MECHANISMS OF INSULIN-DEPENDENT DIABETES LOCUS 9-MEDIATED PROTECTION FROM TYPE 1 DIABETES

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by
Gregory J. Berry

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The dissertation of Gregory J. Berry was reviewed and approved* by the following:

Hanspeter Waldner  
Assistant Professor of Microbiology and Immunology  
Dissertation Advisor  
Chair of Committee

Laura Carrel  
Associate Professor of Biochemistry and Molecular Biology

Todd D. Schell  
Associate Professor of Microbiology and Immunology

Neil D. Christensen  
Professor of Pathology, and Microbiology and Immunology

Jianxun Song  
Assistant Professor of Microbiology and Immunology

Jianming Hu  
Professor of Microbiology and Immunology  
Director of Microbiology and Immunology Graduate Program

*Signatures are on file in the Graduate School
Abstract

There is currently no cure for type 1 diabetes (T1D), a chronic autoimmune disease typically diagnosed in children that results in destruction of the insulin-producing beta cells of the pancreas and hyperglycemia and serious life-long health complications. A cure for this debilitating disease has remained elusive in part due to its complex genetics. Linkage studies have identified over 25 Insulin dependent diabetes (Idd) regions known to be associated with T1D development. We focused on one of these regions, a ~200 gene region called Idd9. This region contains genes known to regulate lymphocyte function and development and can confer greater than 90% protection from T1D in nonobese diabetic (NOD) mice, a spontaneous T1D animal model of human T1D in which the NOD Idd9 is exchanged with the same region from a T1D-resistant mouse strain. The goal of this dissertation was to further elucidate the mechanisms by which Idd9 confers protection from T1D development in NOD mice. We investigated the role of this region in the regulation of islet-specific, autoimmune CD4+ T cells and the role of a sublocus of this region, Idd9.3, in overall T1D development.

To identify the role of Idd9 in the regulation of diabetogenic, islet-specific CD4+ T cells, we developed novel NOD.B10 Idd9 (line 905) congenic mice that harbor predominantly islet-reactive CD4+ T cells expressing the BDC2.5 T cell receptor (BDC-Idd9.905 mice). We compared these BDC-Idd9.905 mice containing the Idd9 from the T1D-resistant C57BL/10 (B10) mouse to BDC2.5 TCR transgenic (BDC2.5) mice containing the NOD-derived T1D-susceptible Idd9. Overall, these
findings identified *Eno1*, *Rbbp4*, and *Mtor* as candidate genes in the regulation of diabetogenic islet-specific CD4+ T cells and suggest their role in T1D pathogenesis.

*Idd9.3* contains 19 genes instead of the ~200 found on full-length *Idd9*, allowing us to more easily focus in on individual genes to identify specific genes and genetic mechanisms contributing to T1D. We used congenic NOD mice containing the T1D-resistant B10 *Idd9.3* (NOD B10 *Idd9.3*) and compared them to NOD mice and found that NOD.B10 *Idd9.3* mice had a vast reduction in B cells. Constitutive expression of MicroRNA-34a (miR-34a), one of the genes on *Idd9.3*, had previously been shown to mediate an early B cell developmental block by repressing the B cell developmental transcription factor Foxp1. We therefore set out to determine whether the B cell development block in NOD.B10 *Idd9.3* mice was controlled by the miR-34a-Foxp1 axis. We investigated miR-34a expression and Foxp1 levels in developing B cells of NOD.B10 *Idd9.3* mice and found a significant increase in miR-34a expression that directly correlated with a decrease in Foxp1 levels. This finding implicated miR-34a and its repression of Foxp1 as a likely mechanism underlying the B cell paucity seen in NOD.B10 *Idd9.3* mice. We also show that reduced B cell numbers and impaired B cell APC function in NOD.B10 *Idd9.3* mice directly correlates with inefficient diabetogenic CD4+ T cell proliferation as compared to NOD mice, likely due to reduced numbers and ability of B cells to act as APCs. Therefore, miR-34a-mediated B cell paucity may contribute to inefficient priming of diabetogenic CD4+ T cells, resulting in T1D protection in NOD.B10 *Idd9.3* mice.
Overall, our studies of *Idd9* and *Idd9.3* have led to the identification of novel *Idd9* candidate genes and identification of novel mechanisms by which these genes may contribute to T1D protection.
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**Abbreviations:**

Ab- antibody

ACK- Ammonium-chloride-potassium

Ago2- Argonate 2

APC- Antigen present cells

ARS2- Arsonate resistance protein 2

BB- Biobreeding

BCR- B cell receptor

BDC2.5 TCR transgenic NOD mouse- BDC

C57BL/10- B10

cDMEM- Complete Dulbecco’s modified eagle medium

cDNA- Complimentary deoxyribonucleic acid

CFSE- Carboxyfluorescein succinimidyl ester

CLP- Common lymphoid progenitor

CPM- counts per minute

cRNA- Complimentary ribonucleic acid

Ct- Cycle threshold

DAVID- Database for Annotation, Visualization, and Integrated Discovery

DC- dendritic cell

DMEM- Dulbecco’s modified eagle medium

ELISA- Enzyme-linked immunosorbent assay

FAC- Functional Annotation Clustering

FACS- Fluorescence-activated cell sorting
FC- Fold change
FO- Follicular
Foxp1- Forkhead box protein P1
GAD- Glutamic acid decarboxylase
GFP- Green fluorescent protein
GMCSF- granulocyte-macrophage colony-stimulating factor
Gp- Glycoprotein
GWAS- Genome-wide association studies
HEL- Hen egg lysozyme
HLA- Human leukocyte antigen
HOX- Homeobox
HSA- Heat-stable antigen
HSCs- Hematopoetic stem cells
HSP60- heat shock protein 60
IA-2- Insulinoma-associated protein 2
IBD- Inflammatory bowel disease
Idd- Insulin-dependent diabetes
Ig- Immunoglobulin
IFN- Interferon
IL- Interleukin
IP- Ingenuity pathway
KRAB- Krueppel-associated box
LCMV- Lymphocytic choriomeningitis virus
mAb- Monoclonal antibody
MHC- Major histocompatibility complex
miR- MicroRNA
miRNAs- MicroRNAs
mRNA- Messenger RNA
MS- Multiple sclerosis
MSCV- Murine stem cell virus
MZ- Marginal zone
NFAT- Nuclear factor of activated T cells
NK- Natural killer
NKT- Natural killer T
NOD- Nonobese diabetic
NON- Nonobese non-diabetic
NOR- Nonobese resistant
PAMPs- Pathogen-associate molecular patterns
Plat-E- Platinum-E
PLN- pancreatic lymph node
RA- Rheumatoid arthritis
RISC- RNA induced silencing complex
RNA- Ribonucleic acid
RPM- Revolutions per minute
RT-PCR- Reverse transcription polymerase chain reaction
SCID- Severe combined immunodeficiency
siRNA- small interfering RNA
SLE- Systemic lupus erythematosus
Sm- Smith
SNPs- Single-nucleotide polymorphisms
SPF- Specific pathogen-free
T1D- Type 1 diabetes
TCR- T cell receptor
Teff- T effector cells
T_{H}- T helper
TLR- Toll-like receptor
TNFR- Tumor necrosis factor receptor
TR- Transitional
Treg- Regulatory T cell
UTR- Untranslated region
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Chapter I: Introduction

Type 1 diabetes (T1D) is a polygenic disease. In fact, linkage studies have identified over 25 Insulin dependent diabetes (Idd) regions known to be associated with T1D development. Our goal was to focus on one of these regions on mouse chromosome 4 called Idd9. This ~200 gene region is known to regulate lymphocyte function and development and can confer almost complete protection from T1D in nonobese diabetic (NOD) mice, a spontaneous T1D animal model of human T1D, when the NOD Idd9 is exchanged with Idd9 from T1D-resistant mice, such as the C57BL/10. **Our goal was to identify genes within this region that contribute to T1D induction and to deduce the mechanisms by which these genes function to mediate disease in NOD mice.** In order to further elucidate the mechanisms by which Idd9 confers protection from T1D development in NOD mice, we investigated the role of this region in the regulation of islet-specific, autoimmune CD4+ T cells and the role of a sublocus of this region, Idd9.3, in overall T1D development.

**Objective #1: Identify Idd9.3-specific genes and genetic mechanisms contributing to T1D.**

Idd9.3 contains 19 genes (instead of the ~200 found on full-length Idd9) and allowed us to focus more closely on these genes and identify specific genes and genetic mechanisms contributing to T1D. We did this by comparing a congenic NOD mouse containing the T1D-resistant B10 Idd9.3 (NOD B10 Idd9.3) to the T1D-susceptible NOD mouse.
**Findings:**

We found that NOD.B10 ldd9.3 mice had a ~5-fold reduction in B cells, a specific lymphocyte population known to be important as antigen presenting cells (APC) in T1D pathogenesis. MicroRNA-34a (miR-34a), one of the genes on ldd9.3, had previously been shown to mediate a B cell developmental block by repressing the B cell developmental transcription factor Foxp1. We investigated miR-34a expression and Foxp1 levels in developing B cells of NOD.B10 ldd9.3 mice and found a significant expression increase in miR-34a that directly correlated with a decrease in Foxp1 levels, implicating miR-34a and its repression of Foxp1 as a likely mechanism underlying the B cell paucity seen in NOD.B10 ldd9.3 mice. Furthermore, we discovered that the B cell APC abilities of NOD.B10 ldd9.3 mice were reduced and we also found a reduction in diabetogenic CD4+ T cell proliferation in NOD.B10 ldd9.3 mice as compared to NOD mice in response to endogenous autoantigen. Our results show that B cell paucity reduced B cell APC function in NOD.B10 ldd9.3 mice directly correlates with inefficient diabetogenic CD4+ T cell proliferation compared to NOD mice and support the concept that diabetogenic CD4+ T cells are inefficiently primed in NOD.B10 ldd9.3 mice due to reduced numbers and ability of B cells to act as APCs. These data also suggest that ldd9.3-encoded miR-34a and its repressive effect on Foxp1 is responsible for the B cell paucity found in NOD.B10 ldd9.3 mice and may contribute to inefficient activation and proliferation of diabetogenic T cells, resulting in T1D resistance.
Objective #2: To identify the role of Idd9 in the regulation of diabetogenic, islet-specific CD4+ T cells.

We developed novel NOD.B10 Idd9 (line 905) congenic mice that harbor predominantly islet-reactive CD4+ T cells expressing the BDC2.5 T cell receptor (BDC-Idd9.905 mice). We compared these BDC-Idd9.905 mice containing the Idd9 from the T1D-resistant C57BL/10 (B10) mouse to BDC2.5 TCR transgenic (BDC2.5) mice containing the NOD-derived T1D-susceptible Idd9. Microarray analysis was done to identify the gene expression profile differences between islet-specific CD4+ T cells from each of these mice followed by bioinformatics analyses to identify gene cluster and network differences between each strain and functional assays to determine the responses and diabetogenic potential of the T cells containing the T1D-resistant B10 Idd9 versus those containing the NOD Idd9.

Findings:

These analyses identified differential expression of Idd9-encoded genes Eno1, Rbbp4, and Mtor, involved in cellular growth and development gene networks. Furthermore, functional analyses identified a significant reduction in the responsiveness and diabetogenic potential of BDC-Idd9.905 CD4+ T cells as compared to those from BDC2.5 mice. Overall, these findings identified Eno1, Rbbp4, and Mtor as candidate genes in the regulation of diabetogenic islet-specific CD4+ T cells and suggest their role in T1D pathogenesis.
Overall, these studies have led to the identification of novel candidate genes on Idd9 and Idd9.3 and identification of novel mechanisms by which these genes function in T1D pathogenesis.
Chapter II: Literature Review

I. Introduction to The Immune System

a. Innate Immunity

The innate immune response is the host’s first line of defense against pathogens. This response occurs rapidly, with an initial response mounted almost immediately upon exposure to a pathogen. Innate immunity is non-adaptive, meaning that it does not rely on specific recognition of microbial antigens by lymphocytes. Instead, an innate immune response is dependent upon the recognition of certain molecular motifs common in many pathogens, referred to as pathogen-associated molecular patterns (PAMPs), by innate immune receptors such as toll-like receptors (TLRs) \(^1\). This non-adaptive response also means that immunological memory is not generated against a pathogen as it is in adaptive immunity \(^2\).

Mononuclear phagocytes, such as macrophages, are able to respond as part of the innate immune response by engulfing and digesting pathogens. Other cells that can respond as part of an innate immune response are neutrophils and dendritic cells (DC). These phagocytic cells are all derived from the myeloid lineage \(^2, 3\) and become activated through their recognition of PAMPs on the pathogens they encounter and phagocytize or endocytose \(^4\). There is substantial crosstalk between innate and adaptive immunity because phagocytic cells such as DCs also play a critical role of presenting antigen to naïve lymphocytes during induction of
the adaptive immune response. For example, activated innate immune cells such as DCs function as professional antigen-presenting cells (APCs) that present major histocompatibility complex (MHC) II and can drive CD4+ T cell differentiation, which is critical for an adaptive immune response.

b. Adaptive Immunity

Adaptive immunity is the next line of defense in an immune response after innate immunity. Unlike the immediate-acting innate immune response, the adaptive immune response will respond over the proceeding days to eliminate a pathogen. T and B lymphocytes are derived from the lymphoid lineage and are the cells that drive an adaptive immune response (2). The adaptive immune response is an antigen-specific response and involves actions such as antibody production by B cells directed against the pathogen and its byproducts, and T cell-mediated responses directed against the pathogen or pathogen-infected cells. When a naïve lymphocyte encounters antigen recognized by its B cell receptor (BCR) or T cell receptor (TCR), steps leading to clonal expansion commence in which this antigen-specific T or B cell replicates, resulting in an antigen-specific clonal population that is able to respond to the pathogen. This initial recognition of antigen occurs in secondary lymphoid organs, such as the spleen and lymph nodes. An antigen-specific response also generates memory cells for future encounters with the same pathogen (5). T or B cell adaptive immune responses require antigens to be presented via activated professional APCs such as DCs, Macrophages, and B cells.
that are activated through recognition of PAMPs (4). In the case of an autoimmune response, a T or B cell response is similar to that directed towards a pathogen, but is instead improperly directed towards the host’s own tissues, resulting in tissue damage.

Humoral immunity is represented by B cells recognizing antigens via their antigen-specific B cell receptor (BCR). These antigens are internalized, processed, and presented by the B cell in the context of surface MHC II molecules to antigen-specific CD4+ T helper (T_H) cells. Activation of B cells for antibody (Ab) production typically results following T_H cell recognition of the antigen being presented by the B cell. As a result, B cells can undergo affinity maturation to switch from lower-affinity immunoglobulin (Ig)M production, differentiating into plasma cells that secrete high affinity IgG, IgA and IgE Abs that neutralize pathogens. Some B cell responses to pathogen can occur independently of T cell help, such as T cell-independent B cell responses to bacterial lipopolysaccharides, but are inefficient at inducing affinity maturation. These antibody-mediated B cell responses form the basis of antibody-mediated humoral immunity (2).

T cells recognize antigens via their antigen-specific T cell receptor (TCR). CD4+ T cells recognize peptide antigens presented by APCs on MHC II molecules, while cytotoxic CD8+ T cells recognize peptide antigens presented on the MHC I. In order for T cells to become activated, they must recognize antigen that is presented by an activated APC. T cell clonal expansion and differentiation, or T cell priming, requires 3 signals delivered by the activated APC. Signal 1 is TCR recognition of the peptide-MHC complex on the APC, signal 2 is co-stimulation of the T cell by co-
stimulatory molecules such as CD80 and CD86 expressed by the activated APC, and signal 3 is typically a chemical (cytokine) signal from the APC. Signal 3 is required by CD4+ T cells to differentiate into their distinct $T_H$ effector cells, such as $T_H1$, $T_H2$, $T_H17$ and regulatory T (Treg) cells (2). Collectively, CD4+ and CD8+ T cell responses make up the T cell mediated adaptive immune response against pathogens. This process is also similar to that which occurs during the development of an autoimmune response. Instead of T cells becoming primed in response to a pathogen, they instead become primed to recognize the host's own tissues, leading to autoimmunity.

II. Basic Concepts in Autoimmunity

a. Introduction to Autoimmune Diseases

Autoimmunity occurs when the immune system recognizes and responds to the host's own cells and tissue. If left unchecked, this response to self will inevitably lead to damage and autoimmune disease development. Since the Nobel Laureate Paul Ehrlich first described autoimmunity as 'horror autotoxicus' at the beginning of the twentieth century, autoimmune diseases have been recognized as a major cause of morbidity and mortality worldwide (6). The National Institutes of Health estimates there are at least 80 known autoimmune disorders. In fact, the United States Department of Health and Human Services reports that autoimmune disorders affect over 23.5 million Americans and are a leading cause of death and
disability in the United States. A study from the Mayo Clinic estimates that 1 in 12 women and 1 in 20 men in the United States will develop an inflammatory autoimmune rheumatic disease in their lifetime (7). While women have a preferential susceptibility to autoimmune disease development, the mechanisms behind this gender discrepancy are unknown (8).

b. Autoimmunity is a Break in Self-Tolerance

During normal lymphocyte development, lymphocytes arise that can respond to proteins made by the host called self-antigens. These self-reactive lymphocytes could continue development in the thymus and bone marrow and subsequently enter the periphery, leading to raging autoimmune disease. Fortunately for the host, there are an array of mechanisms to ‘tolerate’ self-reactive lymphocytes. Together, these mechanisms maintain self-tolerance, a state in which an individual’s immune system does not attack healthy tissues of the body. The first checkpoint is to remove or inactivate self-reactive lymphocytes before they leave their early developmental site of the thymus (T cells), or bone marrow (B cells); this is referred to as central tolerance. The most important mechanism of central tolerance is negative selection, where lymphocytes that have a strong reactivity with self-antigen undergo apoptosis and are deleted during their development. While central tolerance is quite effective at removing strongly self-reactive lymphocytes, there is still a small population of self-reactive lymphocytes that make it through the selection process and out into the periphery. Peripheral tolerance is the second checkpoint to halt
self-reactive lymphocytes that have left the central lymphoid organs and then recognize self-antigens in the periphery. One important mechanism in peripheral tolerance is anergy, where a lymphocyte recognizes self-antigen, but without co-stimulation, leading to cellular inactivation. Other mechanisms of peripheral tolerance include control of self-reactive lymphocytes by regulatory T cells (Tregs) and restriction of lymphocyte access to antigens (2).

c. Organ-Specific vs. Systemic Autoimmune Disorders

Autoimmune disorders are grouped based on their target tissues: ‘organ-specific’ autoimmune disorders and ‘systemic autoimmune’ disorders. Organ-specific autoimmune disorders have one target specific organ that gets attacked by the immune response, such as is the case in type 1 diabetes (T1D) and multiple sclerosis (MS). In type 1 diabetes, the target organ is the pancreas, while in MS the target organ is the central nervous system. Systemic autoimmune disorders include diseases in which a pattern of disseminated disease across multiple organs is seen, such as in systematic lupus erythematosus (SLE) (2).

