealed that insulitis was more severe in diabetic WT animals after poly I:C treatment compared with nondiabetic animals. Islet pathology for all WT animals was associated with a mean insulitis score of 3.11 ± 0.45 (Table 2.1). End-stage insulitis was present in diabetic WT animals, which was associated with distorted islet architecture, shrunken size, and presence of few residual infiltrating lymphocytes (Fig. 2.4B). Moderate insulitis was present (2+) in one WT animal that was normoglycemic at the end of the study (Fig. 2.4B); other WT normoglycemic animals had no evidence of insulitis. In contrast, all normoglycemic Ifnar1<sup>−/−</sup> rats were completely
free of insulitis, with normal islet size and structure (Fig. 2.4B). However, insulitis was observed in the two Ifnar1−/− rats that were diabetic. The mean insulitis score was 0.64 ± 0.43 among all poly I:C–treated Ifnar1−/− animals (Table 2.1). The overall concordance between the diabetes phenotype and insulitis scores agrees with our previous findings (Blankenhorn, Rodemich et al. 2005).

<table>
<thead>
<tr>
<th>Animals assessed1</th>
<th>Diabetic animals (percent total)</th>
<th>Insulitis score2, mean ± S.E.</th>
<th>P value</th>
<th>Serum insulin (pg/ml), mean ± S.E.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>n = 9</td>
<td>n = 7 (78%)</td>
<td>3.11 ± 0.45</td>
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<td></td>
<td></td>
<td></td>
<td>P = 0.0030</td>
<td>334 ± 101</td>
<td></td>
</tr>
<tr>
<td>Ifnar1−/−</td>
<td>n = 11</td>
<td>n = 2 (18%)</td>
<td>0.64 ± 0.43</td>
<td></td>
<td>P = 0.0302</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>918 ± 235</td>
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Table 2.1. Insulitis scores and insulin levels from WT and IFNAR1-deficient rats treated with poly I:C.

Data are n, n (%), or mean ± SE. P values by Mann-Whitney U test. 1Terminal samples from six poly I:C-treated diabetic WT rats were not available for histopathology or serum insulin testing. 2The description of the scoring system is provided in the Methods section.

Immunohistochemical staining on samples from all WT animals revealed abundant glucagon-positive cells, with a marked decrease in insulin-positive cells in diabetic WT rats and moderate loss of insulin-positive cells in normoglycemic poly I:C–treated WT animals (Fig. 2.4B). Both insulin and glucagon were abundant in islets of all normoglycemic poly I:C–treated Ifnar1−/− animals, with some loss of insulin-positive cells only in the small number of Ifnar1−/− diabetic animals (Fig. 2.4B). Insulin was measured in all available terminal serum samples; Table 2.1 shows the mean serum insulin values for WT versus Ifnar1−/− rats regardless of diabetes status. Overall, WT rats had
significantly lower levels of terminal serum insulin compared with Ifnar1−/− rats, as anticipated.

We assessed cytokines and chemokines in organs harvested from WT and Ifnar1−/− animals 4 days after the administration of poly I:C (or saline control). CXCL10 and CCL5, whose expression is mediated by IFN, were significantly decreased in spleens from poly I:C-challenged Ifnar1−/− rats compared with poly I:C-challenged WT rats (Fig. 2.4C). In contrast, differences were not observed in IL-1β or CCL2 in spleens of WT versus Ifnar1−/− rats after poly I:C challenge (Fig. 2.4C). Cytokines and chemokines were also measured in total pancreata, PLNs, and sera. Ifnar1−/− rats challenged with poly I:C had decreased levels of CXCL10, CCL5, and CCL2 in pancreata as well as decreased CXCL10 in sera compared with polyI:C-challenged WT rats (Fig. 2.6A).

2.4.4. Weanling Ifnar1−/− LEW.1WR1 Rats Develop Diabetes at a Low Frequency After Infection with KRV.

KRV infection induces autoimmune diabetes in LEW.1WR1 rats (Tirabassi, Guberski et al. 2010). We conducted KRV infection studies with weanling Ifnar1−/− rats with two goals: 1) to establish whether rats deficient in IFNAR1 could survive infection with this parvovirus and 2) to see whether the frequency of diabetes would be reduced compared with WT rats. Diabetes was observed in 3 of 16 (19%) Ifnar1−/− rats infected with KRV and monitored over a 40-day period (Fig. 2.7A). No other morbidities occurred in these animals over the course of the study. Thus, although Ifnar1−/− rats succumbed to EMCV infection, they tolerated and survived KRV infection. Nine of 17 (53%) WT rats became
Figure 2.6. Cytokines and chemokines are differentially induced in pancreata, pancreatic lymph nodes, and sera of WT and Ifnar1⁻/⁻ rats. A: Samples were collected from WT or Ifnar1⁻/⁻ rats at day 4 following i.p. injection of poly I:C (or saline control). Following poly I:C challenge, Ifnar1⁻/⁻ rats have decreased pancreatic CXCL10 (*, $P = 0.0335$), CCL5 (**, $P = 0.0063$), and CCL2 (**, $P = 0.0081$) as well as decreased serum CXCL10 (****, $P < 0.0001$) compared to WT rats. B: Samples were collected at day 5 following i.p. injection with KRV (or media control). Following KRV infection, Ifnar1⁻/⁻ rats have decreased PLN CCL5 (*, $P = 0.0241$) compared to WT rats. Note that PLN samples were not harvested from all KRV-infected animals due to technical limitations. Values are shown as ng/g pancreas or pancreatic lymph node (PLN) or ng/mL sera. Each point represents a sample from an independent animal and the horizontal bar shows the mean value. $P$ values are unpaired t tests. Error bars represent the S.D.
Figure 2.7. *Ifnar1*−/− rats are partially protected from KRV-induced diabetes and exhibit differential cytokine and chemokine production in spleens after KRV infection. Animals 21–25 days old of either sex were inoculated with KRV or media control as described in the Methods section. A: The frequency of KRV-induced diabetes was reduced in *Ifnar1*−/− rats compared with that in WT rats. *P* = 0.0461 (log-rank test). Blood glucose values for individual WT and *Ifnar1*−/− rats are plotted vs. time. B: *Ifnar1*−/− rats have decreased splenic CXCL10 and CCL5 compared with WT rats at 5 days after KRV infection. Each point represents a sample from an independently infected animal, and the horizontal bar shows the mean value. *P* = 0.0108 for CXCL10; **P* = 0.0041 for CCL5 in WT vs. *Ifnar1*−/− KRV-infected rats (unpaired *t* test). Error bars represent the SD. In contrast, *Ifnar1*−/− rats have increased total splenic IL-1β and CCL2 compared with WT rats at 5 days after KRV infection. Each point represents a sample from an independently infected animal, and the horizontal bar shows the mean value. *P* = 0.0435 for IL-1β; **P* = 0.0096 for CCL2 in WT vs. *Ifnar1*−/− KRV-infected rats (unpaired *t* test). Error bars represent the SD. C: Spleens from KRV-infected WT and *Ifnar1*−/− rats do not have significant differences in viral transcript levels at day 5 postinfection. Copies of KRV transcript levels were measured by RT-qPCR and normalized to *GusB*. Fold induction relative to the uninfected sample is shown.
diabetic after infection with KRV (Fig. 2.7A), a rate consistent with that previously reported in KRV-infected LEW.1WR1 rats (Tirabassi, Guberski et al. 2010). The difference in the frequency of diabetes in WT versus Ifnar1−/− rats was statistically significant ($P = 0.0461$), indicating that the absence of IFNAR1 is partially protective against KRV-induced diabetes. Pancreatic sections from all the normoglycemic KRV-infected Ifnar1−/− rats at the end of the study showed the complete absence of insulitis, whereas all diabetic rats, whether WT or IFNAR1 deficient, had classical end-stage insulitis (data not shown).

### 2.4.5. KRV Differentially Induces Cytokines in WT and Ifnar1−/− LEW.1WR1 Rats

KRV has been reported to be present in spleens of LEW.1WR1 rats after infection by the i.p. route (Alkanani, Hara et al. 2014). To establish whether IFNAR1 deficiency results in altered cytokine responses after KRV challenge, we measured select cytokines and chemokines in spleens collected from weanling rats that were uninfected or 5 days after KRV infection. CXCL10 and CCL5 were each significantly decreased in spleens from KRV-infected Ifnar1−/− rats compared with KRV-infected WT rats (Fig. 2.7B), illustrating an overall diminished type I IFN-driven response in splenic cells after KRV infection in Ifnar1−/− rats. Of note, levels of IL-1β and CCL2 were significantly higher in KRV-infected Ifnar1−/− rat spleens compared with KRV-infected WT rat spleens (Fig. 2.7B). We measured KRV transcript levels in these WT and Ifnar1−/− rat spleens to determine whether viral replication was altered in the absence of functional IFNAR. Although viral transcript levels were slightly (approximately two-fold) higher in Ifnar1−/−
rat spleens compared with WT, the difference was not statistically significant (Fig. 2.7C). Total pancreatic, PLN, and serum cytokines and chemokines were also measured from these animals, but only limited differences were observed between KRV-infected WT and Ifnar1+ rats (Fig. 2.7B).

2.5. Discussion

Collectively, these data underscore the importance of type I IFN-mediated signaling for the development of autoimmune diabetes. The role of IFN signaling has been implicated in the initiation of islet autoimmunity and development of T1D (Crow 2010). A strong IFN gene signature was identified in the peripheral blood of at-risk children before initiation of islet autoimmunity (Ferreira, Guo et al. 2014, Kallionpaa, Elo et al. 2014). The current findings in Ifnar1+ LEW.1WR1 rats firmly define a role for type I IFN and downstream signaling pathways in poly I:C- and KRV-induced diabetes in the rat model and agree with a previous study in which anti-IFN-α antibody administration to T1D-prone BB rats resulted in a trend toward a later onset of poly I:C-induced diabetes (Ewel, Sobel et al. 1992). They also support human GWAS findings for human T1D risk associations with single nucleotide polymorphisms (SNPs) in genes such as IFIHI that participate in IFN-regulated pathways. Specifically, a nonsynonymous SNP in IFIHI resulting in an amino acid change of alanine to threonine at 946 (A946T) of MDA5 is associated with an increased risk of diabetes (Smyth, Cooper et al. 2006). MDA5 recognizes cytoplasmic long double-stranded RNA (dsRNA) intermediates generated during the replication cycle of CVB or intracellularly delivered synthetic dsRNA analog
poly I:C, leading to potent IFN-α/β induction (Wu and Chen 2014). Funabiki et al. (Funabiki, Kato et al. 2014) characterized a constitutively active form of MDA5 caused by the amino acid mutation G821S that results in type I IFN hyperexpression and causes lupus-like nephritis; they also similarly associated hyperexpression of type I IFN with A946T. The resulting increase in IFN is associated with severe autoimmune disease.

In contrast, four rare-variant nonsynonymous SNPs in IFIH1 were found to be protective against T1D in GWAS (Nejentsev, Walker et al. 2009); at least two of these variants, E627X and I923V, are predicted to decrease MDA5 function with loss of type I IFN responses after viral challenge (Shigemoto, Kageyama et al. 2009). Recent data have also shown that NOD mice heterozygous for MDA5 were protected from T1D when infected with CVB4; this protective effect was attributed to a unique type I IFN signature that led to expansion of Tregs at the site of autoimmunity (Lincez, Shanina et al. 2015). Similarly, Ebi2, a regulator of the interferon regulatory factor 7 (IRF7)-driven inflammatory network (IDIN), including human IFIH1 (Heinig, Petretto et al. 2010), was associated with an increased risk of T1D; the specific Ebi2 polymorphism is associated with increased expression of IDIN genes (Heinig, Petretto et al. 2010). Finally, the human Tyk2 gene, which was mapped to the possible T1D susceptibility locus (Mein, Esposito et al. 1998), encodes the IFNAR1-associated molecule tyrosine kinase 2, and its deficiency results in a reduced antiviral response (Shimoda, Kato et al. 2000). These data support the critical contribution of IFN-regulated pathways in the development of T1D following environmental insult.
Rat models are particularly useful for understanding the pathogenesis of T1D, specifically in defining the roles of genetic and environmental factors, including viral infection. The mechanism by which KRV induces diabetes in rats has been partially dissected and involves both innate and adaptive immune responses (Mordes, Zipris et al. 2013). KRV infection of LEW.1WR1 rat primary islets and splenic cells reportedly activates the Toll-like receptor (TLR) 9 signaling pathway, leading to the activation of two major transcription factors, IRF7 and nuclear factor-κB (NF-κB) through the adaptor protein MyD88 (Zipris, Lien et al. 2007, Alkanani, Hara et al. 2014). Treg depletion synergizes with KRV to induce diabetes (Ellerman, Richards et al. 1996). In our studies, the interferon-stimulated response element (ISRE)-regulated chemokines CCL5 and CXCL10 are decreased in spleens of KRV-infected Ifnar1⁻/⁻ rats, whereas NF-κB-driven IL-1β and CCL2 are significantly increased (Fig. 2.7B), confirming roles for both IRF7 and NF-κB in KRV infection. Of importance, IL-1β is an inflammatory cytokine that has long been implicated in the development of T1D (Mandrup-Poulsen, Pickersgill et al. 2010, Moran, Bundy et al. 2013). The robust IL-1β induction suggests that IL-1β may not be a major contributor in the early stages of diabetes in this model. Of note, loss of IFNAR1 does not affect the overall survival of KRV-infected rats in the manner that it affects survival of EMCV-infected rats. Type I IFN production is elicited by several pathways in the infected host, depending on the specific virus, and each virus may have a unique means of antagonizing or evading the IFN response. For example, a murine parvovirus has been shown to efficiently evade host type I IFN (Mattei, Cotmore et al. 2013), so rodent parvoviruses may possess unique mechanisms to counteract IFN-
induced antiviral effectors. Although the impact of type I IFN on KRV replication is not completely defined, ISGs are induced during KRV infection and could contribute to autoimmunity.