III. Type 1 Diabetes

a. Introduction to Type 1 diabetes
Type 1 diabetes (T1D) is an organ-specific autoimmune disorder, caused by immune infiltration of the pancreatic islets of Langerhans and destruction of the insulin-producing pancreatic beta cells, which results in insulitis and hyperglycemia (9). Those afflicted with T1D require lifelong daily insulin injections and frequently experience multiple complications as a result of their disease status such as heart disease, kidney failure, and blindness (10, 11). While the precise mechanisms triggering T1D induction are unclear, it is thought that disease is mediated by the interplay of multiple insulin-dependent diabetes (Idd) loci, unknown environmental factors, and stochastic events leading to the initiation of the autoreactive immune response (12). It has been suggested that viral infections could play a role in T1D induction, but this link is far from proven (13).

b. Type 1 diabetes pathogenesis

The major understanding of disease mechanisms in T1D pathogenesis have been made through the use of animal models of T1D. Studies characterizing the infiltrating cell populations of the pancreatic islets of Langerhans are mostly CD4+ T cells, but also include CD8+ T cells, B cells, DCs, macrophages and natural killer (NK) cells (14, 15). Studies have also revealed that T1D pathogenesis has 2 distinct checkpoints. Checkpoint number 1 is insulitis, which is characterized by the immune infiltration of the pancreatic islets of Langerhans. Checkpoint number 2 is overt diabetes, in which glucose homeostasis is no longer
maintained and hyperglycemia occurs. While checkpoint number 1 is completely penetrant, meaning that all mice get insulitis (immune cell infiltration into the pancreatic islets of Langerhans), checkpoint number 2 is only seen in a subset of mice, suggesting that the breach of tolerance seen in T1D results from the failure of multiple regulatory steps (16).

It has been well established that CD4+ T cells as well as CD8+ T cells play a critical role in T1D induction and progression (17-21). T cell targeting drugs inhibiting T cell activation, such as cyclosporine, or T cell depleting antibodies are effective at ameliorating disease (22-26) (see section 6). In T1D, the critical step of T cell priming occurs in the pancreatic lymph nodes (PLN), the site where diabetogenic T cells first encounter and respond to pancreatic islet antigens (27, 28). In T1D, B cells have been shown to play a critical role in their function as APCs, rather than as antibody producers (29, 30). Also, B cell ablation leads to T1D prevention and reversal in a mouse model of T1D, pointing out their overall importance in T1D pathogenesis (31, 32). Clinical trials using the B cell-depleting antibody rituximab have also shown some effectiveness in treating T1D patients (33). Overall, it is clear that T cells play a central role in T1D pathogenesis and that B cells play a central role in priming autoreactive T cells in T1D.

c. Genetics and Environmental Contributions in Type 1 Diabetes
According to the World Health Organization, approximately 35 million people throughout the world have been diagnosed with T1D. The incidence of T1D throughout the world is rapidly on the rise and is trending towards earlier onset (34). In fact, the incidence of T1D has been doubling approximately every 20 years (35). T1D is currently responsible for 5-10% of total diabetes cases worldwide (36, 37) and is the most common cause of diabetes in children and adolescents (38, 39). Epidemiological studies have revealed that T1D has been increasing by 2-5% worldwide, with 1 in 300 people in the United States being affected by their 18th birthday (36).

The DIAMOND project (40, 41) and the EURODIAB study (42, 43) were done in the late 1980s and early 1990s to look at the incidence of T1D in children. These studies found that the incidence of T1D is highly variable across populations, with a greater than 350-fold difference in disease incidence seen across the 100 populations analyzed. The lowest incidence of 0.1/100,000 per year was seen in the Zunyi region in China, while the highest incidence of 40/100,000 per year was seen in Finland (35, 42), with >20/100,000 per year incidences also found in Sardinia, Sweden, Portugal, the United Kingdom, Canada, and New Zealand (41, 42). These data reveal that genetic variability across populations is likely playing a role in T1D susceptibility. For example, although Finland shows an extremely high T1D incidence, its neighboring country Estonia shows an incidence of only one-quarter of Finland’s (35). Also, Finland and Sardinia, which had two of the highest T1D incidences, are both relatively homogeneous populations, pointing out the role of genetic susceptibility in T1D development. This same comparison also pointed out
the polar-equatorial gradient previously thought to have a significant role in T1D development is not as critical as previously thought, since Finland and Sardinia are 3000 km away from each other and in different global regions and environments, yet both regions show a very high incidence of disease (35). This comparison points out that although environmental components could play a role in T1D development, two of the highest T1D incidences were found in completely different regions, underscoring the importance of genetic susceptibility in T1D development.

IV. Animal Models of T1D

a. Introduction to Rodent Models of T1D

Animal models have been integral to our understanding of T1D. Studying the mechanisms behind T1D pathogenesis in humans is quite challenging because T1D is usually diagnosed when a patient can no longer maintain glucose homeostasis. During the pre-clinical period, a patient is typically asymptomatic because the remaining beta cells are making up for the loss of insulin production, but the initial immune mediated destruction of the beta cells has already commenced (44). Diagnosis well after the initial events of disease induction have occurred means the entire pre-clinical phase of T1D is missed and cannot be studied.

These complexities have necessitated the use of rodent models, which have both a controlled genetic background and environment, to study T1D development. Mouse models, in particular, are excellent genetic models for human diseases such
as T1D because greater than 99% of genes in mice have orthologs in humans and about 90% of the mouse genome is syntenic (in approximately the same chromosomal location) compared to humans (45). T1D pathogenesis in rodent models of T1D that spontaneously develop disease also have a great deal of similarity with human T1D (17). The use of immune-modulating therapies and interventions and pre-clinical T1D drug testing can be extensively studied in these models to initially identify potential treatments that could be beneficial in T1D patients. Two established animal models have advanced our understanding of T1D: the nonobese diabetic (NOD) mouse, the animal model that has revealed the most about T1D pathogenesis over the past 30 years, and the Biobreeding (BB) rat. The proceeding sections will highlight the importance of each rodent T1D models in T1D research.

b. The NOD Mouse

The most established, studied, characterized animal model of T1D is the NOD mouse (17). While other mouse strains such as the C57BL/6 and C57BL/10 are T1D resistant, NOD mice are the only spontaneous mouse model of T1D (14) and will spontaneously develop a high incidence of disease without the need for immunization, or any other experimental manipulations. This strain was developed in Japan while developing a cataract model derived from the outbred Jcl:ICR strain (14, 15). Researchers identified a founder that spontaneously developed T1D and through repetitive brother-sister breedings, established the NOD mouse line, which
has a disease incidence of 60%-80% in females and 20%-30% in males (15, 46). NOD mice that do not develop T1D still typically develop insulitis, demonstrating various checkpoints for T1D development in this model.

T1D onset in NOD mice develops between 12-30 weeks of age. In NOD T1D, mononuclear infiltrates surrounding pancreatic islets are first recognized at 3-4 weeks of age. This is referred to as peri-insulitis. This then progresses to full insulitis by 10 weeks of age. Overt T1D typically develops past 12 weeks of age (17). The range of T1D onset and severity is likely dependent on environment because it has been shown that T1D incidence decreases precipitously in NOD mice when they are exposed to microbial products such as lipopolysaccharides, or kept in conventional housing facilities (47, 48). Conversely, NOD mice kept in a germ-free environment show an increase in disease development (46, 49, 50). This suggests that microbial stimuli may tolerize the NOD immune response and partially protect from T1D development.

T1D in NOD mice share many similarities with human disease. For example, T1D is mediated by autoreactive T cells in both humans and NOD mice (17). Also, the same gender bias is seen in both human and NOD females (17, 51). Also similar to human T1D, environment has been shown to play a contributing role in T1D induction and pathogenesis in the NOD model (46, 49). NOD mice show a tendency towards development of multi-organ autoimmune diseases, mirroring what is found in some human T1D patients (17). Examples include NOD mouse susceptibility to developing a host of other autoimmune disorders, such as Sjogren’s syndrome (52), systematic lupus erythematosus-like disorders (53), autoimmune thyroiditis (54),
autoimmune sialitis (55), and autoimmune polyneuropathy (56). These disease similarities make the NOD mouse an outstanding animal model for human T1D.

T1D models have been developed from the NOD mouse to study specific components of T1D pathogenesis. Numerous groups have used cyclophosphamide, an antiproliferative cancer drug used in chemotherapy, to induce T1D. This treatment is effective in NOD mice in which insulitis has commenced (57, 58). It has been shown that one likely mechanism by which cyclophosphamide works is through depletion of Tregs, resulting in T1D induction in NOD mice (59). Only NOD mice and other strains closely related genetically to the NOD (60) become diabetic with this treatment. Other models of T1D, such as drug-induced disease models, congenic T1D resistance models (covered in 5c,d), and T cell receptor transgenics (covered in 4d) have also been successfully derived from the original NOD mouse model.

c. The Biobreeding (BB) Rat

The biobreeding (BB) rat, another animal model of T1D, was first discovered in 1974 in a Canadian colony of outbred Wistar rats that spontaneously developed hyperglycemia and ketoacidosis (61). The BB rat also develops a high incidence of T1D mediated by the T cell-mediated destruction of the beta cells of the pancreas (62). Also, like the NOD, the BB rat model exhibits immune system perturbations such as Treg depletion and TLR ligation that lead to T1D development (62). One
disadvantage of the BB rat is that it exhibits CD4+ and CD8+ T cell lymphocytopenia, which is not a characteristic of T1D in humans or NOD mice (63).

d. NOD-derived BDC2.5 T Cell Receptor Transgenic Mouse

Transgenic mouse models have been developed to study the contribution of specific cell types, such as diabetogenic CD4+ T cells that target pancreatic autoantigens in T1D pathogenesis. One example is the BDC2.5 TCR transgenic BDC2.5 mouse, which was developed to study autoreactive CD4+ T cell responses in T1D (21). This transgenic model was derived using the diabetogenic BDC2.5 CD4+ T cell clone isolated from a diabetic NOD mouse by Haskins et al. (64, 65). The TCR alpha and beta chains from the BDC2.5 CD4+ T cell clone were subsequently isolated by Katz et al. and used to generate the BDC2.5 TCR transgenic mouse which carries a transgene derived from BDC2.5 CD4+ T cell clone, in which almost all CD4+ T cells express the same TCR as the original T cell clone (21). Since its generation, the BDC2.5 TCR transgenic mouse has been used extensively by many laboratories to study T1D pathogenesis and the function of autoreactive, islet-specific CD4+ T cells in T1D.

V. Genetic Susceptibility to T1D

a. Approaches to Elucidate the Genetics of Polygenic Diseases
Many autoimmune diseases are caused by the interactions of multiple genes working in tandem rather than being caused by a single gene. This is also the case in many autoimmune disorders, such as SLE (66), RA (67) and T1D (44). Genetic approaches have been utilized to investigate individual genetic loci and the gene(s) on these loci that are causing disease: association studies (using a control population that does not exhibit the disease compared to the population that does) and linkage studies (done in related individuals, usually siblings) (68). The approach to use depends on the effect size of the disease (the strength of the association between the disease allele and the disease) and the frequency of that disease in the population. Before the development of genome-wide association studies (GWAS), linkage studies were the primary method for identifying causative genes in disease. These studies were done across families by using designated panels of single nucleotide polymorphisms (SNPs) to identify susceptibility loci in disease (44). Overall, linkage studies tend to be more effective in diseases that have a high effect size, but are less common in the overall population. Linkage studies in T1D have revealed chromosomal regions that are contributing to disease, such as the human leukocyte antigen (HLA) region (44).

Association studies comparing specific SNPs at designated loci between two groups, or whole genome comparisons examining SNPs across the entire genome, such as GWAS, are more effective than linkage in families in diseases commonly found in the population that do not have a large effect size. These studies need a large sample size to directly compare the genotype to the disease phenotype across an entire population. GWAS have also been useful in identifying additional novel loci
in T1D (69). **Figure 2.1** illustrates the strengths of linkage vs. association studies in relation to effect size and the disease frequency in the population.
Figure 2.1: Relative Strengths of Linkage and Association Approaches for Mapping Genes in Complex Disorders.

The chart shows the effect of a disease allele’s frequency in the population and its effect size on the optimal choice of study design. A disease allele that occurs frequently in the population and that has a large effect on disease risk is unlikely to exist. At the opposite end of the spectrum, a disease allele that is rare and has a small effect size is likely to exist but is unlikely to be found — and such alleles would be of limited public health interest. In general, linkage studies are most effective in disorders in which disease alleles are anticipated to have a large effect size but occur infrequently. Association studies are most effective for the detection of alleles that occur frequently but have a small effect size. These are general trends, and there are no specific boundaries in efficacy between the two approaches.

b. The Polygenic Nature of T1D

The T1D is a polygenic disease, meaning no single gene or genes are the causative agents for disease development. Instead, in both humans and NOD mice, multiple genetic factors must come together to contribute to disease. While T1D is found at a higher incidence in some families, its mode of inheritance is not clear (70-73). Through genome-wide association studies across human populations, 26 different loci have been identified that contribute to T1D development, with 19 of them associated with immune regulation (44). Several of these loci are also shared with NOD mice. Susceptibility gene pathways shared between mice and humans include the genes encoding the major histocompatibility complex (MHC), also called human leukocyte antigen (HLA) when referred to exclusively in humans, and non-MHC related genes such as insulin, CTLA-4, IL-2/IL-2a, PTPN22 and NRAMP1 (74). Figure 2.2 illustrates non-MHC associated T1D candidate genes that have been identified in humans.

c. The Major Histocompatibility Complex (MHC)

The most potent T1D susceptibility factor is the MHC. NOD mice have a distinct MHC, H-2\(^e\), which is essential for their T1D development (75, 76). Early linkage studies also showed that the T1D-susceptibility conferred by the MHC was dose-dependent, since NOD mice heterozygous for the MHC had a low penetrance of
**Figure 2.2: Putative Functions of Non–HLA-Associated Loci in Type 1 Diabetes.**

The y axis indicates the best estimate of the odds ratio (the measurement of association between the allele and T1D) for risk alleles at each of the indicated loci on the basis of currently published data. Although not shown, the HLA region has a predicted odds ratio of approximately 6.8. On the x axis are indicated possible candidate genes within genomic regions in which convincing associations with type 1 diabetes have been reported. On the basis of the known functions of these candidate genes, the corresponding bars in the graph depicting odds ratios have been color-coded to suggest possible roles of these loci in susceptibility to type 1 diabetes. At *IL2RA* and *TNFAIP3*, there is evidence of two independent effects on risk with different odds ratios, so these loci both appear twice in the figure.

In NOD mice, at least one H-2^g7 MHC II allele is necessary for T1D development (77, 78) and NOD mice congenic for the MHC from T1D-resistant strains of mice such as the H-2^b MHC from C57BL/10, or the H2^{ab1} from the nonobese non-diabetic (NON) strain do not develop T1D (79, 80). H-2^g7 has a unique binding groove, giving it the ability to bind many different low affinity peptides, such as autoantigens in T1D (81, 82). It has also been shown that if a non-H-2^g7 class II is expressed as a transgene in APCs, T1D development is inhibited in NOD mice (83, 84). Another important feature of T1D development in NOD mice is that APCs are not able to express H2-E MHC class II molecules due to an exon 1 deletion of the H2-Ea^b allele. If H2-E expression is restored using a transgenic system, T1D development is impeded in NOD mice (85).

The contribution of non-MHC genes has also been further defined in NOD mice, because linkage studies can be more easily performed. The first linkage studies in NOD mice showed that while the H-2^g7 is necessary, it is not sufficient to cause disease because C57BL/10 mice expressing the H-2^g7 do not develop T1D (78) (Table 2.1). This points out the importance of non-MHC genes in T1D development. These MHC and non-MHC regions are collectively referred to as insulin dependent diabetes (idd) loci.

A major commonality between T1D-susceptible H-2^g7 in mice and T1D-susceptible (HLA)-DQ2 and HLA-DQ8 in humans is that in both species, there is an amino acid substitution changing aspartic acid to a neutral serine at the beta 57 position (86, 87). In humans, the MHC HLA class II region on chromosome 6p21 accounts for 45% of T1D susceptibility (88). Numerous other genome-wide linkage
Table 2.1: MHC influences in T1D in the NOD mouse model

Table summarizes the T1D incidence in backcrosses of NOD mice to T1D-resistant C57BL/10 mice and interprets what disease results indicated about the genetic control of T1D in NOD mice and the relative contribution of MHC and non-MHC associated alleles.