Figure 2.8 provides an overview of IFN signaling pathways in virus-induced diabetes in the context of this study as well as others (Marroqui, Lopes et al. 2015, de Beeck and Eizirik 2016). Viral nucleic acid structures are recognized by pattern recognition receptors, including MDA5 and TLR3, after viral infection of β-cells, leading to the activation of the key transcription factors IRF3, IRF7, and NF-κB (Ortis, Naamane et al. 2010), which is followed by induction of the transcription and synthesis of IFN-β, a subset of ISGs (Wu and Chen 2014), and inflammatory cytokines; the magnitude of these responses may depend on the genetic susceptibility of the host. IFN-β binds to the type I IFN receptor and exerts its antiviral effects through the trimolecular ISGF3 (IFN-stimulated gene factor 3) complex STAT1-STAT2-IRF9, which binds to the ISRE and induces the expression of ISGs for the recruitment of immune cells (e.g., lymphocytes, monocytes, dendritic cells), leading to insulitis and diabetes. Type I IFNs also affect β-cell survival during infections with viruses linked to human T1D (de Beeck and Eizirik 2016). We show that the absence of functional IFNAR tempers the onset of diabetes.

We found the incidence of diabetes in our WT LEW.1WR1 rats to be comparable to previously published reports: 38% for KRV (Tirabassi, Guberski et al. 2010) and close to 100% for poly I:C (Mordes, Guberski et al. 2005). Of note, IFNAR1 deficiency results in an incidence of diabetes of ~18%, regardless of whether the trigger is poly I:C or KRV. This suggests a common mechanism of diabetes and insulitis in IFNAR1
Figure 2.8. The triggering phase of virus-induced diabetes involves the activation of the host antiviral type I IFN immune signaling pathways. Viral-derived nucleic acid structures or the dsRNA mimetic poly I:C are recognized by pattern recognition receptors, including MDA5/TLR3, resulting in activation of IRF3, IRF7, and NF-κB. IRF3 and IRF7 induce the transcription and synthesis of IFN-β and a subset of ISGs, whereas NF-κB transcribes inflammatory cytokines. Released type I IFN binds to the type I IFN receptor, which comprises IFNAR1 and IFNAR2, and exerts its antiviral effects through the downstream activation and nuclear translocation of the trimolecular ISGF3 complex (STAT1-STAT2-IRF9), which binds to ISRE, inducing the expression of ISGs for recruitment of immune cells. Collectively, this suggests that enhanced inflammatory responses initiated and amplified by type I IFN signaling pathways in response to viral infection contribute to autoimmunity and T1D in genetically predisposed individuals.
deficiency. The mechanism behind diabetes during IFNAR1 deficiency remains undefined but may relate to IFN production that precedes IFNAR1 signaling events or that may depend on TLR-mediated activation of NF-kB [TLR9 for KRV (Zipris, Lien et al. 2007); TLR3 for poly I:C (Alexopoulos, Holt et al. 2001)] with subsequent T-cell recruitment. Virus-induced diabetes that occurs independently of the IFNAR1 pathway can be further explored in the rat model and, perhaps, eventually defined through generation of double-knockout rat lines. We have already dissected important genetic factors in virus-induced diabetes in rats. The genome-encoded T-cell receptor element Vβ13 controls genetic susceptibility to diabetes (Eberwine, Cort et al. 2014); depletion of Vβ13+ T cells prevents poly I:C-induced diabetes in LEW.1WR1 rats (Liu, Cort et al. 2012). Additional genetic studies define diubiquitin as a susceptibility gene for virus-induced diabetes in rats (Cort, Habib et al. 2014). With continued advancements in genomics and in CRISPR-Cas9 genome editing, we anticipate exciting developments in autoimmune diabetes in the near future through rat models of diabetes.

In summary, these data advance our understanding of how innate immunity influences the development of T1D. These studies help us to better understand why certain individuals with specific polymorphisms in IFIH1 are either predisposed to or protected from T1D. Viral infection and innate immune activation may initiate early events in β-cells and/or immune cells that ultimately lead to autoimmune attack and T1D in genetically susceptible individuals. In the long term, findings from these studies could be transitioned to diabetes models involving human islets and human immune cells.
(Gallagher, Brehm et al. 2015) in which type I IFN pathways are disrupted. The current data reinforce the need for novel approaches to diabetes prevention and treatment, such as viral vaccine development (England and Roberts 1981, Hyoty and Knip 2014) or even cytokine-modulating therapies.
Preface to Chapter III

Natasha Qaisar designed experiments for the work presented here. Natasha Qaisar and Glennice Ryan designed the flow cytometry plan. Natasha Qaisar, Glennice Ryan, Suvana Lin, and Basanthi Satish performed the experiments in Chapter III collectively. Dr. John Mordes provided feedback on the statistical analysis. Natasha Qaisar performed the data analysis and wrote Chapter III with mentorship from Dr. Jennifer P. Wang.
CHAPTER III

THE ABSENCE OF IFNAR SIGNALING IS ASSOCIATED WITH ALTERATIONS IN LYMPHOCYTE POPULATIONS DURING VIRAL INFECTION OF RATS

3.1. Abstract

Type I interferon exerts potent immunomodulatory effects on the adaptive immune system through both direct and indirect mechanisms. It has been hypothesized that Kilham rat virus (KRV)-induced diabetes in genetically susceptible rats may involve the activation of adaptive immune responses as well as loss of immune tolerance in the lymphoid organs, in particular spleen and pancreatic lymph nodes. We previously reported that disruption of type I IFN signaling though functional inactivation of the IFNAR receptor protects the rats from poly I:C or KRV-induced diabetes. We generated the hypothesis that IFNAR-deficiency provides protection against diabetes by modulating T lymphocyte populations during the development of autoimmune diabetes. We first demonstrate that functional disruption of IFNAR signaling does not change the baseline levels of lymphocyte populations in Ifnar1⁻/⁻ rats when compared to WT rats. Next, while the percentage and absolute cell number of CD8⁺ T cells in the spleens are similar in both uninfected and KRV-infected WT rats, significant decreases are observed in KRV-infected Ifnar1⁻/⁻ rats. In contrast, the percentage and absolute cell number of splenic CD4⁺CD25⁺FoxP3⁺ regulatory T cells in KRV-infected Ifnar1⁻/⁻ rats are similar to those of both uninfected WT and Ifnar1⁻/⁻ rats, but are significantly decreased in KRV-infected
WT rats. These data suggest that IFNAR signaling modulates the effector T cell and regulatory T cell populations following viral infection, processes that very likely participate in the pathogenesis of diabetes.
3.2 Introduction

In addition to their direct antiviral and antimicrobial protective mechanisms, IFNs potently modulate various functions of immune cells by affecting physiological processes such as cell cycle regulation, survival and differentiation (Gough, Messina et al. 2012, Crouse, Kalinke et al. 2015). Studies have documented essential roles for both induced and constitutive IFN-I signaling in the homeostasis of hematopoietic stem cells (HSCs) in the steady-state and during viral infection (Gough, Messina et al. 2012).

In murine in vivo studies, Essers et al. reported that elevated levels of IFN-α induce the direct exit of WT HSCs from quiescence and their transient proliferation in response to treatment with IFN-α or poly I:C (Essers, Offner et al. 2009). In contrast, Ifnar1−/− HSCs are resistant to IFN-α and remain quiescent in response to treatment with IFN-α or poly I:C, which suggests that IFNAR signaling is required for poly I:C-mediated in vivo proliferation of HSC (Essers, Offner et al. 2009). Importantly, the total number of HSCs in Ifnar1−/− mice is reduced compared to WT mice in the absence of any IFN-α or poly I:C treatment (Essers, Offner et al. 2009, Gough, Messina et al. 2012). Ifnar1−/− mice also have increased levels of myeloid lineage cells in peripheral blood and bone marrow (Hwang, Hertzog et al. 1995) and a reduced turnover rate of the splenic CD8a+ subset of conventional DCs (Mattei, Bracci et al. 2009, Mattei, Schiavoni et al. 2010) or NK cells (Swann, Hayakawa et al. 2007), demonstrating that endogenous IFN signaling is required to maintain an optimal number of innate immune cells.

The physiological consequences of lack of IFN signaling on cells of the adaptive immune system are complex and less clear. Nonetheless, thymic epithelial cells express
the highest levels of constitutively active IFN-β as was observed in a luciferase reporter mouse, engineered to track IFN-β gene expression \textit{in vivo}, suggesting that basal IFN signaling in the thymus may have a role in T cell development or tolerance induction under non-inflammatory conditions (Lienenklaus, Cornitescu et al. 2009). Unexpectedly, unmanipulated IFNAR1-deficient mice have unaltered numbers of CD4+ or CD8+ T cells and B220+ B cells in the peripheral blood and bone marrow (Hwang, Hertzog et al. 1995), which suggests that other compensatory factors contribute to the homeostatic balance of T and B cells in \textit{Ifnar1}^{-/-} animals.

As key drivers of providing protective immunity during viral infections, induced IFN-I may exert their pleiotropic biological effects by influencing immune cells both directly and indirectly. Antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages serve as a prominent cellular connection between innate and adaptive immunity. By promoting DC maturation, migration and antigen presentation, IFN-I indirectly regulates antiviral T cell immunity for effective priming of T cell responses (Hervas-Stubbs, Perez-Gracia et al. 2011). IFN-I facilitates the conversion of plasmacytoid DCs into myeloid-derived DCs (Zuniga, McGavern et al. 2004), and enhances upregulation of MHC class I expression on lymphocytes (Lindahl, Gresser et al. 1976). Specific cell types such as metallophilic macrophages found in spleen inhibit IFN-I signaling by constitutively expressing USP18, a negative regulator of IFN (Honke, Shaabani et al. 2011). This mechanism allows unrestricted virus replication in these macrophages and ensures a source of sufficient viral antigen for promoting effective virus-specific adaptive immune responses (Honke, Shaabani et al. 2011). In addition,
IFN-I signaling in APCs generates cytokines and chemokines that can indirectly modulate cell numbers and activities of the immune system (Crouse, Kalinke et al. 2015). For instance, IL-15 plays a prominent role in proliferation and survival of natural killer (NK) cells and memory T cells in response to IFN-I (Crouse, Kalinke et al. 2015). Similarly, IFN-I can stimulate the production of IFN-γ by both DC (Montoya, Schiavoni et al. 2002) and NK cells (Nguyen, Salazar-Mather et al. 2002), which further promotes the differentiation of T helper 1 (TH1) cells (Swain, McKinstry et al. 2012). Collectively, these studies demonstrate that IFN-I signaling is essential for optimal stimulation, survival, expansion and differentiation of antiviral T cells.

Given their critical immunomodulatory activities during viral infection, it is not surprising that IFN-I signaling plays a major role in regulating antiviral T cell immunity in direct and/or indirect manners, thereby bridging innate and adaptive immunity (Crouse, Kalinke et al. 2015). Proper T cell activation requires three distinct signals. T cell receptor (TCR) engagement of peptide-MHC complex (signal 1) and costimulation between B7 and CD28 (signal 2) are required to initiate proliferation of naïve T cells and specific cytokine (signal 3) to differentiate into effector and memory T cells (Crouse, Kalinke et al. 2015). IFN-I and IL-12 serves as signal 3 cytokines for CD8+ T cell responses, thereby directly influencing T cell activation, proliferation and survival (Curtsinger, Valenzuela et al. 2005). In addition, two recent in vivo studies reported that intrinsic IFNAR signaling on T cells protect antiviral CD8+ T cells from NK cell-mediated attack (Crouse, Bedenikovic et al. 2014, Xu, Grusdat et al. 2014). To definitively demonstrate the important role of direct IFN-I signaling on antiviral T cells
In vivo, Kolumam and colleagues performed adoptive transfer experiments in which lymphocytic choriomeningitis virus (LCMV)-specific naïve CD8+ cells from WT and IFNAR-deficient mice were transferred into WT mice followed by LCMV challenge. They show that IFN-I acts directly on antigen-specific CD8+ T cells, allowing their clonal expansion and the production of optimal numbers of memory CD8+ T cells (Kolumam, Thomas et al. 2005). Furthermore, cDNA microarray analysis of mouse CD8+ T cells in response to IFN-I or IL-12 showed that these cytokines regulated the transcriptional expression of genes which have critical roles in CD8+ T cell survival, function, signaling and migration, and involved histone-dependent chromatin remodeling mechanisms (Agarwal, Raghavan et al. 2009).