_Borrowed from gene-gene interactions in the NOD mouse model of Type 1 diabetes._

_Ridgeway et al. Advances in Immunology 2008._

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Diabetes</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD (H-287)</td>
<td>80% in females</td>
<td>Homozygous expression of the NOD-derived MHC is not sufficient to cause diabetes. Non-MHC NOD-derived genes are also required.</td>
</tr>
<tr>
<td>B10.H-287</td>
<td>None</td>
<td>Homozygous expression of the NOD-derived MHC and one dose of all NOD-derived (or B10-derived) non-MHC susceptibility alleles are not sufficient to cause diabetes. B10-derived, non-MHC protective alleles have an overall dominant protective effect on the T1D phenotype.</td>
</tr>
<tr>
<td>NOD × B10.H-287 F1</td>
<td>None</td>
<td>Homozygous expression of the NOD-derived MHC and a sufficient number of NOD-derived (or B10-derived) non-MHC susceptibility alleles are present in 14% of backcross one females. At least nine non-MHC regions influence T1D susceptibility in this backcross one generation.</td>
</tr>
<tr>
<td>(NOD × B10.H-287 F1) × NOD backcross one generation</td>
<td>14% in females</td>
<td>Homozygous expression of the NOD-derived MHC and, on average, 25% of Idd loci homozygous for susceptibility alleles each F2 mouse, only 0.4% of mice develop T1D. These results also support the hypothesis that in combination B10-derived, non-MHC protective alleles have an overall dominant protective effect on the T1D phenotype.</td>
</tr>
</tbody>
</table>
studies in humans have also reported linkage between the HLA and T1D susceptibility. It is also clear that certain HLA haplotypes are protective from T1D, since 20% of Europeans and Americans carry the protective HLA-DR2, but fewer than 1% of T1D positive children are HLA-DR2 (DQB1*602) positive (89-95). In humans, candidate-gene association studies have also identified multiple non-HLA associated loci, many of which have roles in immune cell function (Figure 2.2) (44). While the odds ratio for these non-HLA associated genes is clearly lower than the HLA region, their combined effect is likely to have a significant outcome in T1D development.

d. Non-MHC Loci

More than 30 Idd loci on 15 different chromosomes other than the MHC have been identified as causing T1D susceptibility in NOD mice (Figure 2.3) (96). The first extensive linkage studies were conducted with NOD mice backcrossed to T1D-resistant C57BL/10 mice that were congenic for the NOD H-2\textsuperscript{g7} MHC (97), allowing researchers to perform segregation analysis and focus on the contribution of non-MHC genes in T1D susceptibility. These backcrosses pointed out the complexity of the gene interactions between the NOD and C57BL/10 genomes and identified several non-MHC regions influencing T1D development (97, 98) ([Table 1] (99).

Congenic mouse models, in which these non-MHC Idd regions in the NOD mouse genome are replaced with Idd regions from T1D-resistant strains of mice,
Figure 2.3: The Idd regions in mouse.

The shaded regions indicate the various Idd regions spread throughout the NOD genome that have been identified through linkage analysis and through the use of congenic mouse strains. Associated human orthologous regions are also identified.

The figure was taken from http://www.t1dbase.org/page/PosterView/MouseIddRegions.


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were first derived in order to confirm that specific Idd loci identified in linkage analyses were in fact contributing to T1D protection (75). The contributions of particular Idd loci to T1D development have been deduced by replacing NOD chromosomal segments with those from T1D-resistant mouse strains, such as the C57BL/6 or C57BL/10 (75), resulting in congenic NOD mouse strains genetically different at the defined Idd genetic locus, but otherwise genetically identical to NOD mice. These congenic mice containing confirmed T1D-resistant Idd loci could then be used to identify which gene(s) on the specific Idd region were responsible for the region’s protective effect. Since their development, these congenic mouse strains have been used extensively to identify the mechanisms behind how specific Idd loci function in T1D protection and to then identify candidate genes on these loci responsible for those mechanisms. Many of the Idd regions have been further characterized and have been used to identify gene(s) that may be contributing to disease pathogenesis through candidate gene analyses.

Since T cells are integral in T1D development, T cell receptor (TCR) transgenic mouse models have also been crossed with the congenic NOD mouse models to investigate the role of specific Idd loci on islet-specific T cell functions. An example of this is the BDC2.5 TCR transgenic model crossed with the NOD.B10 Idd9 (R28) congenic mouse model to investigate the role of Idd9 in the functional differences of islet-specific CD4+ T cells (100). The same congenic/transgenic approach has also been used to study the influence of Idd regions on CD8+ T cell diabetogenicity (101). In our studies of a further narrowed candidate Idd9 region than previously studied, we also utilized this transgenic/congenic model approach
and crossed one of the *Idd9* congeneric NOD mouse strains (line 905) with the BDC2.5 TCR transgenic mouse to investigate the role of *Idd9* in the genetic regulation of islet-specific CD4+ T cells. (see data chapter V).

i. Insulin Dependent Diabetes Locus 9 (*Idd9*)

*Idd9* has been shown to provide almost complete protection from T1D when this region is replaced with *Idd9* from a T1D-resistant mouse strain such as the C57BL/10 mouse (102). *Idd9* is found on the distal arm of mouse chromosome 4 (103) and contains genes known to be important in immune cell development and function. *Idd9* also partially overlaps with *Idd11*, which confers a similar phenotype. The *Idd9* region of >200 genes has been fine-mapped to four subregions, each of which contain T1D candidate genes (102, 104). While the *Idd9* does not have an orthologous T1D region with any known human T1D susceptibility loci, it contains several immunologically relevant genes and genes linked to human disease, such as those coding for *Lck*, *Mtor*, *Masp2*, and *Cd137* in mice and humans (105-107). This region is referred to as line 905 (108-110) (Figure 2.5). Line 905 was derived by selective breeding between the longer segment of line 1104 (R28 in Lyons et al. 2000 (102), see Figure 2.4) and NOD mice to derive a shorter and more defined *Idd9* interval. These NOD.B10 *Idd9* congeneric mice have been used to study the effects of *Idd9* derived genes on T1D development. *Idd9* has been shown to have a significant effect on T cell function. A less inflammatory T cell phenotype making more interleukin (IL)-4 and less interferon (IFN) gamma is found in the islet-
infiltrating T cells of NOD B10.\textit{Idd9} mice as compared to NOD mice (102). The \textit{Idd9} region has also been shown to influence pancreatic beta cell susceptibility through its effect on inflammatory cytokine-mediated killing and cytotoxic CD8+ T cells (111).
FIGURE 2.4: Localization of Idd9.1, Idd9.2 and Idd9.3

Subcongenic strains of NOD mice containing Idd9 intervals from the T1D-resistant C57BL/10 background were developed and assessed for the development of diabetes. Figure shows the cumulative incidence of diabetes in congenic strains of NOD mice containing indicated Idd loci from T1D-resistant C56BL/10 mice over 210 days. The line of congenic mouse, the B10 Idd region they contain, and their representative symbol are indicated at the top of the figure. Regions important to our studies include the R3 (also referred to line 1106 in figure 2.5), which is the B10 Idd9.3 interval, and the R28 (also referred to line 1104 in figure 2.5), which was the subcongenic used to derive the B10 Idd9 mice containing a more defined Idd9 region used in our studies (referred to line 905 in figure 2.5). Disease incidence of other subcongenic Idd9 mice (R73, R11, R15, R38) are not used in our studies.

Borrowed from Lyons et al. Immunity 2000

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**Figure 2.5: Map of Idd9 congenic strains**

The congenic mouse line and the T1D-resistant region (C57BL/6, C57BL/10, or NOR) they contain are indicated. Overlapping regions, previously identified regions, and newly identified regions (in Hamilton-Williams et al. 2013) are indicated. Location of Idd regions based on microsatellite marker analyses is also indicated. Regions important to our studies include the 1106 (referred to line R35 in figure 2.4), which is the B10 *Idd9.3* interval, and the 1104 (referred to line R28 in figure 2.4), which was the subcongenic used to derive the B10 *Idd9* mice containing a more defined *Idd9* region (line 905) used in our studies.

Borrowed from Hamilton-Williams et al. Mamm. Genome 2013

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Another T1D protective function of B10-derived *Idd9* is further demonstrated in TCR transgenic models used to study diabetogenic CD4+ T cells. Islet-specific BDC2.5 CD4+ T cells containing the B10 *Idd9* are not as diabetogenic as those containing the NOD *Idd9* (100). Two potential mechanisms identified in this less diabetogenic phenotype are enhanced anti-inflammatory cytokine production and altered T cell homing (100). In the case of T cell homing, the *Idd9* region has been shown to play a significant role because when transgenic BDC2.5 CD4+ T cells containing the T1D-resistant B10-derived *Idd9* are compared to BDC2.5 CD4+ T cells, the ones containing the T1D-resistant B10 *Idd9* show impaired homing to the pancreas and PLN (100).

In addition to the effects of *Idd9* on T cell function, there is also evidence to support the role of *Idd9* in B cell development and subsequent T1D susceptibility. NOD mice have impaired B cell anergy induction upon antigen stimulation, while NOD mice containing a T1D-resistant *Idd9* have this B cell function restored (112, 113). This is important in T1D because B cells can preferentially serve as a subpopulation of APCs that support the expansion of diabetogenic CD4+ T cells (114).

ii. Subloci of *Idd9*

The *Idd9* region has also been fine mapped to identify specific subregions within this larger region that could have individual protective effects in T1D. These extensive studies resulted in the identification of the following *Idd9* subregions:
Idd9.1, Idd9.2, Idd9.3 (102) and recently Idd9.4 (104). Lyons et al. demonstrated that each B10 Idd9 subregion is able to partially protect NOD mice from T1D (Figure 2.4), indicating that different T1D candidate genes are likely found on these loci. By identifying these smaller subregions, the task of defining the mechanisms by which disease occurs and identifying specific genes contributing to T1D development has become more feasible. Congenic NOD mice containing each of these Idd9 subregions have been used to further investigate each of these regions in T1D protection.

Idd9.1 regulates Treg development and function, leading to T1D protection in mice containing the B10 Idd9.1 (101). This subregion also promotes natural killer T (NKT) cell development, which are deficient in NOD control mice (115). Idd9.1 has been fine-mapped and separated into Idd9.1 and Idd9.4. The tyrosine kinase Lck is one of the candidate genes on Idd9.1 due to its central role in TCR complex signaling (104). Idd9.1 and Idd9.4 also overlap with Idd11 (Figure 2.5). Congenic NOD mouse strains developed from backcrosses with C57BL/6 mice and SJL mice were first used to identify this region (116). Idd11 has been further defined and contains the novel gene of unknown function AK005651, which may play a role in T1D susceptibility due to its decreased expression in the thymus and spleen of NOD mice (117).

Idd9.2 was originally defined as a 5.6-Mb genetic segment containing the candidate genes Cd30 (renamed Tnfrsf8), Tnfr2 (renamed Tnfrsf1b) and Mtor. Its borders were defined as the difference between line 1105 (R11) and line 1106 (R35) (containing the Idd9.3) (102) (See figure 2.5). The candidate gene Cd30 (encoding the CD30 signaling molecule) is a member of the tumor necrosis factor
receptor (TNFR) superfamily (118). The TNFR superfamily members are important mediators in immune response initiation, expansion, and termination. CD30 signaling can stimulate proliferation (119, 120) and cytokine production (121) in T cells, and can also induce apoptosis susceptibility (122). CD30 has been shown to play an important role in peripheral tolerance by controlling autoreactive CD8+T cell expansion in a CD8+T cell TCR transgenic model of T1D (123). CD30 has been shown to have amino acid sequence differences between the NOD and B10 strains (102), although levels of both CD30 mRNA and protein are equivalent between the two strains, pointing out that functionality rather than expression differences would need to account for the role of CD30 in autoimmune development (124). Since the initial work done with Idd9.2, this sublocus has been further refined and reduced in size to a 3.9 Mb region containing more than 40 genes. The congenic NOD mice containing this refined B10 Idd9.2 region are referred to as line 1566 (101, 109) (See figure 2.5). While this reduced locus no longer contains Cd30 or Tnfr2, these genes are still clearly on the full-length Idd9 segment (line 905- containing Idd9.1.2.3) and therefore may still play an important role in T1D development. NOD mice containing the refined B10 Idd9.2 segment are still significantly protected from T1D development, demonstrating this region still contains important genes in T1D pathogenesis (104). This shorter Idd9.2 segment contains the candidate genes masp2 (encoding mannan-binding lectin serine peptidase 2) and Mtor (encoding mammalian target of rapamycin), both of which have roles in immune function.

Idd9.1 and Idd9.2 also overlap with a region in the nonobese resistant (NOR) congenic mouse model in which a NOD Idd locus has been replaced with a T1D-
resistant NOR Idd locus (NR4) and has been shown to reduce pathogenicity in CD4+ T cells and B cells (112, 125, 126) (figure 3-NR4). Hamilton-Williams et al. showed that Idd9.2 and Idd9.3 are able to reduce the expansion of autoreactive CD8+T cells and that this was mediated by CD4+ T cells and nonlymphoid cells (109).

Lyons et al. developed NOD congenic mouse line 1106 (R35) to study the effect of Idd9.3 on T1D development (Figure 2.4). They showed that congenic mice containing the B10-derived Idd9.3 provided 40% protection from T1D compared to NOD mice (102). This region was further defined as a 1.2 Mb region containing 19 known genes, including microRNA (miR) miR-34a (127). A prominent candidate gene previously identified on Idd9.3 is the gene encoding tumor necrosis factor receptor (TNFR) superfamily member and immune signaling molecule CD137(4-1BB/TNFRSF9), which contains amino acid variations in NOD mice compared to C57BL/10 mice (102). NOD mice show reduced T cell proliferation and IL-2 production in response to anti-CD3 plus immobilized 4-1BBL (the natural ligand of CD137) co-stimulation as compared to congenic mice with the B10 Idd9.3 interval (127). NOD B10.Idd9.3 mice also show an accumulation of CD137+ Tregs with age, which secrete soluble CD137 as compared to NOD mice (128). These studies point out a role for Idd9.3 in the regulation of T cell co-stimulation and Treg differentiation. Treatment of NOD mice with soluble CD137 has also been shown to have an ameliorative effect on T1D development, although insulitis is not prevented (129). The production of autoimmune anti-smith (sm) Abs have also been mapped to the Idd9.3 region (130), suggesting that Idd9.3 may be having an influence on B cells as well. Collectively, these studies show the overall importance of Idd9.3 in
immune regulation (130).

VI. T Cells and their Role in T1D Pathogenesis

a. CD4+ T Cells in T1D

CD4+ T cells are essential for T1D induction. This was first shown by Garrison Fathman's group, using a CD4-depleting monoclonal antibody (mAb) (GK1.5) to remove CD4+ T cells in NOD mice (23). CD4+ T cell depletion in 90-110 day old NOD mice in the late stages of insulitis resulted in reversal of lymphocyte infiltration into the pancreatic islets of Langerhans and subsequent T1D prevention (23). The CD4+ T cell requirement for T1D development was further confirmed by the demonstration that diabetogenic CD4+ T cell clones could effectively transfer T1D in young NOD mice (64). CD4+ T cell TCR transgenic models have also been extensively used to specifically study the role of monospecific CD4+ T cells expressing and islet antigen-specific TCR in T1D induction. These studies have shown that diabetogenic CD4+ T cells alone can induce T1D in adoptive transfer models of T1D (21). In addition, if transgenic CD4+ T cells from BDC2.5 TCR transgenic mice are adoptively transferred into NOD severe combined immunodeficiency (SCID) mice, they can clearly induce T1D (see our studies in data chapter IV). Collectively, these studies underline the central role of CD4+ T cells in T1D.

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Although CD4+ T cells are causative agents in T1D, there is also a subset of Foxp3+ regulatory CD4+ T cells (Tregs) known to have an ameliorative effect. The perturbed ratio of Tregs to T effector cells (Teff) has also been implicated in T1D induction because Tregs have been shown to be reduced in number and function in NOD mice pancreata upon T1D induction (131). Tregs in prediabetic NOD mice can inhibit transfer of T1D in an adoptive transfer model using spleen cells from diabetic mice (132) and have been shown to play an ameliorative role when numbers of Tregs are increased as compared to their normal numbers in NOD mice (133-136). In 8.3 CD8+ T cell TCR transgenic mouse, it has also been shown that Tregs can play a role in suppressing diabetogenic CD8+ T cells (101). The reduction of Treg numbers and function in NOD mice is likely another contributing factor in T1D susceptibility.

b. CD8+ T cells in T1D

While CD4+ T cells have been shown to play an indespensible role in T1D development, CD8+ T cells have also been shown to play an important role in disease pathogenesis. Some of the initial evidence pointing to the importance of CD8+ T cells in T1D came from a patient autopsy and newly diagnosed patient biopsy histology data. This histology showed significant CD8+ T cell infiltration into the pancreatic islets of Langerhans (137, 138). The same phenotype was seen in pancreatic graph biopsies from diabetic patient allographs, where insulitis was mostly comprised of CD8+ T cells (139). While these data show that CD8+ T cells
infiltrate the pancreas during the late stages of T1D, their role in early disease pathogenesis before diagnosis is still unclear in humans.

In NOD mice, Miller et al., using an adoptive transfer model of purified CD8+ T cells into irradiated NOD donors, first showed that CD8+ T cells play a role in T1D pathogenesis, but require CD4+ T cell help (140). It has been shown by numerous studies in both the NOD mouse and the BB rat that both CD4+ and CD8+ T cells are the primary mediators of T1D (17, 46, 76, 141). Adoptive transfer models of T1D have also confirmed that both L3T4+ (CD4+) and Lyt-2+ (CD8+) cells are critical for disease induction (142-144). While adoptive transfers of T cells from prediabetic mice into NOD SCID mice require both CD4+ T cells and CD8+ T cells for optimal T1D induction, enriched CD8+ T cells did not transfer T1D (145). Similarly, transfer of T1D into NOD neonates also showed a requirement for both CD4+ and CD8+ T cells (146). In addition, although diabetogenic CD8+ T cell clones isolated from NOD mice are capable of inducing T1D, they require CD4+ T cell help, re-iterating the need for CD4+ T cells in CD8+ T cell models of T1D (147). These studies show that CD8+ T cells require CD4+ T cell help to contribute to T1D development in NOD mouse models of T1D.

A role for CD8+ T cells in late T1D development has been implicated in NOD mice, where if the MHC I expression is inhibited in pancreatic beta cells (148, 149) or APCs (150), T1D does not develop, but insulitis still occurs. Other model systems, such as mice expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein (gp) in the pancreatic beta cells, have also been used to look at autoimmune responses and the effect of MHC haplotype on T1D onset. Ohashi et al.
showed that in a model of LCMV infection to induce an immune response against the gp on pancreatic beta cells, T1D development was dependent upon MHC I expression (151). In a related paper, Laufer et al. showed that if the same LCMV-gp model was crossed with a MHC II deficient mouse, the resulting mice still developed T1D, pointing out lack of a requirement for CD4+ T cells for T1D induction in this model. The histology from their model also showed that the only difference between the pancreatic islets of diabetic mice with or without MHC II was that mice lacking MHC II did not have CD4+ T cells infiltrating their pancreatic lesions (152). The caveats of this model are that the CD8+ T cell response was directed against the viral LCMV-gp antigen on pancreatic beta cells following a LCMV infection and not an actual pancreatic antigen. The robust immune response to gp following LCMV infection likely breaks tolerance and bypasses the need for CD4+ T cells in this model of T1D induction. Overall, these LCMV studies point out a potential pathological role for CD8+ T cells in T1D development and the necessity of APC activation in triggering CD8+ T cell-induced pathology, but the wealth of evidence still highlights the critical role of CD4+ T cells in most models of T1D induction.

c. Autoantigens in T1D

Identification of autoantigens in T1D is one important step in understanding how diabetogenic T cells get activated. These autoantigens are the endogenous antigens to which autoreactive diabetogenic T cells respond during T1D induction. Many of the autoantigens in T1D are still unknown and extensive work will be
required to identify them. Some autoantigens that have been identified in T1D include glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA-2), heat shock protein 60 (HSP 60), and insulin (17).

One method that has been successful in identifying novel T1D autoantigens is through the use of islet-specific transgenic T cells (153, 154). T cells from TCR transgenic mice, such as the BDC2.5 TCR transgenic mouse (21), can be used to identify autoantigens in T1D due to their engineered islet-antigen specific TCR and the fact that their CD4+ T cells contain the receptor that can identify a specific T1D autoantigen. In fact, Chromogranin A was identified as an autoantigen because of its recognition by monospecific islet-antigen specific CD4+ T cells from the BDC2.5 TCR transgenic mouse. Chromogranin A binds atypically to the NOD H-2\textsuperscript{a7} MHC II molecule, which could play a role in it’s ability to activate diabetogenic CD4+ T cells (154).

VII. An Overview of MicroRNA Processing and Consequences of Dysregulation

a. MicroRNA (miRNA) Processing

MicroRNAs (miRNAs) have recently been shown to play a significant role as regulators of gene expression in all known plant and animal genomes (155, 156). These ~22-nucleotide-long, single-stranded, non-coding RNAs function by directly base pairing with the complementary seed sequence of the 3’ untranslated regions (UTR) of target messenger RNA (mRNA) transcripts to repress protein expression
and/or promote degradation of the mRNA transcript (157). Processing of miRNAs begins with the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III. This pri-miRNA is then cleaved in the nucleus by the Drosha-Pasha (DGCR8) microprocessor complex, which is assisted by other factors such as arsenate resistance protein 2 (ARS2) (158). This pre-miRNA then undergoes nuclear export via Exportin-5-Ran-GTP into the cytoplasm. The pre-miRNA is then cleaved to its mature length by the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP. The 3’-5’ messenger strand is then degraded and the 5’-3’ functional strand is loaded with Argonate 2 (Ago2) into the RNA induced silencing complex (RISC). This RISC complex is then able to repress translation through mRNA target cleavage, translational repression, or mRNA deadenylation (159) (Figure 2.6). MiRNAs usually have multiple targets because their short sequence allows them to bind many targets of close complementarity. So far, hundreds of miRNAs have been identified in both the mouse and human genome (157).

b. Regulation of Hematopoetic Development by MicroRNAs

MicroRNAs (miRNAs) have been shown to play an essential role in hematopoietic development through their regulation of gene expression at the post-transcriptional level. Recently, miRNAs have been shown to play an extensive role in the regulation of many developmental processes. In immune cells alone, over 100 different miRNAs are expressed that have the potential to regulate immune cell
Figure 2.6: The “linear” canonical pathway of microRNA processing.

The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded. In this review we discuss the many branches, crossroads and detours in miRNA processing that lead to the conclusion that many different ways exist to generate a mature miRNA.

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development and function and modulate both the innate and adaptive immune responses *(Table 2.2)* (157). Hematopoietic stem cells (HSCs) in the bone marrow are the earliest precursors that give rise to the cells making up the immune system (160). These HSCs are tightly regulated and differentiate into both lymphoid and myeloid cells. Deletion of arsenate resistance protein 2 (ARS2), a pri-miRNA processing factor, is embryonic lethal and its deletion in adult mice causes bone marrow failure. This bone marrow failure is likely caused by important role of miRNAs in HSC regulation (158). Other individual miRNAs have also been shown to play important roles in the regulation of genes that regulate HSC homeostasis, such as the repression Homeobox (HOX) genes (161, 162).

c. Dysregulation of miRNA Expression

Due to their regulatory ability, miRNAs have been implicated in disease from cancers of immunological origin to autoimmunity. In fact, miRNA expression has been shown to be significantly dysregulated in a number of autoimmune disorders such as inflammatory bowel disease (IBD), RA, SLE, and MS (163). This link to autoimmunity is likely due to the important role miRNAs play in the regulation of immune cell development and function (164-166). MiR-155 is a good example of an immunomodulatory miRNA. Mice deficient in miR-155 show impaired function in T cells, B cells, and DCs and have an array of genes dysregulated in their CD4+ T cell population (167). CD4+ T cell differentiation and germinal center formation in B cells is also impaired in these mice (168). MiRNAs also play an important role in B
Table 2.2: Selected microRNAs with roles in the immune system.

Selected microRNAs with immune function, the immune cell types in which they are expressed, and the gene(s) they regulate are shown.

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<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Cell type expressing microRNA</th>
<th>Target genes</th>
</tr>
</thead>
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<tr>
<td>miR-221 and miR-222</td>
<td>HSCs</td>
<td>KIT</td>
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<tr>
<td>miR-10</td>
<td>HSCs</td>
<td>HOX family</td>
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<tr>
<td>miR-126</td>
<td>HSCs</td>
<td>HOXA9 and PLK2</td>
</tr>
<tr>
<td>miR-155</td>
<td>B cells, T cells, macrophages and DCs</td>
<td>SHIP1, PU.1, AID, SOCS1, BACH1, CEBPB, CSFR, TAB2, MAF and JARID2</td>
</tr>
<tr>
<td>miR-150</td>
<td>B cells and T cells</td>
<td>MYB</td>
</tr>
<tr>
<td>miR-17–92</td>
<td>B cells and T cells</td>
<td>BIM and PTEN</td>
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<td>miR-181a</td>
<td>T cells</td>
<td>DUSP5, DUSP6, SHP2 and PTPN22</td>
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<td>T cells</td>
<td>ETS1</td>
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<td>NFIA and SPI1</td>
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<td>NFkB1</td>
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<td>miR-34</td>
<td>DCs and B cells</td>
<td>JAG1, WNT1 and FOXP1</td>
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cell abnormalities, such as leukemia and lymphoma, which was first shown in chronic lymphocytic leukemia (CLL) patients who showed a loss in miR-15a/16-1 expression (169). Overexpression of MiR-21 in mice leads to pre-B cell lymphoma (170) and Hodgkin lymphoma also shows an upregulation of miR-155 (171). All of these findings point out the critical role miRNAs in immunological disorders and highlight the role of miRNAs in the regulation of B cell function and hematopoietic development. The timing of miRNA expression is also critical during hematopoietic development. For example, mature B and T cells express miR-150, but if it is overexpressed in developing B cells, development is impaired (166). Since these same processes are dysregulated in many autoimmune disorders, miRNA-mediated deregulation of gene expression and subsequent impaired function of immune cell subsets is a likely factor in autoimmunity. It is also likely that miRNAs are playing a role in T1D development due to their central role in immune function and development. Our work has also shown that miR-34a may play a role in T1D development (see data chapter V).

VIII. B Cells in T1D

a. B cell Developmental Stages and Regulation

Early B cell development occurs in the bone marrow where the B cell lineage is derived from a common lymphoid progenitor (CLP). Following the CLP in the bone marrow is pre-pro B cell (Hardy fraction A), pro-B cell (Hardy fraction B, C),
pre-B cell (Hardy fraction D), immature/new B cell (Hardy fraction E) and mature B cell (Hardy Fraction F) stages, which are defined by specific cell surface markers (Figure 2.7) (172, 173). This ordered process leads to variable-(diversity)-joining [V(D)J] rearrangement. The pro-B cell stage is where the Ig heavy chain is rearranged, while the Ig light chain is rearranged during the pre-B cell stage. The later immature and mature B cell stages have variable expression of surface IgM. Hardy fraction F in the bone marrow is a pool of cells that have either never left the bone marrow, or have left, continued differentiation in the periphery, and then returned as mature B cells to the bone marrow. The cells that return to the bone marrow as part of the F fraction are mature plasma cells. B cells continue maturing in the periphery, where they develop through the transitional B cell phase (T1-T3) and terminally differentiate into mature B cell subsets including Follicular (FO) B cells as the majority of B cells and Marginal Zone (MZ), as well as B1 peripheral B cells subsets (173). MZ B cells are a splenic population located at the marginal zone between the circulatory system and the lymphoid tissue. They are efficient at capturing antigen because of their role in immune surveillance of the blood (174). FO B cells are present in the spleen and lymph nodes and freely circulate throughout the body and can readily respond to antigens and present them to T cells (175). MZ B cells and FO B cells have both been implicated in T1D pathogenesis. MZ B cells undergo a rapid expansion in NOD mice and cells phenotypically similar to MZ B cells are found in the pancreatic lymph nodes, where they are associated with increased CD4+ T cell activation (176). FO B cells have also been shown to play a role in early T1D, because their depletion inhibits T1D development in NOD.
FIGURE 2.7: Diagram of B lineage development

Hematopoietic stem cells (HSC) in mouse bone marrow through multilineage progenitor (MLP) and common lymphoid progenitor (CLP) to B cell committed stages (Fr. A to F) showing cell surface phenotype, expression of B-lineage genes and of mu heavy chain and kappa light chain are shown. Relative expression levels are indicated by line thickness. Note that only Fr. C is pre-BCR<sup>T</sup>. SLC, surrogate light chain.


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Stages in Mouse Bone Marrow B Lymphopoiesis from Hematopoietic Stem Cells

- AA4.1
- B220
- CD43
- HSA
- BP-1
- cKIT
- IL-7Ra
- CD19
- CD25
- Ig-α/β
- SLC
- Rag-1/2
- TdT
- Mu
- Kappa
mice (177). These experiments have revealed the role peripheral B cells as important APCs in T1D induction.

Early B cell development is tightly regulated by B cell specific transcription factors that drive the expression and repression of genes and subsequent B cell lineage differentiation. Transcription factors are gene regulatory proteins that function to activate or repress gene expression through their direct interaction with DNA. Transcription factors function through their recognition and binding of specific regulatory regions of DNA, such as enhancers, silencers, insulators, and tethering elements (178). This regulatory process is highly conserved (179). In eukaryotes, every developmental stage from embryogenesis to specific checkpoints during hematopoiesis utilizes a specific array of transcription factors to tightly regulate gene expression programs and allow proper development to progress. Multiple transcription factors have been shown to play critical roles in early B cell development. These transcription factors include PU.1, Pax5, E2A, STAT5, early B cell factor-1 (EBF1), Ikaros and Foxp1 (180-182). Figure 2.8 shows the specific transcription factors involved in B cell lineage commitment, with the exception of Foxp1, which functions at the pro- to pre-B cell transition during B cell development.

The Forkhead transcription factor Foxp1 is one of the ‘Foxp’ subfamily of transcription factors. Hu et al. showed that Foxp1 is an essential transcriptional regulator during B cell lymphopoiesis and that the absence of Foxp1 expression leads to defective early B cell development (182). Specifically, a Foxp1 deficiency led to a decrease in B cell lineage genes, such as Pax5, Tcfe2a, and Ebf1 in the B220+
Figure 2.8: Multistep Model of B Cell Development

Successive stages of differentiation from the LMPP (lymphoid-primed multipotent progenitor), ELP (early lymphoid progenitor), pre-pro-B cell, and committed pro- and pre-B cell are depicted. Developmental capacities of the successive stages are indicated. Key transcription factors, growth factor receptors, and cell-surface markers are shown, with important events initiated at a particular stage shown in blue. An arrow pointing upward indicates positive interactions, and t indicates gene repression. RAG1 expression is initiated in the ELP and is maintained until throughout the remaining stages depicted. IRFs, interferon regulatory factor-4 and -8; and preBCR, pre-B cell receptor.

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cells of the fetal liver. There was also a decreased expression of recombination-activating genes 1 and 2 associated with a developmental block at the pro- to pre-B cell transition in the bone marrow. It was found that Foxp1 binds to the Erag enhancer and regulates immunoglobulin heavy chain V(D)J recombination, making Foxp1 expression essential for B-cell lymphopoiesis (182). In fact, Foxp1 was first shown as a critical transcription factor in development because its knockout results in an embryonic lethal condition, with embryos dying at day 14.5 due to outflow tract and heart valve defects (183).

As another layer of regulation downstream of transcription factors, miRNAs also serve a critical regulatory functions during B cell development with miRNAs extensively influencing lineage choice and overcoming developmental checkpoints in both the bone marrow and lymphoid tissues. For example, in B cell development, multiple miRNAs have been shown to regulate the progression of B cell development (Figure 2.9) through their control of critical B cell lineage transcription factors (184). Also, a B cell specific knockout of the dicer gene, directly leading to defective miRNA expression in B cells, led to profound B cell developmental defects and a developmental block occurring at the pro- to pre-B cell transition. Further investigation found that the pro-apoptotic protein Bim was upregulated, which usually gets targeted for degradation by miR-17-92 (185). It has also been shown that if dicer it knocked out in mature B cells, the result is more MZ B cells and less FO B cells in the periphery. This dicer knockout also led to increased autoimmune antibody production and autoimmune disease in a fraction of female mice (186).
Figure 2.9: miRNAs involved in B cell development in the bone marrow and periphery.

B cells development starts in the bone marrow and achieves a remarkable diversity of immunoglobulin loci by V(D)J recombination. Immature B cells migrate to the secondary lymphoid organs where they are activated by specific antigens. Once they are activated, they undergo proliferation and further differentiation into plasma cells that secrete antibodies or memory B cells that can be reactivated with a secondary infection. There are at least three different types of mature B cells: B1 cells, conventional follicular B2 cells and marginal-zone B cells. miRNAs that are involved in different stages of B cell development are indicated above the arrows. Secondary diversification of the immunoglobulin loci is achieved by SHM and CSR at the germinal center.

Abbreviations, CLP: Common lymphoid progenitor; MZB: marginal zone B cells; CB: Centroblasts; CC: Centrocytes; PC: Plasma Cell; Mem: Memory cell; SHM: Somatic Hypermutation; CSR: Class switch recombination.

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B-cell development in the Bone marrow  B-cell development in peripheral lymphoid tissues
Specific miRNAs have also been shown to play a role in B cell development in the periphery. Expression of miR-155 is important in peripheral B cells because while mice that lack its expression have normal immune cell populations, upon immunization they exhibit a defective humoral immune response with impaired germinal center formation and diminished IgG antibody class switching (167). miRNAs have also been shown to play important roles in both the innate and adaptive immune response post-development in the periphery (157, 184). (figure 7).

As previously mentioned, Hu et al. identified Foxp1 as an essential transcriptional regulator in B cell development in 2006 (182). Following this finding, Rao et al. demonstrated that miR-34a is able to target and repress Foxp1. They found that constitutive expression of miR-34a led to a partial block in early B cell development at the pro- to pre-B cell stage and that a miR-34a knockdown led to an increase in B cell numbers. They revealed that the mechanism by which miR-34a blocks B cell development is through it’s targeting of Foxp1 and showed that miR-34a is able bind to and repress Foxp1 expression, subsequently blocking early B cell development (187). From these findings, they concluded that the function of miR-34a in B cell development was due to its repression of Foxp1 because a knockdown of Foxp1 with small interfering RNA (siRNA) leads to the same phenotype seen in mice with miR-34a constitutively expressed (187). These two studies collectively show that miR-34a plays a critical role in B cell development through its repression of the critical B cell development transcription factor Foxp1.
One of the 19 genes located on the \textit{Idd9.3} locus is miR-34a. We showed that T1D-protected NOD.B10 \textit{Idd9.3} mice have an early B cell developmental defect resulting in B-cell paucity in the periphery. Since B cells have been shown to be critical in T1D development in NOD mice (188), the reduction in B cells in secondary lymphoid organs, including the PLN, in NOD.B10 \textit{Idd9.3} mice likely contributes to T1D protection. We further showed that NOD.B10 \textit{Idd9.3} mice had a statistically significant increase in miR-34a expression in their pro- and pre-B cells and that this directly correlated with a reduction in Foxp1 levels in these same cell populations. (see data chapter V)

b. B cells as Antigen Presenting Cells in T1D

While every professional APC subset is able to capture endogenous protein via endocytosis, process it, and present this protein on the MHC II molecule (189), only B cells have the ability to recognize and capture specific endogenous proteins, such as autoantigens. This is the case because the BCR is able to recognize and bind epitopes (190). This unique trait enables antigen-specific B cells to concentrate and present antigen on the MHC II at three orders of magnitude lower concentrations than would be required by other APC types (191). The ability of B cells to concentrate minute levels of protein and preferentially present them via the MHC II makes autoreactive B cells particularly important APCs in autoimmune diseases.