Usually, the successful clearance of pathogens by the T-cell effector (Teff) response is followed by the suppression of the immune response. This suppression is mediated through “regulatory cells” of the immune system and is critical for restoring immune homeostasis. Distinct populations of CD4+ T cells, called regulatory T (Treg) cells, arise in the thymus and acquire the expression of transcription factor, forkhead box protein 3 (FoxP3) (Lu, Barbi et al. 2017). These thymus-derived Treg (tTreg) cells (formerly known as ‘natural Treg cells’) migrate to the periphery where they perform immunosuppressive functions including preventing autoimmune diseases (Lu, Barbi et al. 2017). In contrast, a subset of FoxP3+ Treg cells, called the peripherally-derived Treg cells (pTreg), are extrathymic Treg cells that arise from CD4+FOXP3+ T cells following their exposure to transforming growth factor (TGF)-beta and IL-2 in the microenvironment of peripheral tissues (Lu, Barbi et al. 2017). Originally, the Treg cell
phenotype was characterized as CD4^+CD25^{high} but they are now commonly defined as CD4^+CD25^+FoxP3^+ due to constitutive expression of FoxP3. In humans, Treg cells are also identified by low CD127 expression (Roep and Tree 2014). Both FoxP3^+ tTreg and pTreg perform several immunosuppressive activities through various mechanisms (Vignali, Collison et al. 2008). Several other subpopulations of Treg cells are known to exist under unique stimulatory conditions (Lu, Barbi et al. 2017). Emerging evidence from human and mice studies has implicated the immunomodulatory effects of IFN-I in the homeostasis of Tregs particularly during viral infections and autoimmune disorders (Piconese, Pacella et al. 2015). IFN-I can exert positive effects on Treg cells by supporting their expansion and/or function. For example, IFN-I treatment of multiple sclerosis patients rescues Treg cell frequency and suppressive abilities (de Andres, Arístimuño et al. 2007). In contrast, antagonistic effects of IFN-α such as inhibiting Treg cell proliferation or their suppressive abilities have been reported in humans and mice (Golding, Rosen et al. 2010, Srivastava, Koch et al. 2014).

As mentioned above, absence of IFNAR signaling may result in aberrant regulation of the immune system. This raises the possibility of whether or not *Ifnar1^−/−* rats have an innate numerical defect in the T cell populations. Previous studies in BBDR rats and LEW.1WR1 reported the contribution of KRV-induced activation of cellular and humoral immune responses in the development of autoimmune diabetes. Following challenge with KRV, T cells accumulate in the spleen and pancreatic lymph nodes at 5 days post-infection (dpi). Given the essential role of IFN signaling on antiviral T cell immunity, we sought to test the hypothesis that lack of IFNAR signaling prevents T1D in
Ifnar1⁺⁻⁻ rats by restraining KRV-induced alterations in effector T cells populations. The present study also examines how IFNAR signaling modulates the regulatory T cell population in the spleen and blood during KRV infection.

### 3.3. Materials and Methods

**Rats and Virus.** LEW.1WR1 rats (RT1B/Du) were from Biomere (Worcester, MA). Animals were housed in viral antibody–free conditions, confirmed monthly to be serologically free of rat pathogens (Mordes, Leif et al. 2002), and maintained in accordance with institutional and national guidelines (Institute of Laboratory Animal Resources (U.S.) 1996). KRV-UMass strain was prepared and titered by plaque assay as previously described (Zipris, Hillebrands et al. 2003).

**Virus infection and spleen and blood collection.** Weanling WT and Ifnar1⁺⁻⁻ rats of 21–25 days of age of either sex were infected with a single i.p. dose of KRV-UMass strain (1 X 10⁷ PFU) on day 0. Spleens and blood were collected from age-matched mock-infected (i.e., injected with culture media) or KRV-infected animals at 5 or 12 dpi. Blood was collected from euthanized rats by cardiac puncture and immediately mixed with heparin (100 U/ml blood) to prevent coagulation after isolation. Isolated rat spleens were kept in PBS (Corning) at room temperature immediately prior to further processing.

**Cell isolation.** A single-cell suspension of rat spleens was obtained by mincing and passing through a 40-µm sterile nylon mesh with a 3 mL rubber syringe plunger. Cells
were collected by centrifugation at 1,500 rpm for 3 min. The supernatant was discarded, and cells were resuspended in red blood cell lysis buffer (Sigma) for 7 min at room temperature. After removal of erythrocytes, the splenocytes were washed once with PBS, centrifuged and resuspended in an appropriate volume of cell staining buffer (BioLegend) and were stained with antibodies for flow cytometry.

**Flow cytometry.** For surface staining, 100 µL heparinized whole blood from each rat was stained with fluorochrome-conjugated monoclonal antibodies at 1/100 dilution in cell staining buffer (BioLegend), followed by treatment with the BioLegend RBC Lysis/Fixation solution according to the manufacturer’s protocol. For spleen, 200 µL of splenocytes were aliquoted per well in a 96-well plate and then stained with antibodies diluted 1:100 in Cell Staining Buffer, followed by incubation at 4 °C for 30 min. After incubation, cells were washed and fixed in Fixation Buffer (BioLegend) overnight at 4 °C. For nuclear staining of FoxP3, both peripheral blood and splenocytes were first stained for surface markers as described above, followed by fixation, permeabilization and staining with anti-rat FoxP3 or isotype control using a FoxP3/Foxp3 Transcription Factor Staining Buffer Set (ThermoFisher Scientific) according to the manufacturer’s instructions. Table 3.1 provides a list of all antibodies used in this study. For each staining panel, fluorescence minus one (FMO) controls were used to determine gating boundaries by adding the isotype control antibody corresponding to the fluorescent markers of interest missing from the staining panel as previously described (Verschoor, Lelic et al. 2015). The flow cytometry data were acquired using a BD LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc).
**Absolute counting of rat lymphocytes.** Absolute numbers of rat lymphocytes in peripheral blood and spleen were determined using CountBright™ absolute counting beads for flow cytometry (ThermoFisher) according to the manufacturer’s protocol. Briefly, before running samples on the flow cytometer, 50 µL of counting beads at a fixed concentration were added to 200 µL of stained cells for each sample in a FACS tube. A forward vs. linear side scatter plot was used to distinguish the counting beads from lymphocytes and at least 1000 bead events were acquired as per the manufacturer’s protocol (ThermoFisher Scientific). The cell concentration/µL was calculated as: A/B X C/D, where A = number of cell events; B = number of bead events; C = assigned bead count of the lot (beads/50 µL); and D = volume of the sample (µL).

**Table 3.1. List of rat-specific antibodies used in flow cytometry**

<table>
<thead>
<tr>
<th>Surface markers for rats</th>
<th>Monoclonal antibody</th>
<th>Clone</th>
<th>Isotype</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV421-CD3</td>
<td>1F4</td>
<td>BV421-Mouse BALB/c IgM</td>
<td>BD Biosciences</td>
<td></td>
</tr>
<tr>
<td>FITC-CD4</td>
<td>OX-35</td>
<td>FITC-Mouse IgG2a</td>
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<tr>
<td>PerCP-CD8a</td>
<td>OX-8</td>
<td>PerCP-Mouse IgG1</td>
<td>BioLegend</td>
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</tr>
<tr>
<td>PE-CD25</td>
<td>OX-39</td>
<td>PE-Mouse IgG1</td>
<td>BioLegend</td>
<td></td>
</tr>
</tbody>
</table>

**Nuclear marker for rats**

| eFluor 450-FoxP3         | FJK-16s             | eFluor 450-Rat IgG2a | eBioscience/ThermoFisher Scientific |
**Statistics.** Statistical analysis was performed using Prism 7 software (Graphpad). For all data, statistical significance was determined using the nonparametric Mann-Whitney U test, (unpaired two-tailed) due to the non-normal distribution of data. Statistical significance was considered for $P<0.05$.

### 3.4. Results

#### 3.4.1. The disruption of IFNAR signaling does not affect basal lymphocyte populations in LEW.1WR1 rats.

Given the critical immunomodulatory role of constitutive IFN signaling for immune system homeostasis under steady-state conditions, it is important to determine the baseline levels of T lymphocytes in Ifnar1$^{-/-}$ rats at the weanling stage, i.e., when LEW.1WR1 rats are prone to either KRV- or poly I:C-induced diabetes. For this purpose, we analyzed the percentage and absolute number of T lymphocytes in the spleen and blood and compared these between weanling WT (n=2) and Ifnar1$^{-/-}$ rats (n=2) by flow cytometry after mock-infection of WT and Ifnar1$^{-/-}$ weanling rats (21-25 days). We determined the cell percentage and number for CD3$^{+}$CD4$^{+}$, CD3$^{+}$CD8$^{+}$ T cells, and CD4$^{+}$CD25$^{+}$FoxP3$^{+}$ natural Tregs in the peripheral blood and spleen 5 days after treatment of rats using uninfected cell culture media. A gating strategy based on the isotype and FMO controls for determining CD4$^{+}$ T cells, CD8$^{+}$ T cells, or Tregs in spleen is described in Figure 3.1. A-B. A similar gating strategy was also used for determining these cell populations in blood (not shown). We observed that the percentage and absolute numbers of blood and splenic CD4$^{+}$ lymphocytes, CD8$^{+}$ T lymphocytes, or
Tregs do not differ between weanling WT and Ifnar1/-/ rats (Figure 3.2 A-B), which indicates that the disruption of IFNAR signaling does not alter the baseline levels of T cells in these rats under non-inflammatory conditions in blood and spleen.

**Figure 3.1. Gating strategy for determining splenic CD4+ and CD8+ T cells or Tregs in rats.**

3.1.A. Gating CD4 or CD8 T cells
3.1. Gating strategies for the flow cytometric analysis of T cell subsets in rat spleens. (A) Fluorescent counting beads and lymphocytes gates are depicted. Lymphocytes were gated using the FSC-A/SSC-A window. These were further gated based on the surface expression of CD3; CD3+ cells were further gated for CD4+ or CD8+ T cells or unspecific stained cells (isotype). All gating boundaries were established using FMO and isotype controls as described in the Methods section. (B) Gating strategy for flow cytometric analysis of Tregs in rat spleens. Fluorescent counting beads and lymphocytes gates are depicted. Lymphocytes were gated using the FSC-A/SSC-A window. These were further gated based on surface expression of CD4. CD4+ cells were further gated based on their expression of CD25 and FoxP3 or unspecific stained cells (isotype). All gating boundaries were established using FMO and isotype controls as described in the Methods section. A similar gating strategy was used for flow cytometry analysis of all T cell subsets in the blood (not shown).
Figure 3.2A. WT and IFNAR1⁻/⁻ rats have comparable numbers of basal CD4⁺, CD8⁺, and Tregs.
Figure 3.2. WT and Ifnar1⁻/⁻ rats have comparable levels of T cell subsets in the spleen and blood. The percentage (left) and absolute cell number (right) of CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ T cells and CD4⁺CD25⁺FoxP3⁺ Treg cells in the spleen (A) and blood (B) of WT and Ifnar1⁻/⁻ rats at weanling age (21-25) at 5 days following mock-infection with virus-free culture medium. Each data point represents a sample from an independently mock-infected animal (n=2 for each genotype) and horizontal bars indicate the median value. No significant differences (n.s.) are present between the unpaired groups as determined by the Mann-Whitney U test.
3.4.2. **KRV infection differentially affects the percentage and absolute number of lymphocyte subsets in WT compared to Ifnar1−/− rats.**

Our previous published data (Qaisar, Lin et al. 2017) established that IFNAR deficiency leads to altered cytokine and chemokine levels in the spleen during KRV infection. We therefore tested the possibility that disruption of IFNAR signaling modulates KRV-induced immune responses as early as 5 dpi by altering the frequency of lymphocyte subsets in spleen and blood. For this purpose, we infected weanling WT and Ifnar1−/− rats with KRV and harvested spleens and blood at 5 dpi. No difference was observed in the percentage and absolute count of CD4+ (CD3+CD4+) T cells between KRV-infected WT and Ifnar1−/− rats. However, a significant decrease ($P < 0.03$) in the percentage and absolute number of total T cells (CD3+) and the CD8+ subset (CD3+CD8+) was observed in KRV-infected Ifnar1−/− rats relative to KRV-infected WT rats (Figure 3.3A-B), with the decrease in total T cells possibly associated with the decreased CD8+ T cell values. We performed similar analyses on the peripheral blood but observed no differences in the percentage and absolute number of these lymphocyte subsets (Figure 3.3-C). One blood sample in the KRV-infected Ifnar1−/− group could not be processed due to technical issues.
Figure 3.3.A. Percentage of CD4+ or CD8+ T cells in KRV-infected rat spleens.
Figure 3.3.B. The percentage (left) and absolute count (right) of splenic CD3$^+$ (total T cells), CD4$^+$, or CD8$^+$ T cells in KRV-infected rats.
Figure 3.3. Levels of CD8+ T cells are significantly decreased in spleens of KRV-infected Ifnar1−/− rats compared to WT rats at 5 dpi. Weanling rats (21-25 days old) were mock-infected or infected with KRV (10⁷ PFU) intraperitoneally and spleens were harvested. A single-cell suspension of splenocytes was stained with fluorochrome-conjugated mAb. (A) Flow cytometry analysis of lymphocyte populations in spleen are shown. Lymphocytes were gated for CD3⁺CD4⁺ (X-axis) or CD3⁺CD8⁺ cells (Y-axis) as described in Fig. 3.1A. Numbers indicate the percentage of CD8⁺ (upper left) or CD4⁺ (lower right) cells based on fluorescent intensity. (B) The percentage (left) and absolute cell number (right) of CD3⁺ (top panels) and CD4⁺ T (middle panels) or CD8⁺ T cells (bottom panels) in spleen of WT and Ifnar1−/− rats are shown. While no differences were
observed in the percentage and absolute numbers of CD4+ T cells between infected WT and Ifnar1−/− rats, both percentage and absolute numbers of total T cells (CD3+) and CD8+ T cells were significantly lower (*P<0.03) in KRV-infected Ifnar1−/− rats compared to KRV-infected WT rats. (C) The percentage (left) and absolute cell number (right) of CD3+ (top panels) and CD4+ T (middle panels) or CD8+ T cells (bottom panels) in the peripheral blood of WT and Ifnar1−/− rats are shown. No significant differences were observed in the percentage and absolute numbers of T cells between infected WT and Ifnar1−/− rats. n.s.= not significant. Each data point represents a sample from an independently KRV-infected animal (n=4 for WT, n=3 for Ifnar1−/−), and the horizontal bar shows the median. The Mann-Whitney U test was used to determine statistical significance. Significance was defined as P<0.05.
3.4.3. **KRV infection decreases the percentage and absolute number of splenic Tregs in WT compared to Ifnar1−/− rats.**

Given the observation that Treg depletion induces diabetes in LEW.1WR1 rats (Mordes, Guberski et al. 2005) and that Tregs are crucial in controlling pancreatic autoimmunity and maintaining immune tolerance in NOD mice (Petzold, Riewaldt et al. 2013), we analyzed Treg populations in WT and Ifnar1−/− rats following KRV infection. We observed that the percentage and absolute number of splenic Tregs were significantly decreased ($P<0.03$) in KRV-infected WT rats compared to the KRV-infected Ifnar1−/− rats (Figure 3.4 A-B). In contrast to spleen, the peripheral blood from the KRV-infected WT and Ifnar1−/− rats had no differences in levels of Tregs (Figure 3.4 C).
Figure 3.4.A. The percentage of CD3+CD25+FoxP3+ Tregs in KRV-infected rat spleens.
Figure 3.4. KRV-infected WT rats have a decreased frequency of CD4+CD25+FoxP3+ regulatory T lymphocytes compared to infected Ifnar1−/− rats. Weanling rats (21-25 days old) were mock-infected (cell culture media) or KRV-infected (single dose, 10⁷ PFU) intraperitoneally and spleens were harvested. A single-cell suspension of splenocytes was stained with fluorochrome-conjugated mAb for surface markers (CD4+, CD25+) and nuclear marker (FoxP3). (A) Flow cytometry analysis of the Treg population in spleen is shown. Lymphocytes were first gated for CD4 T cells, which were further gated for expression of CD25+FoxP3+ as described in Fig. 3.1B. Numbers indicate the percentage of CD25+FoxP3+ cells (upper right) within the CD4+ lymphocyte population (not shown) based on fluorescent intensity. (B) The percentage (left) and absolute count (right) of CD4+CD25+FoxP3+ Tregs in spleens of WT and Ifnar1−/− rats are shown. Both percentage and absolute cell number of Treg cells are significantly
reduced in infected WT rats compared with infected Ifnar1<sup>+/−</sup> rats (*P<0.03). (C) No significant difference is observed in the percentage (left) and absolute cell number (right) of Tregs in the peripheral blood of KRV-infected WT and Ifnar1<sup>+/−</sup> rats are shown. n.s=not significant. Each data point represents a sample from an independently virus-infected animal (n=4 for WT, n=3 for Ifnar1<sup>+/−</sup>), and the horizontal bar shows the median. The Mann-Whitney U test was used to determine the statistical significance, which was defined as $P<0.05$. 
3.4.4. KRV persistently replicates in the spleen of infected WT and Ifnar1<sup>−/−</sup> rats

To assess if the differences in the lymphocyte populations in the spleen could be attributed to differences in KRV replication at 5 dpi, we performed quantitative RT-PCR (qRT-PCR) to determine the viral VP2 transcript levels in spleens using KRV-specific primers. Consistent with our previously reported data (Qaisar, Lin et al. 2017), we observed no significant difference in the levels of VP2 transcripts between WT and Ifnar1<sup>−/−</sup> rats during infection (Fig. 3.5). KRV transcript analysis by qRT-PCR on spleens collected on 12 dpi from KRV-infected WT and Ifnar1<sup>−/−</sup> rats show that virus genome copies are similar at days 5 and 12, with no significant difference observed between WT and Ifnar1<sup>−/−</sup> rats (Fig. 3.5).
3.5. Comparison of KRV transcript levels in infected rat spleens 5 and 12 days after KRV challenge.

Figure 3.5. Comparison of KRV transcript levels in spleens of WT and Ifnar1<sup>−/−</sup> rats at 5 and 12 days post-KRV infection. Weanling rats 21-25 days old were treated as follows: WT KRV (n=4) vs. WT mock infected (n=2); Ifnar1<sup>−/−</sup> KRV (n=4) vs. Ifnar1<sup>−/−</sup> mock infected (n=2). Spleens were collected at 5 dpi. The same experimental set up was repeated for a separate experiment for collection of spleens at 12 dpi. Total RNA was extracted from spleen using TRIzol and used for determining the expression level of KRV VP2 transcript levels by quantitative RT-PCR (qRT-PCR). All qRT-PCR values were normalized to GusB. Fold induction relative to the uninfected sample is shown. n.s.=not significant. Each data point represents a sample from an independently mock- or virus-infected animal (n=12 for each genotype), and the horizontal bar shows the median. The Mann-Whitney U test was used to determine the statistical significance, defined as $P<0.05$. 
3.5. Discussion

Due to their ubiquitous cellular expression along with the functional effects exerted through the downstream signaling cascades initiated through their activation, type I IFNs modulate a plethora of immune responses which go beyond their antiviral defenses, encompassing a broad range of direct and indirect immunomodulatory effects on the cells of both innate and adaptive immune systems (Le Bon and Tough 2002). IFNs also play a pathological role in the development of several inflammatory and autoimmune disorders, emphasizing the importance of regulated IFN signaling to maintain critical immune balance (Crouse, Kalinke et al. 2015). Multiple lines of evidence have consistently recognized IFNs as critical components of beta-cell immunopathogenesis and diabetes development (Jean-Baptiste, Xia et al. 2017, Newby and Mathews 2017). Here, we sought to assess the impact of absence of IFNAR-mediated signaling on T cell populations in LEW.1WR1 rats.

To examine the effect of IFNAR deficiency on lymphocyte subpopulations, we analyzed the percentage and absolute numbers of splenic and blood CD3+/CD4+, CD3+/CD8+ T cells, and Tregs in unmanipulated Ifnar1−/− rats. We observed no significant difference in the percentage and absolute numbers of T lymphocyte subpopulations between the weanling WT and Ifnar1−/− rats, indicating that lack of IFNAR signaling does not affect the normal levels of T lymphocytes. In addition, the percentages of splenic CD4+ and CD8+ T cell subsets were in agreement with the previously published data (Mordes, Guberski et al. 2005). Our finding that lack of IFNAR1 signaling does not affect the T cell subsets in rats is consistent with evidence from NOD mice having
normal levels of CD4⁺ and CD8⁺ T lymphocytes and B lymphocytes in spleens and pancreatic or inguinal lymph nodes following disruption of IFNAR1-mediated signaling (Quah, Miranda-Hernandez et al. 2014).

Previous studies on BBDR and LEW.1WR1 rats have provided evidence for the T-cell mediated contribution in the development of autoimmune diabetes following KRV infection (Ellerman, Richards et al. 1996). BBDR rats can be protected against diabetes by treatment with mAb directed against rat T-cell surface markers (CD2, CD5, CD8 and TCRαβ), while LEW.1WR1 rats are protected by mAbs directed against CD5 and TCRαβ (Ellerman, Richards et al. 1996). Additional work by Chung et al. demonstrated that KRV infection of the BBDR rats causes a significant increase in the percentage of splenic CD8 T cells but a decrease in the percentage of CD4⁺ T cells at 10 dpi (Chung, Jun et al. 2000). However, KRV induces an increase in the absolute numbers of both CD8⁺ and CD4⁺ T cells (Chung, Jun et al. 2000). Compared to CD4⁺ T cells, the CD8⁺ T cells are more proliferative, as incorporating BrdU and depleting CD8⁺ T cells with a targeting mAb results in a significant decrease in the incidence of KRV-induced diabetes (Chung, Jun et al. 2000). Similarly, KRV infection also increases the percentage and absolute number of CD8⁺ T cells in the spleen and PLNs of LEW.1WR1 rats 5 dpi (Zipris, Hillebrands et al. 2003, Londono, Komura et al. 2010, Hara, Alkanani et al. 2014).

Given the essential role of IFN signaling on the antiviral T cell immunity and the previously established role of effector T cells in facilitating KRV-induced autoimmune diabetes in BBDR and LEW.1WR1 rats, we hypothesized that the disruption of IFNAR
signaling may prevent T1D in rats by restraining KRV-induced alterations in effector T cells populations. To test this hypothesis, we first examined CD4+ and CD8+ T cell populations in the KRV-infected spleen and blood from WT and Ifnar1−/− rats at 5 dpi. We found that both the percentage and absolute numbers of CD8+ T cells decreased significantly in the spleen of KRV-infected Ifnar1−/− rats compared to the KRV-infected WT rats. In contrast, significant differences in the percentage and absolute numbers of CD4+ T cells in the spleen were not present between KRV-infected WT and Ifnar1−/− rats. In addition, analysis of the blood CD4+ and CD8+ T cells showed that the percentage and absolute numbers of these T cell subsets remained similar, indicating that KRV specifically alters the T cell subsets in the spleen by 5 dpi. These data suggest that one mechanism by which Ifnar1−/− rats are protected from KRV-induced diabetes involves a decreased frequency of splenic CD8+ T cells during the early innate immune response, and raise the possibility that IFNAR signaling mediates changes in the frequency of splenic CD8+ T cells, contributing to the beta-cell specific autoreactive attack in the WT rats following KRV infection.

From earlier studies with BBDP and BBDR rat models, researchers formulated the concept of immunological balance between the effector and regulatory arms of the immune system. This idea, conceptualized as the balance hypothesis of autoimmune diabetes in rats, suggests that autoimmune diabetes in rats results from an imbalance between (1) the cytotoxic effector cells that attack the beta cells, and (2) the regulatory cells that normally protect from the disease (Mordes, Bortell et al. 1996, Mordes, Bortell et al. 2004), and requires the presence of at least two defects in rats. The genetic
predisposition offered through the MHC class I allele constitutes a first defect as it leads to the generation of autoreactive effector cells. The second defect can be genetic or environmental (Mordes, Bortell et al. 2004). In the case of BBDR and LEW.1WR1 rats, the second defect is acquired through immune perturbants such as virus infection or poly I:C stimulation, that result in the amplification of the autoreactive population and/or deficiency of regulatory cell populations (Mordes, Bortell et al. 2004). Here, we address the possibility that the disease protective effect mediated through disruption of IFNAR signaling may involve changes in the Tregs population in the spleen of KRV-infected \( Ifnar1^{-/-} \) rats. Analysis of the percentage and absolute numbers of splenic Tregs from the KRV-infected WT and \( Ifnar1^{-/-} \) rats reveals that KRV significantly reduces the percentage and absolute numbers of Tregs in WT rats compared to the \( Ifnar1^{-/-} \) rats following KRV infection. No such effects are observed for the blood Tregs. These results suggest that absence of IFNAR signaling protects against the failure to maintain normal Treg numbers in spleen following KRV infection. In addition, our results of KRV-induced reduction in the percentage and absolute numbers of Tregs in WT rats are consistent with previous reports in the BBDR and the LEW.1WR1 rats in which infection with KRV caused a reduction in splenic Tregs in BBDR and the LEW.1WR1 rats (Zipris, Hillebrands et al. 2003, Londono, Komura et al. 2010).