The role of B cells in autoimmunity is emphasized by the fact that B cell deficient mice are resistant to many spontaneous and induced autoimmune
diseases, including T1D (30, 177, 192, 193), SLE (194), and autoimmune arthritis (195, 196). The role of B cells in T1D was first described when B cell deficient mice containing a mutation in the immunoglobulin (Ig) $\mu$ gene, resulting in halted B cell development and no mature B cells, were backcrossed onto the NOD background. Curiously, the resulting B cell deficient NOD mice (NOD.Ig $\mu$ null) were completely resistant to T1D (188, 197). These mice were free of insulitis and T1D-free by the end of the 20-week study (188), pointing out the critical role of B cells in activating autoreactive T cells in T1D induction (188). Conversely, T1D susceptibility could be completely restored in NOD.Ig $\mu$ null mice if B cells were restored (192).

There are two main ways B cells are thought to contribute to T1D: as secretors of autoantibodies and as specialized APCs. It is unlikely that B cell antibody secretion directly leads to T1D development since chronic infusion of autoantibodies from diabetic NOD donors into NOD.Ig $\mu$ null mice did not transfer insulitis or T1D development, and is therefore not likely to mediate T1D (192). Also, NOD mice expressing a mutant heavy chain immunoglobulin transgene that were not able to secrete antibodies, but still had membrane bound BCRs so B cells could function as APCs, still developed insulitis and T1D. This experiment separated the role of B cells as APCs from their role as antibody producing cells, showing that their APC role was critical in T1D, while antibody production was not (30). Therefore, the evidence supports the role of B cells as specialized APCs in T1D.

To determine whether BCR-mediated capture of autoantigens was needed for B cells to function as diabetogenic APCs, BCR transgenic NOD mice in which the BCR
recognized the irrelevant hen egg lysozyme (HEL) protein were generated and crossed with NOD.Ig \( \mu \) null mice. The resulting mice only had BCRs that recognized HEL and BCR-mediated uptake of beta cell autoantigens was abrogated. As expected, when B cells were not able to uptake autoantigens via BCR-mediated (autoantigen-specific) endocytosis, mice were extremely T1D-resistant, showing the importance of B cells expressing surface autoantibodies (198). Conversely, transgenic mice in which the BCR preferentially recognizes the T1D insulin autoantigen have an accelerated incidence of T1D (199).

While B cell autoantibody production is not thought to be critical in T1D induction, it has been observed that autoantibodies are present in the serum of T1D patients and individuals at high risk for developing T1D (200, 201). NOD mice have only been shown to generate autoantibodies against insulin (Bonifacio diabetes 2001), whereas T1D autoantibodies identified in human T1D patients include insulin, insulinoma-associated antigen 2 (I-A2, ICA512), glutamic acid decarboxylase 65 (GAD65) (202) and zinc transporter 8 (ZnT8) (203). T1D autoantibodies, especially directed against insulin and GAD65, have also served as reliable markers in T1D diagnosis are highly indicative of likely T1D development in high-risk individuals (204, 205). This underscores the point that although autoantibodies are not thought to contribute to disease, they are indicative of the presence of autoreactive, diabetogenic B cells specific for T1D autoantigens that could serve as APCs to diabetogenic T cells.
Chapter III: Accelerated T1D induction in mice by adoptive transfer of diabetogenic CD4+ T cells


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I. Brief Background

In the NOD mouse model of T1D, spontaneous disease development typically takes at least 3 months. A method to more rapidly induce T1D for the study of disease pathogenesis and therapeutic interventions allows researchers to collect more data in less time, moving the discovery process forward.

II. Hypothesis

The transfer of BDC2.5 TCR transgenic CD4+ T cells into NOD SCID mice will accelerate T1D induction.

III. Overall findings and significance

The adoptive transfer of islet-specific CD4+ T cells induced rapid T1D within 2 weeks, making this a rapid and efficient protocol to induce T1D in mice for pathogenesis studies or therapeutic interventions. Other major advantages of this
technique are that isolation of adoptive transfer of diabetogenic T cells can be done in one day and irradiation of recipient mice is not required.
Accelerated Type 1 Diabetes Induction in Mice by Adoptive Transfer of Diabetogenic CD4+ T Cells

Gregory Berry, Hanspeter Waldner
Department of Microbiology & Immunology, Pennsylvania State University College of Medicine

Correspondence to: Hanspeter Waldner at huw10@psu.edu

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Abstract

The nonobese diabetic (NOD) mouse spontaneously develops autoimmune diabetes after 12 weeks of age and is the most extensively studied animal model of human Type 1 diabetes (T1D). Cell transfer studies in irradiated recipient mice have established that T cells are pivotal in T1D pathogenesis in this model. We describe herein a simple method to rapidly induce T1D by adoptive transfer of purified, primary CD4+ T cells from pre-diabetic NOD mice transgenic for the islet-specific T cell receptor (TCR) BDC2.5 into NOD.SCID recipient mice. The major advantages of this technique are that isolation and adoptive transfer of diabetogenic T cells can be complete within the same day, irradiation of the recipients is not required, and a high incidence of T1D is elicited within 2 weeks after T cell transfer. Thus, studies of pathogenesis and therapeutic interventions in T1D can proceed at a faster rate than with methods that rely on heterogenous T cell populations or clones derived from diabetic NOD mice.

Video Link

The video component of this article can be found at http://www.jove.com/video/50389/

Introduction

The NOD mouse develops autoimmune diabetes spontaneously and has been widely used as an animal model for human T1D. Pathogenesis of T1D in NOD mice is characterized by infiltration, beginning at 3-4 weeks of age, of the pancreatic islets of Langerhans by dendritic cells and macrophages, followed by T and B cells. This phase of non-destructive peri-insulitis leads to a slow, progressive destruction of insulin-producing pancreatic β cells, resulting in overt diabetes by 4-6 months of age. Transfer of splenocytes, CD4+, or CD8+ T cells from diabetic NOD mice have been shown to mediate diabetes in immunocompromised NOD mice, indicating that islet-reactive T cells play a central role in T1D pathogenesis. Depending on the experimental conditions, diabetes developed in recipient mice slowly, over several weeks in these studies. Similarly, various T cell clones, derived by time-consuming and costly culturing of diabetogenic T cells, have been reported to mediate diabetes several weeks after transfer into recipient mice. With the availability of transgenic mice expressing TCRs derived from CD4- or CD8-restricted diabetogenic T cell clones, several laboratories have subsequently shown that splenic T cells from such mice were able to transfer diabetes to recipients. Specifically, BDC2.5 NOD mice are transgenic for the BDC2.5 TCR, which is specific for chromogranin A, a protein in pancreatic beta cells. Transfer of in vitro-activated or un-activated whole or fractionated spleen cells from overtly diabetic or prediabetic BDC2.5 mice transferred diabetes to neonatal or immunodeficient NOD mice at varying efficiencies.

We describe a simple method that utilizes purified transgenic CD4+ T cells from pre-diabetic BDC2.5 mice to induce T1D in recipient mice at high efficiency and consistency. Large numbers of naive, islet antigen-specific CD4+ T cells are isolated from these mice by fluorescence-activated cell sorting (FACS) for CD4+CD62L+ T cells expressing the transgenic TCR Vβ4 chain. Purified transgenic T cells are then transferred without activation into NOD.SCID mice, which lack functional T and B cells and are insulin- and diabetes-free. The recipient mice are monitored for elevated concentrations of urine glucose indicating T1D, which develops rapidly within two weeks after the T cell transfer.

In contrast to other methods that transfer diabetogenic T cells with heterogenous specificities, our protocol uses FACS-sorted CD4+ T cells that almost exclusively express the diabetogenic BDC2.5 TCR. Due to their homogeneity, only small numbers of transferred T cells (~1x10^5 cells/mouse) are required for rapid T1D development within 2 weeks at 100% incidence. Another advantage of our protocol is that irradiation of recipient mice is not necessary as it is for some other methods. A potential limitation of this method is that it does not allow the investigation into the contribution of both CD4 and CD8 T cell subsets or specifically CD8 T cells in diabetes.

The described protocol will be useful for studying rapid T1D development, mediated by naïve, monospecific CD4+ T cells, as well as therapeutic strategies to intervene in homing of islet antigen-specific Th cells to the target organ.
Protocol

1. Isolation of T Cells from Spleen and Lymph Nodes of BDC2.5 Mice

1. Use 6-week-old pre-diabetic female BDC2.5 mice as donors of diabetogenic CD4+ T cells. Mice should be diabetes-free as determined by urine glucose measurement (see below).
2. Euthanize each mouse using CO2 asphyxiation and remove the spleen, axillary and brachial lymph nodes under sterile conditions. To remove the spleen, soak the fur with 70% ethanol, then cut and retract skin. The spleen will be visible as a dark red organ on the left side of the mouse. Make a 1-inch incision in the peritoneum using the small scissors and gently grasp the spleen in the center with a pair of small tweezers. Carefully trim the connective tissue and attached fat as much as possible and remove the spleen.
3. Collect the spleens and lymph nodes in 10 ml Dulbecco’s-Modified Eagle’s Medium (DMEM) in a 15 ml conical tube on ice.
4. Prepare a single cell suspension by using the end of a sterile 10 ml syringe plunger to gently press the lymphoid organs through 70 μm cell strainers (one spleen per strainer and 4-6 lymph nodes per strainer) into the same 50 ml conical tube. During the process, rinse each strainer with 1 ml DMEM several times to maximize the recovery of cells from the strainer.
5. Transfer the collected cells from the 50 ml conical tube into a 15 ml conical tube and centrifuge at 300-400 x g for 7 min at RT.
6. To lyse red blood cells, discard the supernatant, re-suspend the cells in 5 ml Ammonium-Chloride-Potassium (ACK) buffer (pH 7.2), and incubate the ACK-cell suspension at RT for 5 min.
7. Add 10 ml of DMEM to the ACK-cell suspension and centrifuge the tube as above. Wash the cell pellet once in 10 ml DMEM.

2. Fluorescence-activated Cell Sorting of Diabetogenic CD4+ T Cells from BDC2.5 Mice

1. Re-suspend cells in 5 ml FACS staining buffer and count the number of viable cells (using a phase contrast microscope and a hemocytometer) by trypan blue dye exclusion.
2. Using FACS buffer, adjust the cell suspension volume to 5 x 10^7 cells/ml. Remove ~1 x 10^6 cells per staining control (1 no stain, 3 single stains). Stain the sample for non-activated transgenic CD4+ T cells with anti-CD4 (APC), anti-TCR Vβ4 (FITC) and anti-CD62L (PE) monoclonal antibodies (mAb) in a 15 ml tube. Perform cell staining using the mAb concentrations suggested by the manufacturer for 20-30 min at 4 °C in the dark.
3. Wash the sample and single stain controls with FACS buffer at >3X the staining reaction volume. Centrifuge the tube at 300-400 x g, remove the supernatant, and re-suspend the cells in FACS buffer (1-2 x 10^7 cells/ml for sorting sample and 300 μl for single stain controls) and store on ice until the next step.
4. Pass the cell sample through a 35 μm cell strainer cap tube to remove cell clumps. Sort the samples for CD4+ TCR Vβ4+CD62L+ cells on a cell sorter with a trained operator. Collect the sorted cells into 3 ml DMEM in a 15 ml tube. Allow 2.5-3 hr, including setup, to sort 1.5 x 10^8 cells (the approximate number of cells obtained from 3 donor mice) at a sort rate of 2 x 10^4 total events/sec. Expect ~2.5 x 10^6 non-activated transgenic CD4+ T cells per mouse after cell sorting.
5. Record the absolute number of sorted cells at the end of the sort and centrifuge them for 7 min at 300-400 x g. Discard the supernatant and re-suspend the cells (2 x 10^5 cells/ml) in sterile phosphate-buffered saline (PBS) (Mg^{2+}/Ca^{2+} free) for the adoptive T cell transfer.

3. Adoptive Transfer of Diabetogenic CD4+ T Cells from BDC2.5 Mice

1. Using a 1 ml syringe and an 18-½ gauge needle, gently re-suspend the FACS-purified CD4+ T cells and load the 1 ml syringe. In preparation for injection, replace the 18-½ gauge needle with a 27½ gauge needle.
2. Expose 6-8 week-old female NOD.SCID recipient mice to a heating lamp until they exhibit grooming behavior.
3. Restrain recipient mice in a restrainer and wipe their tail with 70% (v/v) ethanol to disinfect the injection site.
4. Inject 1-2 x 10^6 FACS sorted cells/mouse (in up to 500 μl PBS) into either of the lateral tail veins. Do not force the plunger. If the needle is located appropriately in the vein, the injection will take place with almost no resistance.

4. Monitoring Recipient Mice for Hyperglycemia and T1D

1. Beginning five days after the T cell transfer, NOD.SCID recipient mice are monitored daily for elevated urine glucose using reagent strips (Bayer Diastix) according to the manufacturer’s instructions. Mice with two consecutive urine glucose readings >250 mg/dl are considered diabetic.

Representative Results

Our results show the isolation of transgenic BDC2.5 cells expressing CD62L, which is critical for T cells to home to secondary lymphoid organs such as pancreatic lymph nodes. Our findings further demonstrate the potent ability of this monospecific T cell population to transfer rapidly and efficiently T1D to NOD.SCID recipient mice.
Isolation of diabetogenic CD4+ T cells from BDC2.5 mice is shown in Figure 2. Approximately 5 x 10^7 cells from pooled spleen and lymph nodes were obtained per donor mouse before cell sorting. Following the flow cytometric sort, ~2.5 x 10^6 naive transgenic CD4+ T cells (CD4+TCR Vβ4+CD62L+) were obtained from each BDC2.5 donor mouse by using the indicated gating strategy.

As expected, adoptive transfer of small numbers of CD4+TCR Vβ4+CD62L+ cells (~1 x 10^6 cells/mouse) from BDC2.5 donor mice induced T1D in all NOD.SCID recipients (100% incidence) by day 11, as determined by elevated levels of urine glucose (Figure 3). In comparison, transfer of heterogeneous CD3+ BDC2.5 T cells required 3-5 fold more cells to induce T1D in 80% NOD.SCID recipients by 3 weeks.

These data demonstrate that adoptive transfer of small numbers of FACS-purified BDC2.5 transgenic CD4+CD62L+ T cells into NOD.SCID mice induced T1D more rapidly and efficiently.

Figure 1. Sequence of experimental events. Spleen and lymph nodes were collected from BDC2.5 mice. Naive transgenic CD4+T cells (CD4+TCR Vβ4+CD62L+) were isolated by FACS. T cells were re-suspended in PBS and injected intravenously into NOD.SCID recipients, which were subsequently monitored for elevated urine glucose indicating T1D.

Figure 2. Gating strategy to sort CD4+TCR Vβ4+CD62L+ spleen and lymph node-derived cells from BDC2.5 mice by FACS. A single-cell suspension of pooled spleens and lymph nodes was stained with fluorescently-labeled anti-CD4 (APC), anti-TCR Vβ4 (FITC), and anti-CD62L (PE) mAb. The dot plot on the left shows the CD4+TCR Vβ4+ cell population that was used to gate the CD62L+ cells, shown in the right dot plot. Boxed cells represent percentages of gated cell populations.
Figure 3. Adoptive transfer of diabetogenic CD4+ T cells. FACS-isolated CD4+TCR Vβ4+CD62L+ cells from BDC2.5 mice were intravenously injected (1.3 x 10^6 cells/mouse) into NOD.SCID recipient mice (n=5). Mice were monitored for T1D by measuring urine glucose concentrations in the recipients. Mice with two consecutive readings of >250 mg/dl were considered diabetic.

Discussion

T1D can be induced in recipient mice at varying efficiencies by adoptive transfer of whole spleen cells or T cell subsets from diabetic NOD mice or mice transgenic for TCRs derived from diabetogenic T cell clones. We report herein a reproducible method to induce T1D in recipient mice within two weeks at 100% incidence by transferring FACS-purified CD62L+ BDC2.5 transgenic CD4+ T cells into NOD.SCID mice.

Specific advantages of the BDC2.5 T cell transfer model described here include the very short induction time of T1D compared to months for spontaneous diabetes and up to several weeks for diabetes transfer by diabetogenic T cell subsets or T cell clones. In addition, due to their homogeneity, only small numbers of purified BDC2.5 transgenic T cells are required to transfer T1D with high efficiency and consistency. In contrast to some other T cell transfer methods, in vitro activation of transgenic BDC2.5 T cells prior to transfer is not necessary in our protocol. Another advantage of our monospecific T cell transfer method is that novel therapeutic strategies to intervene in the homing of islet antigen-specific T cell to the target organ can be investigated more selectively than with other protocols that transfer diabetes with heterogenous T cell populations.

A potential limitation of our method is that it is not suited to determine the contribution of both CD4+ and CD8+ T cell subsets in T1D pathogenesis because monospecific, diabetogenic CD4+ T cells are used to transfer diabetes.

In the absence of a FACS instrument, BDC2.5 transgenic T cells may be isolated by column-purification using PE-labeled anti-TCR Vβ4 mAb and anti-PE magnetic beads followed by CD4+CD62L+ magnetic beads (Miltenyi).