Our other observation is that KRV can replicate to a similar extent in the spleens of both the WT and \( Ifnar1^{-/-} \) rats at both day 5 and day 12. This is intriguing because KRV-infected spleens of \( Ifnar1^{-/-} \) rats at day 5 have reduced levels of ISGs such as CXCL10 and CCL5 (Qaisar, Lin et al. 2017) as well as detectable changes in the T cell
populations compared to KRV-infected WT rats as discussed above. Studies on parvovirus-induced IFN signaling are few, but one report with murine parvovirus showed the induction of a limited IFN signaling response (Mattei, Cotmore et al. 2013), suggesting that this particular parvovirus can efficiently evade antiviral defense imposed by IFN signaling. Considering this available evidence, it may be plausible to speculate that IFN signaling does not have a major potent effect on KRV persistent replication in infected cells due to KRV’s ability to subvert the specific antiviral defense mechanism, but the PAMPs from KRV (both structural proteins as well as replication products) serve as a sustained source for activation of IFNAR signaling and downstream ISGs and proinflammatory cytokines in WT rats to break the immune balance in favor of effector T cells. In contrast, in the absence of IFNAR signaling, viral replication remains unaffected, but the lack of IFNAR signaling disrupts the forward feedback required to induce the diabetogenic immune responses such as expression of IFN-dependent ISGs and the resulting activation of autoreactive T cells.

In summary, we examined the effect of disruption of early innate immune responses mediated through IFNAR signaling on the development of virus-induced diabetes in rats. Our preliminary data support our hypothesis that absence of IFNAR signaling prevents the upregulation of CD8+ T cells with concomitant protection against loss of Treg cells following viral infection. These protective shifts help in maintaining the finely-tuned immune balance, leading to suppression of KRV-induced autoimmune diabetes in the Ifnar1−/− rats. The main limitation of this pilot study is its small sample size (see Methods) which may undermine the statistically significant differences observed in T cell numbers
between spleens from WT and Ifnar1−/− rats following KRV infection. Therefore, further validation of these data with a larger cohort of animals is necessary and will be performed in the near future.
Preface to Chapter IV

Contribution summary:

Natasha Qaisar and Dr. Jennifer Wang designed the experiments and performed data analysis. Natasha Qaisar, Suvana Lin, Basanthi Satish performed the rat infection experiments in Chapter IV collectively. Riccardo Racicot prepared RNA-seq libraries. Natasha Qaisar analyzed RNA-seq data under the guidance of Dr. Alper Kucukural (Bioinformatics Core, UMass Medical School). Natasha Qaisar wrote Chapter IV under the mentorship of Dr. Jennifer P. Wang.
CHAPTER IV

TRANSCRIPTOMIC ANALYSIS REVEALS AN ASSOCIATION BETWEEN VIRUS-INDUCED EARLY ACTIVATION OF THE INNATE IMMUNE SYSTEM AND AUTOIMMUNE DIABETES IN RATS

4.1 Abstract

Early damage to the pancreatic beta cells governed by virus-induced inflammatory responses has been postulated to be an underlying factor in islet autoimmunity and the development of T1D. To determine if such early innate immune mechanisms are activated by Kilham rat virus (KRV) infection in the LEW.1WR1 rats, we performed a comprehensive analysis of gene expression in spleens from KRV-infected WT and Ifnar1−/− rats as well as mock-infected WT and Ifnar1−/− rats using a bulk RNA-sequencing approach. We identified robust hyperexpression of numerous IFN-I-induced genes in spleens from KRV-infected WT rats compared to mock-infected WT rats at 5 dpi, including Oas1, Irf7, Isg15, Mx2, Ifit3, Igtp, and Dhx58. Hyperexpression of several inflammatory cytokines and chemokines for T-cell and monocyte recruitment and MHC class I genes was also evident, providing for a direct association between the IFN-I pathway and MHC class I. Only a subset of interferon stimulated genes, MHC class I genes, and inflammatory genes were increased in KRV-infected Ifnar1−/− rats compared to mock-infected Ifnar1−/− rats. Finally, MHC class II genes were induced in KRV-infected Ifnar1−/− rats compared to KRV-infected WT rats. Our comprehensive analysis of
differential early gene expression between WT and Ifnar1−/− rats highlights the importance of IFN-I and MHC class I in diabetes development and suggests that the early induction of inflammatory pathways is pro-diabetic.
4.2 Introduction

It is hypothesized that a combination of genetic, immune and environmental factors triggers a destructive process leading to the development of T1D (Pugliese 2017). Multiple pathological mechanisms contributed by innate and adaptive immune systems are involved in the destruction of the beta cells (Wallberg and Cooke 2013). Essential components of this destructive process include proinflammatory cytokines and chemokines such as IFN-I, activation of beta-cell-reactive T cells, and failure of immune tolerance (Wallberg and Cooke 2013, Tai, Wong et al. 2016).

Most of our understanding of initial stages of T1D is based on studies involving pancreatic tissue of deceased T1D-patients or data obtained from autoimmune diabetes animal models (Pugliese 2016). Substantial evidence obtained through these studies strengthens the hypothesis that beta cells possibly undergo critical pathological changes before the manifestation of the symptomatic phase of T1D (Richardson, Morgan et al. 2014). One of the hallmarks of these early pathological changes towards initial beta cell dysfunction is insulitis, an inflammatory lesion of the islets, which coincides with the appearance of autoantibodies against the beta-cell antigens (Pugliese 2017). Insulitis is often characterized by the infiltration of the islets by CD4 and CD8 T cells, B cells, macrophages, NK cells, and DCs, but cytotoxic CD8 T cells constitute the most abundant and predominant T cell population (Coppieters, Dotta et al. 2012, Pugliese 2016, Morgan 2017). These CD8 T cells could attack beta cells hyperexpressing HLA class I molecules, the latter being another major histopathologic feature of the insulitis observed in T1D pancreatic samples (Bottazzo, Dean et al. 1985, Foulis and Farquharson 1986, Foulis,
Liddle et al. 1986, Foulis, Farquharson et al. 1987, Foulis, Farquharson et al. 1987, Richardson, Rodriguez-Calvo et al. 2016). Viruses associated with T1D may serve as precipitating factors in the elevated expression of HLA class I (and class II) molecules (Krogvold, Edwin et al. 2015, Hyoty 2016), possibly through the activation of IFN signaling pathways.

IFN-Is have been insinuated to play an immunopathogenic role in modulating beta cell destruction and/or the development of T1D (Jean-Baptiste, Xia et al. 2017). Accordingly, elevated IFN-I (in particular IFN-α) expression has been detected in pancreatic islets from human T1D patients (Foulis, Farquharson et al. 1987, Somoza, Vargas et al. 1994, Huang, Yuang et al. 1995, Dotta, Censini et al. 2007) and NOD mice (Erlich, Valdes et al. 2008, Li and McDevitt 2011). Similarly, increased levels of serum IFN-α were documented in T1D patients (Chehadeh, Weill et al. 2000, Mavragani, Niewold et al. 2013). Studies with the BBDP rat model demonstrated that poly I:C-induced diabetes was associated with augmented serum IFN-α levels, leading to increased incidence and accelerated onset of diabetes (Ewel, Sobel et al. 1992). Further studies on dose-dependent responses of poly I:C-induced diabetes in BBDP rats associated elevated levels of serum IFN-α with significantly earlier onset of diabetes (Sobel, Ewel et al. 1994). In contrast, increased poly I:C-induced IFN-α was also documented in normal Wistar rats but these rats did not become diabetic, suggesting that the immunopathological potential of IFN requires the existence of other genetic factors conferring immunological susceptibility (Sobel, Ewel et al. 1994).
The pleiotropic effects displayed by IFN-I result from encoding a broad spectrum of ISGs whose protein products primarily act to restrict viral entry, replication, and release, but may also function to regulate the production of various cytokines and chemokines to regulate innate and adaptive immune responses in various biological and pathological settings, or may have pro-apoptotic effects (Gonzalez-Navajas, Lee et al. 2012). Some ISG-encoded antiviral proteins include myxovirus resistance 1 (MX1), ISG15, IFN-inducible double-stranded RNA-dependent protein kinase (PKR; encoded by EIF2AK2), 2′-5′-oligoadenylate synthetase (OAS), IFN-induced transmembrane proteins (IFITMs), apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC), and tripartite motifs (TRIMs) (McNab, Mayer-Barber et al. 2015). Additionally, innate viral sensors such as MDA5, RIG-I, and TLR3 are also instantly hyperexpressed as ISGs for enhanced viral recognition and activation of IFN signaling (McNab, Mayer-Barber et al. 2015). Importantly, enhanced expression of ISGs can thus constitute an interferon gene signature that can act as a surrogate for IFN-I activation and is detectable by transcriptomic analysis (Banchereau, Cepika et al. 2017).

Evidence from both human and animal pancreatic islets reveals the presence of ISGs and proinflammatory cytokines. In accordance, both ISGs (Lundberg, Krogvold et al. 2016) and cytokines (Roep, Kleijwegt et al. 2010, Waugh, Snell-Bergeon et al. 2017) have been detected in the pancreatic islets from T1D patients. Similarly, elevated expression of IFN-α and the IFN-I gene signature in pancreatic lymph nodes is associated with the induction of T1D in NOD mice (Erlich, Valdes et al. 2008). These studies suggest that a primary damage to pancreatic islets due to a dysregulated type I IFN
signaling pathway (possibly initiated through viral infections) plays a diabetogenic role in the early pathogenesis events in T1D (Jean-Baptiste, Xia et al. 2017, Eizirik and Op de Beeck 2018).

In this chapter, we use an RNA-seq approach with the goal to characterize gene expression induced by KRV preceding islet autoimmune attack in the LEW.1WR1 rats. We hypothesized that failure to activate ISGs and proinflammatory cytokines in the KRV-infected Ifnar1−/− rats confers protection against the autoreactive attack against beta cells. We identify a distinctive group of ISGs, MHC class I genes and inflammatory pathway genes uniquely hyperexpressed in WT rat spleens following KRV infection compared to uninfected WT rats. In contrast, relatively few of these pathways are induced in KRV-infected Ifnar1−/− rats compared to mock-infected Ifnar1−/− rats. These data underscore mechanistic links between KRV-induced IFN-signaling, inflammation, and the initiation of islet autoimmunity and diabetes in rats.

4.3. Materials and methods

**Virus infection of rats and spleen collection and total RNA preparation.** Weanling WT and Ifnar1−/− rats of 21-25 days of age of either sex were infected with a single i.p. dose of KRV-UMass strain (1 X 10⁷ PFU) on day 0. Spleens were harvested from mock-infected (i.e., injected with culture media) WT rats (n=2) and Ifnar1−/− rats (n=2) or KRV-infected WT rats (n=4) and Ifnar1−/− rats (n=4) at 5 dpi. Spleens were homogenized in 1 mL TRIzol reagent using TissueRuptor (Qiagen) and total RNA was extracted following
TRIzol method (Invitrogen). RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop).

**RNA-seq library preparation and sequencing.** Approximately 10 μg total RNA for each of the 12 rat samples was treated with TurboDNase (ThermoFisher Scientific) and ribosomal RNA (rRNA) depletion was performed using a Ribo-Zero™ Gold rRNA removal kit (Illumina). For RNA-seq, we prepared strand-specific libraries by following the protocol from Zhang and colleagues (Zhang, Theurkauf et al. 2012). The quality of the prepared libraries was confirmed using the Advanced Analytical Technologies, Inc. Fragment Analyzer through the Molecular Biology Core Lab (UMass Medical School). The 12 libraries were pooled and sequenced with paired end reads (75 bp each) using Illumina NextSeq 500 according to the manufacturer’s specifications. RNA-seq data can be accessed at the NCBI Gene Expression Omnibus (GEO) repository with accession number GSE114322.

**RNA-seq data analysis.** All raw sequencing reads were processed using an in-house pipeline (Dolphin) at the University of Massachusetts Medical School. The clean read pairs were aligned to the rat reference genome and rRNA sequences were filtered from the mapped reads. The RNA-Seq by Expectation Maximization (RSEM) method was used to determine the expression levels of genes by providing mapped transcript counts in the RNA-seq reads (Li and Dewey 2011). The differentially expressed genes (DEGs) were identified using DESeq2 and significant differences in genes were identified as fold
change >2 between uninfected and virus-infected samples and if the adjusted $P$ value (P adj) was <0.05 (Love, Huber et al. 2014). Biological functional annotation and associated pathways of detected DEGs were identified using Gene Ontology (GO, http://geneontology.org) and DAVID Bioinformatics Resources 6.8 (Huang da, Sherman et al. 2009) databases. These functional enrichment analyses were performed using the rat reference genome and gene IDs, adjusted $P$-values and fold changes of the DEGs. To determine potential interactions among increased or decreased DEGs, we constructed protein-protein interaction networks using the STRING online tool (http://string-db.org).

**4.4. RESULTS**

**4.4.1. Alignment of the RNA-seq reads to the rat genome**

The number of total reads aligned with RSEM is summarized in Table 4.1. Greater than 10 million raw reads were generated for each library. After removing reads aligning to rRNA, the remaining reads were aligned to the rat reference genome by the RSEM method. Based on RSEM estimation, the percentage of aligned reads was between 9-37% for all libraries. We detected transcripts for at least 13,968 rat genes with a variable read count number per gene for each library based on the RSEM calculation. These data were then further processed for gene expression analysis.
Table 4.1. Summary of reads successfully aligned to the rat genome

<table>
<thead>
<tr>
<th>Library Name</th>
<th>Library description</th>
<th>Total Reads</th>
<th>Reads After Filtering (%)</th>
<th>Reads Aligned (RSEM) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6</td>
<td>Mock-WT</td>
<td>12,336,981</td>
<td>9,797,948 (79.42)</td>
<td>3,232,731 (26.20)</td>
</tr>
<tr>
<td>S8</td>
<td>Mock-WT</td>
<td>15,304,673</td>
<td>12,506,341 (81.72)</td>
<td>4,531,245 (29.61)</td>
</tr>
<tr>
<td>S1</td>
<td>KRV-WT</td>
<td>20,453,395</td>
<td>8,982,715 (43.92)</td>
<td>1,836,354 (8.98)</td>
</tr>
<tr>
<td>S2</td>
<td>KRV-WT</td>
<td>9,557,388</td>
<td>8,550,933 (89.47)</td>
<td>3,531,557 (36.95)</td>
</tr>
<tr>
<td>S10</td>
<td>KRV-WT</td>
<td>10,459,706</td>
<td>7,946,920 (75.98)</td>
<td>2,329,575 (22.27)</td>
</tr>
<tr>
<td>S11</td>
<td>KRV-WT</td>
<td>14,693,221</td>
<td>13,749,923 (93.58)</td>
<td>5,130,274 (34.92)</td>
</tr>
<tr>
<td>S3</td>
<td>Mock-IFNAR1−/−</td>
<td>15,536,139</td>
<td>8,403,418 (88.98)</td>
<td>5,266,845 (33.90)</td>
</tr>
<tr>
<td>S4</td>
<td>Mock-IFNAR1−/−</td>
<td>9,444,036</td>
<td>8,403,418 (88.98)</td>
<td>2,598,485 (27.51)</td>
</tr>
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<td>S5</td>
<td>KRV-IFNAR1−/−</td>
<td>10,434,695</td>
<td>7,337,797 (70.32)</td>
<td>1,170,931 (10.49)</td>
</tr>
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<td>S7</td>
<td>KRV-IFNAR1−/−</td>
<td>16,453,919</td>
<td>11,547,107 (70.18)</td>
<td>3,147,184 (19.13)</td>
</tr>
<tr>
<td>S9</td>
<td>KRV-IFNAR1−/−</td>
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<td>17,705,481 (94.34)</td>
<td>5,923,691 (31.56)</td>
</tr>
<tr>
<td>S12</td>
<td>KRV-IFNAR1−/−</td>
<td>11,161,108</td>
<td>5,334,003 (47.79)</td>
<td>1,170,931 (10.49)</td>
</tr>
</tbody>
</table>

4.4.2. Identification of differentially expressed genes in rat spleens with KRV infection.

We detected differential gene expression levels and their significance levels (P-values) by using DSEQ by making three comparison groups: (1) KRV-infected WT and uninfected WT, (2) KRV-infected Ifnar1−/− and uninfected Ifnar1−/−, and (3) KRV-infected Ifnar1−/− and KRV-infected WT rats. If the fold-change of gene expression was ≥2 and the P-value was <0.05, the DEGs were identified and further analyzed. Based on this criterion, we identified 375 DEGs in KRV-infected WT vs. uninfected WT rats (Figure 4.1): a total of 307 DEGs were increased and 68 were decreased following KRV infection. A comparatively greater number of DEGs, 975, was detected in KRV-infected
Ifnar1\(^{-/-}\) vs. uninfected Ifnar1\(^{+/+}\) rats, with 497 increased and 478 decreased DEGs following KRV infection. A comparison of KRV-infected Ifnar1\(^{-/-}\) to KRV-infected WT rats showed 271 DEGs, of which 143 were increased and 128 were decreased in KRV-infected Ifnar1\(^{-/-}\) rats (Figure 4.1).

Table 4.2 shows the top 20 most significantly increased and decreased genes in the spleens for the three comparator groups. The most increased gene (~37-fold) in KRV-infected WT vs. mock-infected WT animals is diubiquitin (Ubd), followed by hexokinase 3 (Hk3) and immunoresponsive gene 1 (Irg1). The majority of the remaining genes were immune-related genes belonging to the IFN-I pathway such as ISGs, as well as chemokines, cytokines, complement, and granzymes. Similar to the KRV-infected WT rats, the top three most highly induced genes in KRV-infected Ifnar1\(^{-/-}\) rats vs. mock-
infected Ifnar1<sup>+/−</sup> rats are Irg1, Hk3, and Ubd. We found a predominance of ISGs such as Oas1a, Irf7, Isg15, Mx2, Ifit3, Igtp, and Dhx58 to be highly induced by KRV in WT compared to Ifnar1<sup>+/−</sup> rats. Finally, a comparison between KRV-infected Ifnar1<sup>+/−</sup> versus KRV-infected WT rats reveals a relative decrease in specific ISGs in KRV-infected Ifnar1<sup>+/−</sup> rats.
Table 4.2. Top 20 most significantly differentially expressed genes in the spleens of either WT or Ifnar1−/− rats.

<table>
<thead>
<tr>
<th>Gene</th>
<th>log2 fold change</th>
<th>p value</th>
<th>Gene</th>
<th>log2 fold change</th>
<th>p value</th>
<th>Gene</th>
<th>log2 fold change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubd</td>
<td>5.21</td>
<td>2.04E-122</td>
<td>Irg1</td>
<td>6.58</td>
<td>1.90E-73</td>
<td>Cyp4b1</td>
<td>2.56</td>
<td>6.79E-21</td>
</tr>
<tr>
<td>Hk3</td>
<td>4.77</td>
<td>6.08E-79</td>
<td>Hk3</td>
<td>5.97</td>
<td>3.79E-101</td>
<td>Ass1</td>
<td>2.38</td>
<td>1.20E-51</td>
</tr>
<tr>
<td>Irg1</td>
<td>4.55</td>
<td>2.95E-51</td>
<td>Ubd</td>
<td>5.59</td>
<td>7.08E-126</td>
<td>Ccl9</td>
<td>2.31</td>
<td>8.20E-21</td>
</tr>
<tr>
<td>Oas1a</td>
<td>4.20</td>
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4.4.3. *Enrichment of functions and pathways in rat spleens*

We performed GO enrichment analysis for the DEGs in the three comparator groups described above. The goal was to significantly enrich the increased or decreased DEGs against the rat genome background. The results here are filtered DEGs which significantly enriched into biological processes against the rat genome using the Fisher's exact test with a false discovery rate (FDR) correction (P<0.05) in the GO database. GO functional enrichment analysis clustered all increased or decreased DEG obtained from the three comparator groups into the following commonly overrepresented (GO term) biological processes: biological regulation, cellular component organization, cellular process, developmental process, immune system process, localization, locomotion, metabolic process, multicellular organismal process, and response to stimulus. Most GO terms belonging to the immune system category included subcategories designated as antigen processing and presentation, and immune response. We noted that antiviral defense genes and corresponding immune genes were subcategorized in the GO term response to stimulus (Figure 4.2).

To examine how DEGs enrich into functional pathways, we performed KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis using the DAVID (Database for Annotation, Visualization and Integration Discovery) bioinformatics tool. Table 4.3 presents a list of top 10 pathways enriched for the increased or decreased genes. Notably, functional pathways commonly enriched in the increased genes set are directly related to antiviral innate immune responses, antigen processing and presentation, T1D, and cell adhesion molecules. In contrast, commonly enriched
pathways for the decreased genes similarly corresponded to inflammatory pathways and antiviral defense mechanism, and were particularly noticeable in KRV-IFNAR1^-/- vs. mock or KRV-WT vs. KRV-IFNAR1^-/- rats. No such significant enrichment for pathways was observed in KRV-WT vs. mock-WT.

**Figure 4.2. Gene ontology annotation enrichment analysis.** The enriched biological process (GO term) categorization for the most differentially expressed genes in the spleens from the 3 groups. The upper panel shows categories increased and the lower panel shows categories decreased.
Table 4.3. Top 10 most significantly enriched KEGG pathways in the spleens of either WT or Ifnar1⁻/⁻ rats following infection.

### Top 10 KEGG enriched pathways for increased genes

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<tr>
<th>Pathway Term</th>
<th>KRV-WT vs. mock-WT P value</th>
<th>KRV-IFNAR1⁻/⁻ vs. mock-IFNAR1⁻/⁻ P value</th>
<th>KRV-IFNAR1⁻/⁻ vs. KRV-WT P value</th>
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### Top 10 KEGG enriched pathways for decreased genes

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4.4.4. Innate immune response and MHC class I pathways are differentially induced in KRV-infected WT rats compared to mock-infected WT rats

A total of 286 of 307 genes map to the rat genome and are enhanced during KRV infection of WT rats. Infection of WT LEW.1WR1 rats results in ~40% diabetes (see Chapter II). Gene induction in spleens for all animals in each treatment group was highly consistent. We observed an overabundance of genes related to immune antiviral defense mechanisms directed by IFN-I signaling (Figure 4.3A). These include genes from several ISG families such as *Isg15, Ifit2* or *3, Mx1* or *2, Oas2* or *3, Ifi44, Ifi47*, and *Ifi27l2b*. In addition, components of antigen processing and presentation by MHC class I represented by *RT1-A1* or *A2, RT1-CE5* or *CE10, RT1-S3, RT1-T24-3* or *4, Tap1* or *2, and B2m* are highly induced following KRV infection. Furthermore, elevated expression of nucleic acids sensors such as *Ifih1, Ddx58, TLR7*, and *Zbp1* is also observed. An additional set of genes comprising of *Tlr4, Cma1/Mcpt3, Casp1*, and *Gbp5* were categorized into the IL-1β pathway (inflammatory). The activation and regulation of adaptive immune system including CD4 or CD8 T cell activation, regulation, migration, and differentiation are also induced. These observations are further supported by the induced genes corresponding to chemokines and cytokines for T cell or monocyte recruitment such as *Cxcl11, Cxcl9, Ccl7, Ccl5, Ccl12, Cxcl16, Ccl2, Cxcl10* and members of the JAK-STAT pathway such as *IFNγ, Socs1* or *3, and Irf1*. The 68 genes that are decreased, shown in Figure 4.3B, do not strongly cluster into broad categories.
Figure 4.3. Bulk RNA-seq analysis of genes induced in spleens from KRV-infected WT rats compared to mock-infected WT rats 5 dpi. Weanling 21-25 day-old rats were treated as follows: KRV-WT (n=4) vs. mock-WT (n=2). A. Increased genes, 307 total. B. Decreased genes, 68 total. Network interactions were determined with STRING.
4.4.5. Enrichment of genes in KRV-infected Ifnar1−/− rats compared to mock-infected Ifnar1−/− rats

We observed strong differences in the GO enrichment of genes in Ifnar1−/− rats following KRV infection. Infection of Ifnar1−/− rats results in ~19% diabetes (see Chapter II). Of 497 significantly increased genes, 463 map to the rat genome. The inflammatory response to antigenic stimulus comprising of TNF, IL1rn, Ahcy, and IL-1β is the most enriched pathway, followed by enrichment for non-immune related pathways such as receptor and ion signaling, drug metabolism, and wound healing. A noticeable enrichment for genes broadly related to MHC class I, T cell activation, complement activation, the IL-1β pathway, innate immunity, and monocyte recruitment is observed (Figure 4.4A). Of 478 significantly decreased genes, a small cluster of chemokines is apparent (Figure 4.4B).
Figure 4.4. Bulk RNA-seq analysis for gene expression profiles induced in spleens from KRV-infected Ifnar1⁻⁻ rats compared to mock-infected Ifnar1⁻⁻ rats 5 dpi. Weanling rats of 21-25 day old were treated as follows: KRV-IFNAR1⁻⁻ (n=4) vs. mock-IFNAR1⁺⁺ (n=2). A. Increased genes, top 200 of 497 total shown. B. Decreased genes, top 200 of 478 total shown. Network interactions were determined with STRING.
4.4.6. Differentially expressed genes in KRV-infected Ifnar1⁻/⁻ rats compared to KRV-infected WT rats

Next, we compared KRV-infected Ifnar1⁻/⁻ rats, which have a lower frequency of diabetes (~19%), to KRV-infected WT rats, which have a greater frequency of diabetes (~40%) (see Chapter II). A total of 134 out of 143 increased genes map to the rat reference genome. The top categories of significantly increased genes in KRV-infected Ifnar1⁻/⁻ rats relative to KRV-infected WT rats are related to antigen processing and presentation via MHC class II (RT1-Ba, -Db1, -Da, -DMA, -Bb, and Cd74), neutrophil regulation (Ptafr, Cd177), granulocyte migration (Cx3cl1, C5ar1, Epx, Cd177, Lgals3, Ccl9, Ccl24), cellular response to IL-1 pathway, negative regulation of T cell activation, and response to IFN-γ (Figure 4.5A).