It should be noted that FACS-purified, as well as column-purified T cells, will include a minor population of CD4+ T cells that do not express the transgenic TCR due to incomplete allelic exclusion of endogenous TCR genes \(^{15}\). The purified T cell fraction may also include CD4+CD25+ Treg cells that may suppress T1D development in recipients. Since both T cell populations have been reported to increase in BDC2.5 mice with age, we recommend using BDC2.5 mice that are not older than 6 weeks \(^{22,23}\). CD4+CD25+ Treg cells can alternatively be excluded from BDC2.5 T cells by FACS-sorting or separation with CD4+CD25+ magnetic beads (Miltenyi) in older BDC2.5 mice.

Alternatively, or in addition to T1D monitoring by urine glucose measurements, blood glucose levels can be determined more accurately in recipient mice using a handheld glucometer.

Critical steps within this protocol include the age of BDC2.5 donor and NOD.SCID recipient mice. Using young BDC2.5 mice (6 weeks) will ensure that they are diabetes-free and that the frequency of both endogenous non-transgenic T cells and Treg cells are low, as pointed out above. It is also important to use young (<12 weeks) NOD.SCID mice as recipient mice because this strain is prone to developing thymomas that manifest themselves after 20 weeks of age \(^{24}\) and may confound T1D development following adoptive T cell transfer.

Future applications of the described method include investigation in CD4 T cell-mediated mechanisms of T1D pathogenesis and novel strategies to intervene selectively in disease induction, which is not feasible in humans.

Disclosures

All mice were housed at the Penn State College of Medicine specific pathogen-free (SPF) facility in accordance with the guidelines of the Penn State Institutional Animal Care and Use Committee.

The authors declare that they have no competing financial interests.
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References

Chapter IV: The type 1 diabetes resistance locus B10 *Idd9.3* mediates impaired B-cell lymphopoiesis and implicates microRNA-34a in diabetes protection.

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I. Brief Background

NOD.B10 *Idd9.3* mice are NOD mice congenic for the 19-gene *Idd9.3* region from T1D-resistant C57BL/10 mice and are significantly protected from T1D compared to NOD mice. We investigated how B10 *Idd9.3* mediates T1D protection in NOD.B10 *Idd9.3* mice and found a profound B cell paucity in the spleen and pancreatic lymph nodes. Since B cells are known to play an important role in T1D development as cells that present autoantigen to CD4+ T cells, we investigated how B10 *Idd9.3* could be causing a paucity of B cells in NOD.B10 *Idd9.3* mice. We also investigated how a paucity of B cells in NOD.B10 *Idd9.3* mice related to T1D protection in these mice.

II. Hypothesis

B10 *Idd9.3* mediates T1D protection through it’s regulation of diabetogenic CD4+ T cells and/or antigen-presenting cells in NOD.B10 *Idd9.3* mice.
III. Overall findings and significance

The B cell paucity in NOD.B10 *Idd9.3* mice correlates with increased miR-34a-expression early in B cell development. Mir-34a has previously been shown to repress expression of Foxp1, a critical B cell development transcription factor. In NOD.B10 *Idd9.3* mice, increased miR-34a expression correlates with reduced Foxp1 levels and is therefore the likely mechanism by which B10 *Idd9.3* mediates B cell paucity. We also found that NOD.B10 *Idd9.3* B cells are less efficient APCs as compared to NOD B cells. Furthermore, adoptively transferred diabetogenic CD4+ T cells proliferate less in the pancreatic lymph nodes of NOD.B10 *Idd9.3* mice as compared to NOD mice, presumably due to less B cells being present as APCs and reduced B cell APC functionality in NOD.B10 *Idd9.3* mice. These findings point out a likely mechanism behind the B10 *Idd9.3*-mediated T1D protection seen in these congenic mice.
The type 1 diabetes resistance locus B10 Idd9.3 mediates impaired B-cell lymphopoiesis and implicates microRNA-34a in diabetes protection

Gregory J. Berry¹, Lynn R. Budgell², Timothy K. Cooper², Neil D. Christensen² and Hanspeter Waldner¹

¹ Department of Microbiology and Immunology, College of Medicine, Pennsylvania State University, Hershey, PA, USA
² Department of Pathology, College of Medicine, Pennsylvania State University, Hershey, PA, USA

NOD.B10 Idd9.3 mice are congenic for the insulin-dependent diabetes (idd) Idd9.3 locus, which confers significant type 1 diabetes (T1D) protection and encodes 19 genes, including microRNA (miR)-34a, from T1D-resistant C57BL/10 mice. B cells have been shown to play a critical role in the priming of autoantigen-specific CD4⁺ T cells in T1D pathogenesis in non-obese diabetic (NOD) mice. We show that early B-cell development is impaired in NOD.B10 Idd9.3 mice, resulting in the profound reduction of transitional and mature splenic B cells as compared with NOD mice. Molecular analysis revealed that miR-34a expression was significantly higher in B-cell progenitors and marginal zone B cells from NOD.B10 Idd9.3 mice than in NOD mice. Furthermore, miR-34a expression in these cell populations inversely correlated with levels of Foxp1, an essential regulator of B-cell lymphopoiesis, which is directly repressed by miR-34a. In addition, we show that islet-specific CD4⁺ T cells proliferated inefficiently when primed by NOD.B10 Idd9.3 B cells in vitro or in response to endogenous autoantigen in NOD.B10 Idd9.3 mice. Thus, Idd9.3-encoded miR-34a is a likely candidate in negatively regulating B-cell lymphopoiesis, which may contribute to inefficient expansion of islet-specific CD4⁺ T cells and to T1D protection in NOD.B10 Idd9.3 mice.

Keywords: B lymphocytes · Foxp1 · Idd locus 9.3 · microRNA-34a · Type 1 diabetes

Introduction

Type 1 diabetes (T1D) is an autoimmune disease resulting from the immune-mediated destruction of insulin-producing pancreatic beta cells [1]. Non-obese diabetic (NOD) mice share many features of T1D with humans and serve as a valuable model in elucidating mechanisms of T1D pathogenesis [2]. Numerous studies have shown that self-reactive CD4⁺ and CD8⁺ T cells play critical roles in T1D induction [2–6]. However, it has become evident that B cells also participate in the disease process. For example, NOD mice that are deficient in B cells or that were treated chronically with anti-IgM antibodies are protected from T1D development [7, 8]. In addition, B-cell depletion by treating NOD mice with antibodies against B cell-specific molecules such as CD20 or B lymphocyte stimulator attenuates or reverses T1D [9, 10]. Furthermore, B-cell depletion with anti-CD20 mAb (Rituximab) in T1D patients has shown therapeutic effects in recent clinical
trials [11]. Notably, B cells contribute to T1D pathogenesis in NOD mice mainly in their function as antigen-presenting cells (APCs) for self-reactive CD4⁺ T cells, but not as producers of auto-antibodies [8, 12].

T1D in mice and humans is a polygenic autoimmune disease with more than 20 identified insulin-dependent diabetes (Idd) loci predisposing one to disease [1, 13]. NOD mice with defined Idd loci, including Idd9, from T1D-resistant C57BL/6 or C57BL/10 mice introgressed in their genome, have been studied to elucidate the mechanisms by which they contribute to T1D pathogenesis [14–17]. NOD.B10 Idd9.3 congenic mice carry a 1.2 Mb genetic region derived from C57BL/10 mice on chromosome 4 and show a 40% reduction in T1D incidence as compared with NOD mice [18]. The Idd9.3 region contains 19 known genes, including the gene encoding the costimulator CD137, which was recently implicated in contributing to B10 Idd9.3-mediated diabetes protection by promoting the accumulation of CD137⁺ regulatory T (Treg) cells that produce soluble CD137 in aged mice [19].

We have identified a novel mechanism by which B10 Idd9.3 negatively affects B-cell development in NOD. B10 Idd9.3 mice and thereby may contribute to inefficient autoantigen-driven CD4⁺ T-cell proliferation and T1D development in these mice. We show that the B10-derived Idd9.3 impaired central B-cell development, resulting in a profound reduction in mature B cells, including marginal zone (MZ) and follicular (FO) B cells. Idd9.3 encodes miR-34a, a noncoding RNA that directly targets Foxp1 mRNA and represses Foxp1 expression [20]. We found that miR-34a expression was significantly upregulated in NOD.B10 Idd9.3 B-cell precursors and MZ B cells as compared with NOD mice, which inversely correlated with their levels of Foxp1, an essential regulator of early B-cell lymphopoiesis [21]. Notably, B-cell paucity and inefficient priming of islet-specific CD4⁺ T cells by NOD.B10 Idd9.3 B cells in vitro was associated with impaired proliferation of these T cells in response to endogenous autoantigen in pancreatic lymph node (PLN) of NOD.B10 Idd9.3 mice.

In summary, our data indicate that B10-derived Idd9.3 mediates impaired B-cell development, which appears to be regulated by the miR-34a-Foxp1 axis and leads to inefficient proliferation of islet-specific CD4⁺ T cells, which may contribute to T1D protection in NOD.B10 Idd9.3 mice. The data suggest miR-34a as a likely candidate gene in controlling this B10 Idd9.3-mediated effect in NOD.B10 Idd9.3 mice.

Results

Profound reduction of peripheral B cells in NOD.B10 Idd9.3 mice

NOD.B10 Idd9.3 congenic mice exhibit a 40% reduction in T1D incidence as compared with T1D-susceptible NOD mice [18, 22]. To investigate the differences in peripheral immune cell populations that may contribute to differential T1D protection between the two strains, we analyzed the frequency and number of major splenic lymphocyte and myeloid cell subsets such as T, B, and GR-1⁺CD11b⁺ myeloid cells by flow cytometry. Although spleens from both NOD.B10 Idd9.3 and NOD mice were comparable in size, NOD.B10 Idd9.3 spleens showed significantly reduced cellularity as compared with NOD spleens. Furthermore, the frequency of splenic CD19⁺ B cells was greatly reduced in NOD.B10 Idd9.3 mice as compared with NOD mice (Fig. 1A). This reduction correlated with an approximately sevenfold reduction in the number of peripheral B cells in NOD.B10 Idd9.3 mice (Fig. 1B). Although, the frequency of CD4⁺ T cells, CD8⁺ T cells, and GR-1⁺CD11b⁺ myeloid cells was increased in NOD.B10 Idd9.3 spleens, the total number of these cell subsets did not differ significantly between the two strains due to the decreased cellularity of NOD.B10 Idd9.3 spleens (Fig. 1B and data not shown). Notably, analysis of the PLNs, where islet antigens are presented to islet antigen-specific CD4⁺ T cells [23, 24], revealed an approximately ninefold reduction in CD19⁺ B cells in NOD.B10 Idd9.3 mice as compared with NOD mice (Fig. 1C). Histological analysis further determined that NOD.B10 Idd9.3 spleens had a reduced overall amount of white pulp that lacked a discrete follicular mantle or marginal zones and exhibited markedly fewer and smaller B-cell follicles as compared with NOD spleens (Fig. 1D).

B10 Idd9.3 mediates paucity in transitional and mature splenic B-cell subsets

Immature B cells migrate from the bone marrow (BM) to the spleen as transitional (TR) B cells, where they give rise to two mature B-cell subsets, FO and MZ B cells [25, 26]. A minor subset of B cells, B1 cells, also seeds the spleen and peritoneal cavities [27]. To determine whether peripheral B-cell reduction in NOD.B10 Idd9.3 mice was due to the lack of specific B-cell subsets, we assessed the frequency and number of TR, FO, MZ, and B1 subsets within the spleens of 7–10 weeks old NOD.B10 Idd9.3 and NOD mice. TR B cells in C57BL/6 mice can be resolved into the three subpopulations T1, T2, and T3 using the anti-A4.1 (CD93) antibody. However, as previously reported by others [28], the AA4.1 antibody stained B cells from NOD and NOD.B10 Idd9.3 mice poorly (data not shown). Thus, we consolidated the TR B-cell subsets of NOD and NOD.B10 Idd9.3 mice into a TR B-cell pool, defined as B220⁺ IgM⁺ CD21⁺ CD43⁻ cells (Fig. 2A) [28, 29]. We found that the cell number of each examined splenic B-cell subset was significantly reduced in NOD.B10 Idd9.3 mice as compared with NOD mice (Fig. 2B). Notably, the MZ B-cell population showed the most striking reduction with ~33-fold fewer cells in NOD.B10 Idd9.3 mice than in NOD mice (7.7 × 10⁴ ± 3.4 × 10⁴ versus 2.5 × 10⁵ ± 8.9 × 10⁴, p = 0.002). Although the reduction in TR, FO, and B1 B cells was smaller than in MZ B cells, B-cell numbers in these subsets were still greatly diminished (~5–8-fold) in NOD.B10 Idd9.3 mice as compared with NOD mice. Thus, these findings show that B10 Idd9.3 mediates a significant reduction in the TR and mature B-cell compartments in the spleen of NOD.B10 Idd9.3 mice.
Figure 1. Reduction of peripheral B cells in NOD.B10 ldd9.3 mice. NOD and NOD.B10 ldd9.3 splenocytes were stained with antibodies for the indicated markers of cell subsets and analyzed by flow cytometry. Dot plots, representative of seven independent experiments, show percentages of CD3+ and CD19+ (A) splenocytes and (C) pancreatic lymph nodes from live-gated cells. (B) Cell numbers of the indicated cell subsets and total cell numbers in spleens from individual mice are shown. Data represent mean ± SD of 12–20 samples (except for CD8 in NOD and GR1–1/CD11b where n = 6) pooled from greater than ten independent experiments. (D) Representative H&E (left panel, 20× magnification) and B220-specific (right panel, 100× magnification) staining of NOD and NOD.B10 ldd9.3 spleen sections are shown. Control staining was done with secondary antibody only. Images are representative of at least six individual spleens/group.

Impaired development of NOD.B10 ldd9.3 B-cell progenitors

To investigate whether the B10 ldd9.3-mediated reduction in peripheral B cells originates in the BM, we determined the frequency and number of cells in the B220+CD43+ pro-B-cell and B220+CD43− pre-B-cell pools in NOD.B10 ldd9.3 and NOD BM by flow cytometry. The number of total BM-derived cells from NOD.B10 ldd9.3 and NOD mice was comparable (Fig. 3A). However, the frequency and number of both B-cell progenitor pools were markedly reduced in NOD.B10 ldd9.3 as compared with NOD mice (Fig. 3B and C). B220+CD43− BM cells from NOD mice contained a distinct population of mature recirculating B220hi cells, in contrast to NOD.B10 ldd9.3 BM (Fig. 3B). These data indicate that the B10 ldd9.3 locus perturbs B-cell development in the BM of NOD.B10 ldd9.3 mice.

To determine which B-cell progenitor population in the BM is affected by the B10 ldd9.3-mediated effect, we used the cell surface markers BP-1 and heat-stable antigen (HSA) (CD24) to fractionate B220+CD43− cells into three distinct progenitor subpopulations that are defined as Hardy fractions A–C [30]. NOD.B10 ldd9.3 BM showed significantly reduced numbers (∼2–3-fold) of early pro-B cells (HSA+BP-1−) as compared with NOD BM (Fig. 4A). The more mature B220+CD43− BM cells can be divided into pre-B cells (B220+IgM−), immature B cells (B220−IgM+), and mature B cells (B220+IgM+) and are defined as Hardy fractions D–F [30]. Among these CD43-negative B cell subpopulations, pre-B cells (Hardy fraction D (CD43−B220+IgM−)) and immature B cells (Hardy fraction E (CD43−B220+IgM+)) were reduced by approximately twofold in NOD.B10 ldd9.3 mice compared with NOD mice. We detected an even greater reduction (∼18-fold) in the number of mature B cells (Hardy fraction F (CD43−B220+IgM+)) in NOD.B10 ldd9.3 mice (Fig. 4B). Since these cells mainly represent mature B cells recirculating back to the BM from the periphery, this finding is consistent with the observed paucity of mature splenic B cells in NOD.B10 ldd9.3 mice (Fig. 1). Taken together, these data demonstrate that B-cell development in the BM of NOD.B10 ldd9.3 mice is significantly impaired beginning at the early pro-B-cell stage, resulting in a profound reduction in mature B cells.

The B10 ldd9.3 locus impairs B-cell development via an intrinsic effect on hematopoietic progenitors

To determine whether the B10 ldd9.3-mediated effect on B-cell development is mediated by hematopoietic progenitor cells or the
BM stroma, we generated reciprocal BM chimeric mice by reconstituting irradiated mice with NOD- or NOD.B10 Idd9.3-derived BM cells. Thus, BM cells from NOD.B10 Idd9.3 mice expressing the Cd45.1 allotype were transferred into lethally irradiated NOD.B6-Ptprc<sup>−/−</sup> mice containing irradiated NOD- or NOD.B10 BM stroma, we generated reciprocal BM chimeric mice by reconstitution of NOD.B10 Idd9.3 mice and cells within this gate were resolved into IgM<sup>−</sup>CD21<sup>−</sup> and IgM<sup>+</sup>CD21<sup>hi</sup> B-cell pools (second column). These cell populations were further divided into the following B-cell subsets: marginal zone (MZ) (CD21<sup>hi</sup>CD23<sup>−</sup>), follicular (FO), and FO progenitors (CD21<sup>int</sup>CD23<sup>−</sup>) (third column), transitional (TR) (CD43<sup>+</sup>), and B1 (CD43<sup>−</sup>CD23<sup>−</sup>) (fourth column) B cells. Numbers in plots refer to percentages of gated cells. Plots are representative of four independent experiments. (B) Absolute number of B-cell subsets as defined in (A) per spleen from indicated mice were calculated by multiplying the percentages obtained in (A) by the total number of spleen cells and are shown as the mean ± SD representative of four independent experiments. Significance was determined by Student’s t-test.