A total of 118 out of 128 decreased genes map to the rat genome. We found that inflammatory genes and IFN-I genes are differentially decreased in KRV-infected Ifnar1⁻/⁻ rats compared to KRV-infected WT rats. A functional analysis of these genes showed that top pathways significantly decreased in Ifnar1⁻/⁻ rats relative to KRV-infected WT rats include innate and adaptive immune responses, antiviral responses, and chemokines and cytokines production: regulation of effector T cells (Lgals9, Il12rb2, Xcl1, Ifng), Isg15 conjugation (Isg15, Uba7, Ube2l6), positive regulation of IFN-I response (Dhx58, Irf7, Ifih1, Flt3), antiviral ISGs/innate immune regulation (Eif2ak2, Oasl, Isg20, Mx2, Fam111a, Isg15, Oasl1b, Rsad2, Oas3, Ifih1, Irf7), and positive regulation of chemokine and cytokine (Ccl21, Pbbp, Lgals9, Cxcl13, Cxcl17, Xcl1) (Fig. 4.5B).
Fig. 4.5. Bulk RNA-seq analysis of gene expression profile of spleens from KRV-infected Ifnar1−/− rats vs. KRV-infected WT rats at 5 dpi. Weanling rats of 21-25 day old were treated as follows: KRV-infected-IFNAR1−/− (n=4) vs. KRV-WT (n=4). **A.** Increased genes, 143 total. **B.** Decreased genes, 128 total. Network interactions were determined with STRING.
4.5 Discussion

With a focused goal of identifying candidate genes and pathways involved in regulating immune responses at the early stage of T1D, we employed bulk RNA-seq to compare gene expression profiles in the spleens of WT vs Ifnar1−/− rats after 5 days of KRV infection. Our finding of elevated expression of ISGs in the spleens from KRV-infected WT rats compared to mock-infected WT rats implicate that a KRV-activated IFN-I signaling pathway is playing an active role in the initiation of islet autoimmunity.

Specifically, the anticipated overexpression of ISGs (Oas1, Irf7, Isg15, Mx2, Ifit3, Igtp, and Dhx58) is reminiscent of similar observations made from studies with isolated human islets from recent onset T1D patients (Lundberg, Krogvold et al. 2016) or those treated with enteroviral infection (Nyalwidhe, Gallagher et al. 2017) or recombinant IFN-I (Lind, Richardson et al. 2013). Moreover, we observed significant enrichment of multiple genes of MHC class I and chemoattractants for T-cell and monocyte recruitment, providing evidence in support of IFN-I mediated hyperexpression of MHC class I and modulation of adaptive immune responses following viral infection. In contrast, we found that only a subset of ISGs, MHC I genes, and inflammatory genes were increased in KRV-infected Ifnar1−/− rats relative to mock-infected Ifnar1−/− rats. Although nearly seven times more genes were significantly decreased in this comparator group 2, only a minority (~35%) of these significantly decreased genes enriched for immune-related or response to stimulus pathways, with the remaining vast majority corresponding to a diverse set of biological processes (Figure 4.1).
An unexpected finding from our analysis was the significantly increased expression of several MHC class II genes in the KRV-infected Ifnar1−/− rats relative to KRV-infected WT rats. Compared to IFN-I effects on MHC class I, limited literature is available on the IFN-I effect on MHC class II. Nevertheless, earlier studies documented that IFN-beta blocks the induction of MHC class II by IFN-γ (Lu, Riley et al. 1995). Subsequent work showed that IFN-α treatment caused downregulation of MHC-class II in human pancreatic islets in a manner that involves the transactivator CIITA, which is important for regulation of MHC class II expression (Harris, Malanga et al. 2006, Pisapia, Pozzo et al. 2012). Accordingly, we found modest but significantly increased expression of CIITA (log₂ fold change ~1.17) in the comparator group 3. Considering these previous studies, it can be postulated that upregulation of MHC class II genes is due to the absence of inhibitory effects mediated through the IFN-I signaling pathway.

Amongst the inflammatory pathways, the IL-1β pathway significantly emerged as a common inflammatory pathway in comparator groups 1 and 2. Given that 19% of our Ifnar1−/− rats turn diabetic upon challenge with poly I:C or KRV, it is possible that signaling mediated through the IL-1β pathway plays an important role in promoting and sustaining inflammatory conditions, likely leading to enhanced beta cell inflammation and resulting T1D. Therefore, generation of caspase-1 (Casp1) knockout LEW.1WR1 rats, which would not be able to process pro-IL-1β (Kuida, Lippke et al. 1995), can prove useful for mechanistic understanding of these additional inflammatory pathways.

The findings discussed here are entirely derived from rat spleens, which are reportedly comprised of B cells (50%), T cells (36%), CD4+ (23%) and CD8+ (12%) T
cells and Treg cells (Morris and Komocsar 1997, Mordes, Guberski et al. 2005); however, information on proportions of monocytes, NK cells, granulocytes, DCs and pDCs is highly inconsistently reported in rats, and therefore it will be important to determine which cell type(s) in spleen play critical roles in regulating these early innate immune responses. To determine if dysregulated innate immune responses during the early stage play a role in beta cell pathogenesis, single-cell RNA-seq will be a more powerful strategy (compared to bulk-RNA-seq) in dissecting the role of both islet cells and resident immune cells and other uncharacterized populations of cells. In addition, scRNA-seq can also be used further to systematically dissect the population of effector and/or regulatory immune cells during the early stage of diabetes in the rat model.
A complex process dictated by genetic, environmental and immunological factors initiates islet inflammation and progressive and selective loss of the functional beta cell mass, resulting in hyperglycemia and the eventual clinical onset of T1D (Atkinson, Eisenbarth et al. 2014). Both innate and adaptive immune mechanisms contribute to the (auto)immune-mediated targeted destruction of the beta cells, with innate immune mechanisms primarily operating at the initiation phase of the beta-cell autoimmunity. As a first line of defense, innate immunity is triggered following an invasion by pathogens. Environmental factors, including enteroviral infections, are considered prime suspects for contributing to the development of T1D and may be a component in the initiation of beta-cell autoimmunity and development of T1D. Consistent with the virus hypothesis is the strong evidence for the presence of IFN-I and IFN-induced inflammatory mediators in the pancreata of T1D patients, indicating the diabetogenic potential of IFN in the islet autoimmunity.

In support of the causal relationship between virus and T1D, several genes in the IFN-I signaling network, including *IFIH1* and *TYK2*, are linked with predisposition to T1D (Storling and Pociot 2017). This intriguing link between genes in the IFN-I pathway and T1D substantiate the hypothesis that IFN-I signaling induced by viral infections in the pancreatic islets is a significant pathognomonic feature of early beta cell inflammation and subsequent instigation of the autoimmune attack against the beta cells. However, definitive studies on the role of virus-induced IFN-I signaling pathway in the
development of T1D are few, with some providing conflicted results. In this dissertation, I endeavored to answer the fundamental question of how virus-induced innate immune responses, in particular IFN-I signaling pathway and inflammatory pathways, contribute to the pathogenesis of T1D. The work presented here provides the missing fundamental connection between viruses and T1D pathogenesis, initiated and orchestrated through the innate immune mechanisms, specifically IFN-I signaling.

In Chapter II of this dissertation, I attempt to investigate the role of the IFN-I signaling pathway in the initiation and early progression of T1D by utilizing the LEW.1WR1 rat model. I hypothesize that the functional disruption of the IFNAR signaling in rats eliminates further propagation of the IFN-I signaling pathway and the induction of ISGs expression. As a consequence, IFNAR-deficient rats would be protected from diabetes when challenged with either KRV or poly I:C. For this purpose, we generated \textit{Ifnar1}^{-/-} LEW.1WR1 rats using CRISPR-Cas9 gene editing technology. Challenging \textit{Ifnar1}^{+/+} rats with either poly I:C or KRV protected the rats from diabetes and reduced the incidence of insulitis compared to WT rats. Of note, ~18% of the \textit{Ifnar1}^{-/-} rats become diabetic irrespective of the trigger being used to induce diabetes (KRV or poly I:C), implying the potential role of common, IFNAR1-independent mechanisms operating in rats.

As mentioned above, existing evidence on the role of IFNAR signaling in T1D report conflicting data. Blocking of IFNAR signaling through administration of neutralizing mAb against IFNAR1 in NOD mice at 2-3 weeks of age significantly diminished the incidence of T1D (Li, Xu et al. 2008). In contrast, diabetes incidence was
reported to be similar between NOD and NOD.IFNAR1−/− mice even though IFN production was observed, indicating that IFN signaling is not a critical component for the initiation and progression of diabetes in NOD mice (Quah, Miranda-Hernandez et al. 2014). These conflicting results show that, although IFN can be produced and may contribute to the early inflammation of the islets, the full impact of its potential on progression to T1D is dependent on the intrinsic constituents of the host such as combination of immunological and genetic factors. Unlike NOD mice, which can spontaneously develop diabetes and virus infections can protect against diabetes (Oldstone 1988, Atkinson and Leiter 1999), a perpetual source of innate immune stimulation such as viral replication might be a critical determinant for IFN-I-mediated progression to human T1D.

More than 50 genetic loci are thought to influence the risk of developing T1D. Interestingly, a significant proportion of these genes are immune-related and are expressed in human islets (Storling and Pociot 2017). These mounting data challenge the previous paradigm that beta cells are not active participants in their own demise (Soleimanpour and Stoffers 2013). Instead, it has been suggested that beta cells engage in a crosstalk with the immune system to initiate an autoreactive response (Soleimanpour and Stoffers 2013). Therefore, it is ideal to determine whether and how direct beta-cell specific contributions actively participate in self-demise following KRV or poly I:C challenge in the rat model. Whether or not IFN-signaling and proinflammatory pathways originating from the islets are the prominent players in initiating the autoreactivity against the beta cells can be addressed in LEW.1WR1 rats. Likewise, determining the role of
hematopoietic compartment as a primary source of IFN-I and proinflammatory pathways can add to our understanding of the mechanism(s) of protection in the Ifnar1^-/- rats against T1D. By virtue of intrinsic differences, KRV and poly I:C may initiate different sets of innate immune pathways in which IFNAR-dependent and/or IFNAR-independent responses play a dominant role.

Beyond their conventional direct antiviral defense mechanisms, IFN-I signaling exerts several immunomodulatory activities, including regulation of innate immune activation and adaptive immunity (Crouse, Kalinke et al. 2015). With respect to antiviral T cell responses, IFN-I signaling can directly or indirectly stimulate or inhibit their activation, expansion, differentiation, and survival (Crouse, Kalinke et al. 2015). Evidence indicates that KRV-induced diabetes in the BBDR rats is a consequence of a finely tuned immune balance of T cells (Chung, Jun et al. 2000). Therefore, I further examined the impact of functional disruption of IFNAR signaling on T cell populations following immune perturbation.

In Chapter III of this dissertation, I hypothesized that IFNAR-deficiency provides protection against diabetes by modulating T lymphocyte populations during the development of T1D following viral challenge. We established that Ifnar1^-/- rats harbor normal levels of peripheral T lymphocytes in unmanipulated form. Although the percentage and absolute cell number of CD8^+ T cells in the spleens remain similar in both uninfected and KRV-infected WT rats, significant decreases are observed in KRV-infected Ifnar1^-/- rats. The percentage and absolute cell number of splenic Treg cells in KRV-infected Ifnar1^-/- rats were similar to those of both uninfected WT and Ifnar1^-/- rats,
but are significantly reduced in KRV-infected WT rats. In their aggregate, these results suggest that during early stage of virus-induced diabetes, IFNAR signaling affects CD8$^+$ T cells and Treg cells. Correspondingly, our preliminary data support the innate to adaptive connection modulated through IFNAR-dependent signaling during KRV-induced diabetes in the LEW.1WR1 rats. In the context of ‘the balance hypothesis of autoimmune diabetes in rats’ illustrated in Figure 5.1, we propose that although Ifnar1$^{-/-}$ rats harbor preexisting populations of beta-cell-specific autoreactive T cells, absence of IFNAR signaling speculatively reduces the pool of autoreactive T cells with a concomitant protection against loss of Treg populations, thus tipping the balance in favor of an overall protective effect against unleashing autoreactive attack against the islets during KRV infection.

What remains unclear is how IFN-I induced modulation affects the immune balance in the rats following viral infection. Given the critical role of IFN-I signaling for the generation of effector and memory cells during viral infection (Kolumam, Thomas et al. 2005), it can be hypothesized that absence of intrinsic IFN-I signaling in CD8$^+$ T cells can directly affect the cytotoxic effector response, leading to reduced incidence of insulitis and diabetes. In vitro studies involving IFN-I or KRV treatment of Ifnar1$^{-/-}$ CD8$^+$ T cells and cytokine assays (measurement of IFN-$\gamma$, granzyme B) can be performed to assess the impact of absence of IFNAR-dependent signaling on the cytotoxicity of Ifnar1$^{-/-}$ CD8$^+$ T cells. In vivo adoptive transfer of purified CD8$^+$ T cells from the non-diabetic, KRV-infected Ifnar1$^{-/-}$ rats to the CD8$^+$ T cells-depleted WT rats can be conducted to
Figure 5.1. Schematic representation of the utilization of balance hypothesis of autoimmune diabetes in the LEW.1WR1 rats in the absence of IFNAR signaling. Originally proposed in BBDR and BBDP rats and adapted from (Mordes, Bortell et al. 2004). An equilibrium exists between “aggressive” and “regulatory” forces depicted by effector T cells (Teffs) and regulatory T cells (Tregs), respectively. In the WT LEW.1WR1 rats, this balance is disturbed due to KRV infection, leading to IFNAR signaling-mediated shifts in favor of beta-cell-specific preexisting autoreactive Teffs. However, the absence of IFNAR signaling prevents the imbalance between Teffs and Tregs during KRV-induced breakdown of immune balance in favor of overall protection against autoreactive attack against the beta cells and therefore allows preventions against overt diabetes manifestation.
determine if intrinsic IFNAR-dependent signaling is critical for the regulation of the autoimmune attack in rats.

The role of Treg cells has been a subject of intense research in diabetes and other autoimmune disorders (Grant, Liberal et al. 2015, Hull, Peakman et al. 2017). Building off the seminal work done in the rat models of autoimmune diabetes (Mordes, Bortell et al. 2004), we further explored the effect of IFNAR-dependent signaling on the Treg cells during KRV infection. Our preliminary studies show that Ifnar1−/− rats are protected from KRV-induced decrease in the number and percentage of Treg cells in WT rats. These studies can be further expanded to determine if IFNAR-dependent signaling directly or indirectly regulates Treg cells number and/or function as previously explored other viral infections (Piconese, Pacella et al. 2015).

Although both CD8+ and CD4+ T cells are the active participants in inflammation and destruction of beta cells, evidence that multiple deficits in the innate immune responses contributed by macrophages, DCs, and NK cells points at the role of dysregulated innate immune responses in the initiation of inflammation in the beta cells at the initial stage (Ferretti and La Cava 2016). In line with the role of innate immune cells in the beta cell inflammation and destruction, studies have established the importance of IFN-I in promoting DCs and macrophages (Gessani, Conti et al. 2014). A specialized subset of DCs known as plasmacytoid DCs (pDCs) secrete copious amounts of IFN-I (α subtype) during inflammatory conditions such as viral infection or autoimmune disorders (Siegal, Kadowaki et al. 1999, Marshak-Rothstein 2006). Evidence from human T1D patients and studies in NOD mice implicates the role of pDCs
in T1D pathogenesis (Li, Xu et al. 2008, Allen, Pang et al. 2009, Li and McDevitt 2011). A significant delay in the onset and incidence of T1D is observed with pDCs depletion in NOD mice, indicating the prominent role of pDCs as initiators of the T1D development (Li and McDevitt 2011). In addition, blocking IFNAR1 in NOD mice results in a significant increase in the number of tolerogenic immature DCs and the production of the immunoregulatory cytokine IL-10 in PLNs or spleen. It is pertinent to characterize the role of pDCs in our rat model to assess if pDC-derived IFN-I is a predominant initiator of T1D in rats. This can be done by mAb-mediated depletion of pDCs in rats and then assessing the incidence of diabetes following KRV or poly I:C challenge. Previously, the population of pDCs in rat have been phenotypically characterized by producing large quantities of IFN-I and TLR7 and TLR9 expression (Hubert, Voisine et al. 2004). Alternatively, macrophages can serve as prime initiators of the IFN-I induced T1D pathogenesis, as depletion of macrophages led to enhanced protection from insulitis and T1D in KRV and poly I:C-treated BBDR rats (Chung, Jun et al. 1997). Collectively, these future studies can provide a mechanistic understanding of the immunomodulatory effects of IFN-I on effector T cells during viral infection, mediated as a consequence of partnership between the IFN-I and DCs and/or macrophages, thereby providing a bridge between the innate and adaptive immune systems in our rat model during inflammation.

To increase the incidence of KRV-induced diabetes in our rat model, we recently performed a combined treatment regimen involving both poly I:C and KRV. Treatment of WT LEW.1WR1 rats with three doses of poly I:C followed by KRV has been shown to induce ~100% diabetes (see Figure 5.2, panel C). Surprisingly, our recent studies using
combined treatment regimen with poly I:C + KRV in Ifnar1−/− rats resulted in frequency of diabetes similar to the WT rats. This unanticipated but interesting result shows that a brief pretreatment with poly I:C followed by KRV infection induces diabetes in an IFNAR-independent manner, possibly by activating inflammatory pathways such as an IL-1-mediated pathway, therefore emphasizing the need for investigating the contributions of multiple innate immune inflammatory pathways in the pathogenesis of T1D in this rat model.

Figure 5.2. IFNAR1 deficiency protects rats from poly I:C-induced diabetes and KRV-induced diabetes. Rats 21-25 days old were challenged with poly I:C (1 µg/g) by i.p. injection 3X/week for 3 weeks. A. The incidence of poly I:C-induced diabetes was significantly reduced in Ifnar1−/− rats compared to WT rats (P<0.0001, log-rank test). B. Ifnar1−/− rats are only partly protected from KRV-induced diabetes. Animals 21-25 days old of either sex were inoculated i.p. with KRV (10^7 PFU). C. Ifnar1−/− rats were pretreated with poly I:C (2 µg/g) i.p. on days -3, -2, and -1 then inoculated i.p. with KRV (10^7 PFU). Nearly all animals became diabetic.

It has been documented that IL-1β mediates apoptosis of human islet cells in combination with TNF-α and IFN-γ (Delaney, Pavlovic et al. 1997). In addition, IL-1β together with IL-18, have been documented to contribute to Fas-mediated destruction of pancreatic beta cells in NOD mice (Amrani, Verdaguer et al. 2000, Schott, Haskell et al. 2004). The inactive forms of IL-1β and IL-18 are cleaved by caspase-1, a cysteine
protease, to biologically active forms. As one of the important mediators of beta cell dysfunction (Mandrup-Poulsen 1996), the role of IL-1β in type 1 diabetes has remained a subject of much interest and has been investigated in NOD mice (Schott, Haskell et al. 2004) and LEW.1WR1 rats (Hara, Alkanani et al. 2014). Therefore, it is relevant to examine the essential role of caspase-1-mediated IL-1β (and IL-18) signaling in the development of T1D in rats. This can be achieved by developing caspase-1 knockout rats using CRISPR-Cas9 and then assessing the incidence of diabetes by challenging with poly I:C 3x + KRV. Coupled with the knowledge gained from the disruption of IFNAR-dependent signaling, such additional studies will further advance our understanding of how different innate immune pathways predominantly regulate the beta cell inflammation and progression to T1D.

Several hypotheses have been proposed to explain the mechanism for the potential interaction between viruses and host that might contribute to pathogenesis of T1D. Among these, direct virus-induced lysis of the beta cell has been proposed because some viruses are capable of causing severe damage to *in vitro* cultured pancreatic islets and *in vivo* animal models. In contrast, in the immune-mediated T1D that occurs in rats, the involvement of virus-induced innate immune activation eliciting islet autoimmunity has been hypothesized. In the case of KRV, evidence is lacking for direct interaction of KRV with islets, in particular beta cells. Therefore, additional studies focusing on KRV-specific innate immune activation in the beta cell need to be conducted. How poly I:C treatment of rat establishes insulitis and the role of IFN signaling in islets contributes to the development of T1D requires investigation. One approach to address this is to
perform islet transplant experiments, engrafting $Ifnar1^{-/-}$ islets into WT rats (and vice versa) and challenging with either KRV or poly I:C. Specifically, islets can be transplanted from weanling WT LEW.1WR1 donor rats into weanling streptozotocin (STZ)-treated, hyperglycemic $Ifnar1^{-/-}$ LEW.1WR1 recipient rats to restore normoglycemia. Conversely, islets can be transplanted from weanling $Ifnar1^{-/-}$ LEW.1WR1 donor rats into weanling STZ-treated, hyperglycemic WT recipient rats to restore normoglycemia. Subsequently, the islet-recipient rats will be challenged with poly I:C or KRV and monitored for diabetes development to determine if intrinsic IFN-I signaling in islets contribute to diabetes development.

Mounting recent evidence has implicated the dominant role of IFN-α in the pathogenesis of a rare group of human autoinflammatory and autoimmune disorders called IFN-I interferonopathies. Recently, a similar connection between virus-initiated local IFN-I interferonopathy within the pancreas and islet microenvironment has been implicated in the pathogenesis of T1D (Jean-Baptiste, Xia et al. 2017). RNA-seq and immunohistochemical staining can be used to confirm whether rat islets generate IFN-α in response to KRV infection and if this induces MHC class I in islets. Furthermore, few case reports have documented that treatment of hepatitis C virus (Fabris, Floreani et al. 2003) or hairy cell leukemia with IFN-α therapy (Guerci, Guerci et al. 1994) is associated with T1D development. To determine if IFN-α directly contributes to diabetes development in rats, treatment of weanling WT and $Ifnar1^{-/-}$ rats with exogenous recombinant rat IFN-α can be further conducted. It is expected that WT rats will be predisposed to diabetes, whereas $Ifnar1^{-/-}$ rats will be relatively protected from diabetes
development following treatment with recombinant rat IFN-α therapy.

Finally, in Chapter IV of this dissertation, we used bulk RNA-seq for transcriptomic analysis of spleens from WT and Ifnar1−/− rats following KRV challenge 5 dpi. In this exploratory study, we identified significant overrepresentation of biological function groups and networks related to IFN-I signaling pathways, such as ISGs, operating in the WT rats following KRV infection compared to Ifnar1−/− rats following KRV infection. Novel findings from our data include significantly increased MHC class II genes in KRV-IFNAR−/− rats compared to KRV-WT rats. We also noticed significant enrichment of MHC class I and pathways involved in activation of effector T cell responses, providing further evidence for a role for effector T cell responses. In addition, genes in the IL-1β pathway are commonly enriched both in the infected WT and Ifnar1−/− rats, insinuating that an IL-1β-mediated pathway is actively modulated by KRV during T1D pathogenesis. Further validation of the expression of candidate increased or decreased by qRT-PCR, immunostaining, or flow cytometry will be important to confirm these analyses.

These RNA-seq data provide us with an unbiased view of transcriptional landscape of rat spleens following viral infection and how IFNAR-dependent signaling regulates multiple immune-related pathways. Our RNA-seq data are also highly relevant to the emerging interest in the application of transcriptomics as powerful tool for the development of diagnostic or predictive biomarkers and immunomonitoring for several human IFN-I signaling-mediated dysregulated autoimmune and autoinflammatory diseases including T1D (Banchereau, Cepika et al. 2017). In summary, application of
approaches described in this dissertation has the potential to reveal previously unidentified pathways and targets for autoimmune diabetes.
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