Figure 2. Reduction in splenic B-cell subpopulations in NOD.B10 Idd9.3 mice. (A) Live spleen cells from NOD and NOD.B10 Idd9.3 mice (7–10 weeks old; n = 4/strain) were analyzed for B-cell markers by flow cytometry. Cells were gated for total B cells (B220<sup>+</sup>IgM<sup>+</sup>) (first column) and cells within this gate were further divided into the following B-cell subsets defined in (A) per spleen from indicated mice were calculated by multiplying the percentages obtained in (A) by the total number of spleen cells and are shown as the mean ± SD representative of four independent experiments. Significance was determined by Student’s t-test.

Differential expression of miR-34a in progenitor and mature B cells in NOD.B10 Idd9.3 and NOD mice

The Idd9.3 genetic interval contains 19 genes, including microRNA (miR)-34a, which was recently shown to play a critical role in early B-cell development [20]. We hypothesized that the observed differences in central B-cell development between NOD and NOD.B10 Idd9.3 mice were associated with differential miR-34a expression in their B-cell progenitors. To test this hypothesis, we conducted quantitative RT-PCR analysis of miR-34a in fluorescence activated cell sorted (FACS)-purified pro-B (B220<sup>+</sup>CD43<sup>−</sup>IgM<sup>−</sup>) and pre-B (B220<sup>+</sup>CD43<sup>−</sup>IgM<sup>+</sup>) cells from NOD.B10 Idd9.3 and NOD mice. Our analysis revealed that miR-34a expression was approximately fivefold and twofold higher in NOD.B10 Idd9.3 pro-B and pre-B cells, respectively as compared with NOD B-cell progenitors (Fig. 6A). Notably, increased miR-34a expression in B-cell progenitors correlated with their impaired development in the NOD.B10 Idd9.3 BM. These data are consistent with a recent study showing that miR-34a overexpression resulted in a B-cell developmental block at the pro-B cell to pre-B cell transition in mice [20].
Impaired B-cell development in NOD.B10 Idd9.3 mice. BM cells from NOD and NOD.B10 Idd9.3 mice (6–10 weeks old) were counted, stained for indicated markers and analyzed by flow cytometry. (A) Total number of BM cells from individual mice is shown; each symbol represents an individual animal and horizontal bars represent the mean. Data are representative of four independent experiments. (B) Representative dot plot, from four independent experiments, showing B220$^{+}$CD43$^{-}$ and B220$^{+}$CD43$^{-}$ cells in boxes with numbers representing percentages of live-gated cells. (C) Absolute numbers of B220$^{+}$CD43$^{+}$ and B220$^{+}$CD43$^{-}$ BM cells from NOD mice and NOD.B10 Idd9.3 mice ($n$ = 6 each, representative of four independent experiments) were calculated by multiplying the percentages obtained as in (B) by the total number of BM cells and are shown as the mean + SD. Statistical significance was determined by Student’s t-test.

Because C57BL/10 mice, the donor of the Idd9.3 in NOD.B10 Idd9.3 mice, display no reported deficiencies in central B-cell development, we determined whether miR-34a expression in B-cell progenitors of this strain was decreased relative to NOD.B10 Idd9.3 mice. Our quantitative RT-PCR analysis demonstrated that C57BL/10 B-cell progenitor cells did indeed express significantly less miR-34a than those of NOD.B10 Idd9.3 mice (Supporting Information Fig. 1A).

Although miR-34a plays an important role in central B-cell development, its role in splenic B-cell differentiation has not been established. To investigate this role, we determined miR-34a expression in FACS-sorted MZ and FO B cells from spleens of NOD.B10 Idd9.3 mice and NOD mice by quantitative RT-PCR. These analyses demonstrated differential expression of miR-34a in these B-cell subsets from NOD.B10 Idd9.3 mice and NOD mice. While NOD.B10 Idd9.3 MZ B cells showed much higher (~25-fold) miR-34a expression than NOD mice, miR-34a expression was downregulated (~10-fold) in NOD.B10 Idd9.3 FO B cells as compared with NOD mice (Fig. 6B). Taken together, these data show that upregulation of B10-derived miR-34a was associated with impaired central B-cell development and with MZ B-cell paucity in the spleen of NOD.B10 Idd9.3 mice.

Reduced Foxp1 levels in NOD.B10 Idd9.3 B-cell progenitors and MZ B cells

Reductions in Foxp1 expression are associated with impaired central B-cell development and with MZ B-cell paucity in the spleen of NOD.B10 Idd9.3 mice. MicroRNAs regulate gene expression posttranscriptionally by binding to 3′ UTR sequences of their target mRNA, leading to
repressed expression of target protein [31]. To investigate the molecular mechanism by which increased expression of B10-derived miR-34a may affect early B-cell development in NOD.B10 Idd9.3 mice, we examined miR-34a targets that were experimentally supported in a publically available miRNA target database (TarBase) [32]. Our search revealed that Foxp1, an essential transcriptional regulator of B-cell lymphopoiesis, is a direct target of miR-34a. To investigate the role of Foxp1 in the B10 Idd9.3-mediated effect on B-cell progenitors, we determined Foxp1 expression in FACS-purified pro-B cells (B220^+CD43^-IgM^-) and pre-B cells (B220^+CD43^-IgM^+) from BM of NOD and NOD.B10 Idd9.3 mice by intracellular antibody staining for Foxp1. Flow cytometric analysis showed that Foxp1 mean fluorescent intensity (MFI) in pro-B and pre-B cells of NOD.B10 Idd9.3 mice was lower than in NOD as well as in C57BL/10 B-cell progenitors (Fig. 7A and Supporting information Fig. 1B). We next investigated whether Foxp1 levels in NOD.B10 Idd9.3 and NOD FO and MZ B cells correlated with their miR-34a expression. Foxp1 levels in peripheral B-cell subsets between NOD and NOD.B10 Idd9.3 mice displayed smaller differences than in BM B-cell precursors. Specifically, Foxp1 was markedly decreased in NOD.B10 Idd9.3 MZ B cells as compared with NOD mice. In contrast, levels of Foxp1 were higher in NOD.B10 Idd9.3 FO B cells than in NOD mice (Fig. 7B).

Taken together, our findings demonstrate that elevated miR-34a expression in NOD.B10 Idd9.3 B-cell progenitors and MZ B cells inversely correlates with Foxp1 levels in these cells. These results suggest that elevated expression of B10-derived miR-34a on the NOD genetic background leads to decreased Foxp1 levels in developing B cells, which thereby may impair B-cell lymphopoiesis and maturation of MZ B cells in NOD.B10 Idd9.3 mice.

Figure 5. B10 Idd9.3-mediated effect is intrinsic to B-cell progenitors. Flow cytometric analysis of lymphocytes from (A, B) irradiated NOD-CD45.2 and NOD.B10 Idd9.3 mice reciprocally reconstituted with CD45.1^+ or CD45.2^+ donor BM cells and (C, D) from irradiated NOD SCID mice, reconstituted with a 1:1 mixture of NOD-CD45.2 and NOD.B10 Idd9.3 donor BM cells. (A, C) BM cells and (B, D) spleen cells from recipients were gated on CD45.1^+ or CD45.2^+ cells and donor-derived B220^+ IgM^+ B cells are shown. Data are representative of two and one experiment with three to four (A, B) and eight (C, D) recipients each. Numbers in plots represent percentages of gated cells.
Inefficient proliferation of islet-specific CD4+ T cells in NOD.B10 Idd9.3 mice

Previous studies have demonstrated a critical role for B cells in CD4+ T cell priming [33]. Furthermore, B cells have been implicated in T1D pathogenesis because genetic or antibody-mediated depletion of B cells in NOD mice resulted in T1D protection [7, 8, 10]. We first investigated whether the function of B cells mediated depletion of B cells in NOD mice inhibited proliferation of adoptively transferred BDC2.5 T cells [35]. Taken together, our data suggest that inefficient T-cell priming by peripheral CD19+ B cells combined with B-cell paucity may impair proliferation of islet-specific CD4+ T cells and thereby may contribute to T1D protection in NOD.B10 Idd9.3 mice.

Figure 6. Elevated expression of miR-34a in NOD.B10 Idd9.3 B-cell progenitors and MZ B cells. (A) Pro-B cells (B220+CD43+IgM−), pre-B cells (B220+CD43+IgM−), (B) MZ B cells (B220+IgM+CD21hiCD23−) and FO B cells (B220+IgM+CD21hiCD23+) were FACs-sorted from pooled NOD and NOD.B10 Idd9.3 BM cells and spleens from 7–8 weeks old mice, respectively. The expression of miR-34a relative to SS rRNA was determined by qRT-PCR and is shown as the mean of triplicate reactions ± SD. Data from at least two independent experiments with similar results are shown. Statistical significance was determined by Student’s t-test.

Figure 7. Reduced Foxp1 levels in NOD.B10 Idd9.3 B-cell progenitors and MZ B cells. BM and spleen cells from NOD and NOD.B10 Idd9.3 mice (7–8 weeks old) were stained with antibodies for surface expression of indicated B-cell populations followed by intracellular staining with a Foxp1-specific antibody. Histograms show staining with anti-Foxp1 (filled histograms) or secondary antibody (nonfilled histogram) in (A) gated pro-B (B220+CD43+IgM−) and pre-B cells (B220+CD43+IgM−) and (B) MZ B cells (B220+IgM+CD21hiCD23−) and FO B cells (B220+IgM+CD21hiCD23+) from NOD (black) and NOD.B10 Idd9.3 (gray) mice. Numbers in histograms represent geometric mean fluorescence intensity (MFI) of Foxp1. Representative data of three experiments are shown.
Evidence that Foxp1-deficient mice have a profound defect in early B-cell development [21] indicates that early B-cell development in the BM depends on the availability of the transcription factor Foxp1. Conversely, constitutive expression of Foxp1 was reported to increase the MZ B-cell compartment in mice [36]. Our analyses showed markedly reduced levels of Foxp1 in NOD.B10 Idd9.3 B-cell progenitor cells and MZ B cells as compared with NOD mice, suggesting that downregulation of Foxp1 in B-cell progenitors and MZ B cells may contribute to impaired B-cell lymphopoiesis and greatly reduced MZ B-cell maturation in NOD.B10 Idd9.3 mice. Foxp1 is a direct target of miR-34a, a microRNA, which posttranscriptionally represses Foxp1 expression [20]. Importantly, miR-34a is encoded by Idd9.3, and thus may directly contribute to the B10 Idd9.3-mediated effect by inhibiting Foxp1 expression in developing B cells in the BM and the spleen. Indeed, we found an inverse correlation between Foxp1 levels and miR-34a expression in B-cell progenitors and MZ B cells of NOD.B10 Idd9.3 and NOD mice. These data suggest that elevated miR-34a expression resulted in repression of Foxp1 in NOD.B10 Idd9.3 B-cell progenitors and MZ B cells, resulting in the observed reduction in mature B cells in NOD.B10 Idd9.3 mice. This model is supported by recent data from others in which constitutive overexpression of miR-34a in mice resulted in a B-cell developmental block and reduction in peripheral B cells [20].

The precise mechanism for the observed differential expression of miR-34a between NOD.B10 Idd9.3 and NOD B-cell progenitors and MZ B cells is unknown. Epigenetic changes in the promoter region of miR-34a between NOD.B10 Idd9.3 and NOD mice may affect the transcriptional activity of the miR-34a promoter and thereby may lead to differences in miR-34a expression [37]. Alternatively, single nucleotide polymorphisms (SNPs) within miR-34a may modify its structure and increase the binding of mature miR-34a to Foxp1 mRNA, thus leading to increased Foxp1 repression. To investigate potential miR-34a sequence variations between NOD and NOD.B10 Idd9.3 mice, we queried whole genome sequencing data publically available from the Welcome Trust Sanger Institute (www.sanger.ac.uk) between the NOD strain and C57BL/6 strain, which is identical by descent with the C57BL/10 strain throughout much of its genome, including the Idd9.3 region [19]. However, our analysis did not reveal any sequence variants between NOD and B6/B10-derived miR-34a, suggesting that differential repression of Foxp1 in B-cell progenitors and mature B cells of these strains is not mediated by a sequence variation in miR-34a.

Figure 8. Impaired priming of BDC2.5 CD4+ T cells by NOD.B10 Idd9.3 B cells in vitro and in NOD.B10 Idd9.3 mice. Irradiated splenic CD19+ B cells from NOD and NOD.B10 Idd9.3 mice were used to stimulate purified BDC2.5 CD4+ T cells (ratio 5:1) with BDC2.5 mimotope p79. (A) CD4+ T-cell proliferation was determined after 72 h, following an overnight pulse with [3H]-thymidine and shown as counts per minute (CPM). Data are shown as mean ± SEM representative of three independent experiments. Statistical significance was determined by Student’s t-test. (B) BDC2.5 CD4+ T cells were labeled with CFSE and transferred into NOD.B10 Idd9.3 and NOD recipients. CFSE dilution of CD4+ T cells from pancreatic and axillary/brachial lymph nodes was analyzed 90 h after T-cell transfer by flow cytometry. Numbers in histogram represent percentages of CD4-gated CFSE+ cells. Data from one of three independent experiments with similar results are shown.
mechanism underlying increased anti-Sm autoantibody production in NOD.B10 Idd9.3 mice remains to be determined.

B cells play an important role in T1D pathogenesis [7, 8, 10, 12, 40]. Consistent with this role, treatment of T1D patients with anti-human CD20 antibody (Rituximab), which induces rapid depletion of FO, but not MZ B cells [41], has proven to be partially effective in T1D clinical trials [11]. Although the mechanisms by which B cells contribute to T1D pathogenesis are not fully understood, their role as antigen-specific APCs has clearly been implicated [12, 42]. We demonstrate that the function of NOD.B10 Idd9.3 CD19+ B cells to prime islet-specific BDC2.5 CD4+ T cells in vitro was inferior to that of NOD B cells. Notably, BDC2.5 CD4+ T-cell proliferation in response to endogenous autoantigens in the PLN of NOD.B10 Idd9.3 mice was inefficient as compared with NOD mice. Thus, the defective T-cell priming function of B cells as well as greatly reduced frequencies of MZ and FO B cells, both of which have been implicated in T1D pathogenesis [43, 44], may decrease spontaneous activation and proliferation of islet-specific CD4+ T cells in NOD.B10 Idd9.3 mice, contributing to their T1D protection.

Recently, subcongenic analyses of non-obese resistant (NOR) mice revealed that three regions on distal chromosome 4 derived from C57BLKS mice control the diabetogenic activity of B cells and contribute to T1D resistance in NOR mice [45]. However, in contrast to the B10 Idd9.3 region, NOR subloci were not reported to affect B-cell development. Taken together, the study described herein and the study by Stolp et al. [45] support the concept that genes within the distal chromosome 4 region in NOD.B10 Idd9.3 and NOR mice control the development of B cells and thereby may regulate T1D development in these strains.

In conclusion, we demonstrate that B10 Idd9.3 mediates impaired B-cell lymphopoiesis that appears to be regulated by the miR-34a-Foxp1 axis and results in paucity of mature B cells with impaired capacity to prime islet-specific CD4+ T cells, which may contribute to T1D protection in NOD.B10 Idd9.3 mice. From a clinical perspective, our results may aid the development of novel miR-34a-based therapeutic strategies that target B cells in T1D patients. Specifically, these novel therapies may impair the development of FO as well as greatly reduced frequencies of MZ and FO B cells, both of which have been implicated in T1D pathogenesis [43, 44], may decrease spontaneous activation and proliferation of islet-specific CD4+ T cells in NOD.B10 Idd9.3 mice, contributing to their T1D protection.

Materials and methods

Mice

NOD.B10-Idd9R3S (referred to as NOD.B10 Idd9.3) mice [18] and BDC2.5 TCR transgenic (referred to as BDC2.5) NOD mice [6] were originally obtained from Drs. Linda Wicker (University of Cambridge, UK) and Diane Mathis (Harvard University), respectively, and maintained in our facility. NOD mice were purchased from Taconic. NOD.B6-Prkrcre (referred to as NOD-CD45.2) mice [14], NOD.CB17-Prkrcre/J (referred to as NOD SCID) and C57Bl/10SnJ mice were purchased from The Jackson Laboratory. Female mice (6–10 weeks old) were used in all experiments unless where indicated otherwise. To verify the purity of the genetic background of the NOD.B10 Idd9.3 mice in our colony, genome scanning analysis was performed by Taconic, using a mouse 1449 single-nucleotide polymorphism (SNP) panel. None of the mice (n = 4) examined contained non-NOD SNPs outside of the B10 Idd9.3 congenic region. In addition, genomic DNA of these mice was analyzed and confirmed for the homozygous presence of the B10 Idd9.3 genetic interval by PCR using three microsatellite markers that differentiate NOD and B10 genomic segments between the markers AL606914 (TGCA), D4Mit226, and D4Mit42 as described previously [17]. All mice were housed at the Pennsylvania State College of Medicine specific pathogen-free (SPF) facility in accordance with Penn State Institutional Animal Care and Use Committee guidelines.

Histology and immunohistochemistry

Spleens were fixed in 10% v/v formalin/PBS, embedded in paraffin, and H&E stained. For CD45R/B220 analysis, spleens were immediately snap-frozen in liquid nitrogen. Frozen tissue was cryosectioned (8 µm), fixed in ice-cold acetone and rehydrated in PBS. Non-specific staining was blocked with 3% w/v BSA/PBS. Serial sections were incubated with CD45R/B220 mAb (1:200; BioLegend #103201) (RT, 1 h). CD45R/B220 was detected with IgG-HRP (1:200; Santa Cruz; sc3823) and NovaRed substrate (Vector Labs; SK-4800). Spleens from at least six mice per strain were stained and examined by a pathologist blinded to genotype.

Flow cytometry and intracellular staining

Single-cell suspensions of organs (1 × 10⁶ cells/sample) were stained (20 min, 4°C) with the following fluorophore-conjugated antibodies: CD4 (RM4–5), CD45R/B220 (RA3–6B2), CD43 (S7), GR-1 (RB6–8C5), CD11b (M1/70), CD45.1 (A20), CD45.2 (104), CD3e (145–2C11), CD8 (S3–6.7), CD19 (1D3), HSA/CD24 (M1–69), IgM (RMM-1) (all from BD Biosciences, San Jose, CA), and BP-1 (6C3; BioLegend). For intracellular staining, cells were fixed with 10% w/v formaldehyde (15 min, RT), permeabilized with 0.1% w/v Triton X-100 (30 min, RT), resuspended and incubated (10 min) in ice-cold 50% v/v methanol in PBS. Cells were subsequently stained with Foxp1 (1:200; #2005, Cell Signaling Technology), followed by IgG (H’L), P(ab’)² fragments (1:1000; #4414, Cell Signaling Technology), 30 min each. Fluorescent staining was assessed on a LSR II cytometer and the data were analyzed using FlowJo software (Tree Star).

Generation of BM chimeric mice

BM cells were harvested from 6–9 week-old female NOD, NOD-CD45.2 or NOD.B10 Idd9.3 donor mice and depleted of T cells using anti-CD3 and anti-CD8 magnetic beads (Miltenyi Biotech GmbH). NOD-CD45.2 or NOD.B10 Idd9.3 mice and NOD SCID mice were gamma-irradiated with 1200 rads and 300 rads from a Co source, respectively. Irradiated mice were injected the following day with T cell-depleted BM cells pooled from two to three
donors (of a distinct CD45 allotype) or they received a mixture of equal numbers of NOD-CD45.2 and NOD.B10 Idd9.3 BM cells (5 × 10^6 cells/mouse). The recipient mice were maintained on water containing 1.5% v/v sulfamethoxazole/trimethoprim for 2 weeks and analyzed 8 weeks after BM transplantation.

**FACS sorting of B cells**

Following RBC lysis, pooled BM cells from the femurs/tibias and splenocytes of 3–4 NOD, NOD.B10 Idd9.3 or C57Bl/10SnJ mice were stained (20 min on ice) with the following fluorophore-conjugated antibodies (all BD Bioscience): IgM (RM-1), CD45R/B220 (RA3–6B2), and CD43 (S7), CD21/CD35 (7G6), and CD23 (B3B4). Pro B cells (IgM^−B220^+CD43^−^), Pre B cells (IgM^−B220^+CD43^+^), MZ B cells (B220^+IgM^−CD21^−^CD23^−^), FO B cells (B220^+IgM^+CD21^hi^CD23^+^), TR cells (B220^+IgM^+CD21^−^CD23^−^), and B1 cells (B220^+IgM^+CD21^−^CD23^−^) were sorted on a BD FACSaria III cell sorter using DIVA software.

**RNA isolation and real-time RT-PCR**

Total RNA was extracted from FACS-sorted cells, using the RNeasy kit (Qiagen) and reverse transcribed (40 ng), using the miRCURY LNA Universal RT microRNA PCR kit (Exiqon). Quantitative PCR was performed using FastStart SYBR Green master mix (Roche) and the Opticon 2 DNA Engine (Biorad) according to manufacturer’s instructions with the following conditions: 95°C for 10 min, 40 cycles at 95°C for 10 s, 60°C for 1 min using PCR primers for miR-34a and 5S rRNA (Exiqon product no. 204486 and 203906, respectively). PCR specificity was analyzed by melting curve analysis. Mean fold change in mRNA expression of triplicate samples, normalized to 5S rRNA, was calculated using the comparative cycle threshold (CT) method (2^{-ΔΔCT} method) [46].

**T-cell proliferation assays**

CD4^+^ T cells from BDC2.5 NOD mice and CD19^+^ B cells from NOD.B10 Idd9.3 and NOD mice were purified to >95% purity using magnetic microbeads according to manufacturer’s instructions (Miltenyi Biotech GmbH). CD19^+^ B cells were irradiated (3200 rads) and cultured in triplicate wells of 96-well plates with purified BDC2.5 CD4^+^ T cells (5:1 ratio, 1.2 × 10^5 cells/well) in the presence of BDC mimotope p79 in DMEM supplemented with 10% v/v FCS, 100 units/ml penicillin, 100 units/l streptomycin, 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, and 1× MEM nonessential amino acids at 37°C for 72 h. T-cell proliferation was determined by [3H] thymidine uptake assay as previously described [17].

**Adoptive T-cell transfer**

CD4^+^ T cells were purified to >95% purity from spleen and lymph nodes of BDC2.5 NOD mice as described above and labeled with CFSE (3 μM; Invitrogen–Molecular Probes) according to manufacturer’s instructions. 5 × 10^6 CFSE-labeled cells were injected (i.v.) into 5–8 weeks old NOD and NOD.B10 Idd9.3 recipients. CD4^+^ T cells from pancreatic and axillary/brachial lymph nodes were analyzed for CFSE dilution 90 h after T cell transfer by flow cytometry.

**Statistical analysis**

Statistical significance of data was determined by two-tailed, unpaired Student’s t-test. Values of p < 0.05 were considered to be statistically significant.

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Abbreviations: FO: follicular · Foxp1: forkhead box protein P1 · HSA: heat-stable antigen · idd: insulin-dependent diabetes · miR: microRNA · MZ: marginal zone · NOR: non-obese resistant · PLN: pancreatic lymph node · SNP: single nucleotide polymorphism · T1D: type 1 diabetes · TR: transitional

Full correspondence: Dr. Hanspeter Waldner, Department of Microbiology & Immunology, Penn State College of Medicine, 500 University Dr., Hershey, PA 17033–2390, USA
e-mail: huw10@psu.edu

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Supplemental Figure 1: Levels of miR-34a and Foxp1 in B-cell progenitors of C57BL/10 and NOD.B10 Idd9.3 mice.

(A) FACS-sorted pro-B cells (B220^+CD43^+IgM^-) and pre-B cells (B220^+CD43^-IgM^-) from C57BL/10 and NOD.B10 Idd9.3 mice (7-8 weeks old) were analyzed for miR-34a expression by qRT-PCR and (B) for Foxp1 levels by intracellular antibody staining. (A) MiR-34a expression relative to 5S rRNA is shown as the mean of triplicate samples ± S.D. of the mean (error bars). (B) Histograms show representative staining with anti-Foxp1 (filled) or secondary antibody only (non-filled) in gated pro-B and pre-B cells from C57BL/10 (black) and NOD.B10 Idd9.3 mice (grey). Numbers represent geometric mean fluorescence intensity (MFI) of Foxp1. Representative data of three experiments are shown. *p < 0.02 (Student’s t test).
Chapter V: Genome wide expression analysis of islet-specific CD4+ T cells identifies Idd9 candidate genes controlling diabetogenic T cell function and type 1 diabetes development

I. Brief Background

NOD.B10 Idd9 congenic mice, in which the NOD Idd9 chromosomal region is replaced by Idd9 from T1D-resistant C57BL/10 mice, are significantly protected from T1D development. Idd9 is a 31 Mb region on chromosome 4, but the genes and pathways conferring T1D development or T1D protection remain to be fully elucidated. We have developed novel NOD.B10-Idd9 (line 905) congenic mice that harbor predominantly islet-reactive CD4+ T cells expressing the BDC2.5 T cell receptor (BDC-Idd9.905 mice). We used microarrays to analyze the gene expression profiles of ex-vivo and antigen-activated CD4+ T cells from these novel mice and non-congenic BDC2.5 NOD controls to establish functional links between the Idd9 genotype and its phenotype.

II. Hypothesis

B10 Idd9 contains genes and molecular pathways that will diminish the diabetogenic potential of islet-specific CD4+ T cells by limiting their proliferation and pro-inflammatory responses.
III. Overall findings and significance

Our analyses identified *Eno1, Rbbp4* and *Mtor* as downregulated in BDC-*Idd9.905* CD4+ T cells and demonstrated that responsiveness and diabetogenic function of BDC-*Idd9.905* CD4+ T cells were significantly reduced compared to those of BDC2.5 mice. These findings suggest that differential expression of these *Idd9* candidate genes modulate *Idd9*-dependent T1D susceptibility by controlling the diabetogenic activity of islet-specific CD4+ T cells.
Genome-wide gene expression analysis of islet-specific CD4+ T cells identifies genes controlling type 1 diabetes development

Gregory J. Berry*, Christine Frielle*, Anna C. Salzberg†, Daniel B. Rainbow‡, Linda S. Wicker‡ and Hanspeter Waldner*

* Department of Microbiology & Immunology, College of Medicine, Pennsylvania State University, Hershey, PA 17033, USA
† Public Health Sciences, College of Medicine, Pennsylvania State University, Hershey, PA 17033, USA
‡ Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke’s Hospital, Cambridge CB2 0XY, UK

Address correspondence:
Dr. Hanspeter Waldner Department of Microbiology & Immunology, Pennsylvania State University College of Medicine, 500 University Dr., Hershey, PA 17033-2390, USA.
Tel.: +1 717 531 0003
E-mail: huw10@psu.edu

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Abstract

Type 1 diabetes (T1D) is a polygenic disease with multiple insulin dependent diabetes loci (ldd) predisposing humans and non-obese diabetic (NOD) mice to disease. NOD.B10 ldd9 congenic mice, in which the NOD ldd9 chromosomal region is replaced by the ldd9 from T1D-resistant C57BL/10 mice, are significantly protected from T1D development. However, the genes and pathways conferring T1D development or protection by ldd9 remain to be fully elucidated. We have developed novel NOD.B10-Idd9 (line 905) congenic mice that predominantly harbor islet-reactive CD4+ T cells expressing the BDC2.5 T cell receptor (BDC-Idd9.905 mice). To establish functional links between the ldd9 genotype and its phenotype, we used microarray analyses to investigate the gene expression profiles of ex vivo and antigen-activated CD4+ T cells from these mice and BDC2.5 (BDC) NOD controls. Among the differentially expressed genes, those located within the ldd9 region were greatly enriched in islet-specific CD4+ T cells. Bioinformatics analyses of differentially expressed genes between BDC-Idd9.905 and BDC CD4+ T cells identified Eno1, Rbbp4 and Mtor, all of which are encoded by ldd9 and part of gene networks involved in cellular growth and development. As predicted, proliferation and Th1/Th17 responses of islet-specific CD4+ T cells from BDC-Idd9.905 mice following antigen stimulation in vitro were reduced compared to BDC mice. Furthermore, proliferative responses to endogenous autoantigen and diabetogenic function were impaired in BDC-Idd9.905 CD4+ T cells. These findings suggest that differential expression of the
identified \textit{Idd9} genes contributed to \textit{Idd9}-dependent T1D susceptibility by controlling the diabetogenic activity of islet-specific CD4+ T cells.

\section*{1. Introduction}

Type 1 diabetes (T1D) is a T cell-mediated organ-specific autoimmune disease, resulting from the destruction of the insulin-producing pancreatic beta cells \cite{1}. In humans and non-obese diabetic mice (NOD), a well-established mouse model for T1D \cite{2}, multiple genetic regions, insulin dependent diabetes (\textit{Idd}) loci, confer risk of T1D development \cite{3}. Various laboratories have used mice congenic for a number of such insulin dependent diabetes (\textit{Idd}) loci derived either from T1D-susceptible or T1D-resistant strains to study the mechanisms by which these loci regulate T1D development and to identify causative genes \cite{4-10}.

Congenic fine-mapping approaches can be combined with DNA microarray expression analysis in models of complex trait diseases to simultaneously investigate gene expression within, as well as outside of a congenic region of interest. This approach successfully identified \textit{Cd36} as an insulin resistance gene \cite{11}. In T1D two studies profiled longitudinal gene expression in naive spleen cells from NOD mice and NOD.\textit{Idd} congenic mice \cite{12, 13}. The findings of these studies were less informative than expected, suggesting that activated specific lymphocyte populations are a better subject for investigation. Accordingly, CD3-stimulated CD4+ T cells were profiled in NOD.\textit{Idd3/5} congenic mice, which identified two new T1D candidate genes \cite{14}. 

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Fine mapping of the *ldd9* region identified four subregions that independently confer partial protection from T1D: *ldd9.1*, *ldd9.2*, *ldd9.3* and *ldd9.4* (15). The *ldd9.1* subregion partially overlaps *ldd11*, a T1D-protective interval defined in NOD.B6 congenic mouse strains (16). Consistent with the notion that *ldd9* encodes a number of immunologically relevant genes, NOD mice congenic for *ldd9* from the T1D-resistant B10 or NOR strains display various immune-related phenotypical differences (4, 6-10, 17-19).

NOD.B10 *ldd9* congenic mice have the NOD-derived *ldd9* region of chromosome 4 replaced with the *ldd9* from T1D-resistant C57BL/10 mice, resulting in significant T1D protection (4). Differentially expressed genes within the *ldd9* region may contribute to these differences. Alternatively, but not exclusively, altered expression of *ldd9* genes could lead to perturbations in the expression of genes shared by both strains, but located outside of this congenic region. To identify genes and molecular pathways that potentially control the diabetogenic potential of islet-specific CD4+ T cells, we conducted microarray expression analysis of *ex vivo* or antigen-stimulated CD4+ T cells from newly generated BDC2.5 TCR transgenic NOD mice that contain the C57BL/10SnJ derived *ldd9* region (line 905) (hereafter referred to as BDC-*ldd9*.905 mice) (17) and BDC2.5 TCR transgenic NOD mice (20) (hereafter referred to as BDC mice).

In this study, we report that genes involved in cellular growth and development showed significantly reduced expression in islet-specific CD4+ T cells from BDC-*ldd9*.905 mice compared to BDC control mice. Among these genes *Eno1*, *Rbbp4*, and *Mtor* were identified as novel *ldd9* candidate genes.
Consistent with these results, functional analyses of CD4+ T cells from BDC-\textit{Idd9.905} mice showed diminished proliferative and Th1 and Th17 cytokine responses following antigen-specific stimulation \textit{in vitro} compared to BDC control mice. In addition, BDC-\textit{Idd9.905} CD4+ T cells were impaired in their proliferation to endogenous autoantigen and in their diabetogenic function. Our findings confirm the validity of using microarray gene expression analysis in CD4+ T cells from BDC-\textit{Idd9.905} congenic mice to identify \textit{Idd9} candidate genes and molecular mechanisms that control islet-specific CD4+ T cell functions.
2. Material and Methods

Mice

NOD.B10 *Idd9* (NOD.B10-*Idd9*R905) mice were originally obtained from Taconic and the congenic interval present in the strain has been described previously (15, 17). BDC2.5 TCR transgenic NOD mice (20) were originally obtained D. Mathis and C. Benoist (Joslin Diabetes Center, Boston, MA). Both strains were maintained in our facility. NOD mice were purchased from Taconic. Mating male BDC2.5 TCR transgenic NOD mice with female NOD.B10 *Idd9* mice generated BDC2.5 TCR transgenic NOD mice containing the B10 *Idd9*R905 interval. BDC2.5 TCR transgenic F1 litters were identified by flow cytometric analysis of PBLs antibody-stained for CD4 and TCRVβ4 as described previously (7) and then crossed with NOD.B10 *Idd9* mice. Transgenic F2 litters were screened for the homozygous presence of the B10 *Idd9* interval by PCR using microsatellite markers to differentiate between the NOD and B10 genomic segments between markers *D4Mit258* and *D4Mit42* as described previously (7). All mice were housed at the Pennsylvania State College of Medicine specific pathogen-free (SPF) facility in accordance with Pennsylvania State Institutional Animal Care and Use Committee guidelines.

Microarray and real-time RT-PCR analysis

Three independent samples of single cell suspensions from two spleens pooled from BDC or BDC-*Idd9*.905 mice were separated into two batches each. The first
batch of spleen cells (ex vivo) was stained with anti-CD4 (RM4-5) and anti-TCRVβ4 (KT4) antibodies (BD Biosciences, San Jose, CA) for 20 min at 4°C, washed and subsequently sorted for CD4+TCRVβ4+ (transgenic) T cells on a BD FACS Aria III. The second batch of spleen cells (1x10^6 cells/ml) was stimulated with BDC2.5 mimotope p79 (1 µg/ml) in culture medium for 48 hours at 37°C. Stimulated spleen cells were then stained and sorted for CD4+TCRVβ4+ T cells as described for the first batch. Total RNA was extracted from sorted ex vivo or p79-stimulated BDC and BDC-Idd9.905 CD4+TCRVβ4+ cells using the RNeasy kit (Qiagen). RNA quality and concentration was assessed using an Agilent 2100 Bioanalyzer with RNA Nano LabChip (Agilent, Santa Clara, CA).

Microarray analysis was performed using the Illumina MouseWG-6 v2.0 R3 Expression BeadChip Kit (Illumina, San Diego, CA) at the Pennsylvania State University College of Medicine Genome Sciences Facility. Thus, cRNA was synthesized by TotalPrep Amplification (Ambion, Austin, TX) from 500 ng of RNA according to manufacturer’s instructions. In vitro transcription (IVT) was employed to generate multiple copies of biotinylated cRNA. The labeled cRNA was purified using filtration, quantified by NanoDrop, and volume-adjusted to 750 ng/sample. Samples were fragmented, and denatured before they were hybridized to MouseWG-6 v2.0 R3 Expression BeadChips for 18 hours at 58°C. Following hybridization, the chips were washed and fluorescently labeled. Beadchips were scanned with a BeadArray Reader and resultant scan data were extracted with GenomeStudio 1.0 (Illumina, San Diego, CA) (Illumina). Analysis of expression data was performed using GeneSpring Gx11 software (Agilent
Technologies, Santa Clara, CA). Expression for a transcript in a sample was considered Present/Marginal if the detection p-value was <0.15. Transcripts were then further filtered for signal level >100 in at least 50% of the values in one of the six samples. If a transcript/probe did not meet these cutoffs it was excluded from further analysis. Genelists were obtained through volcano plots between non-averaged group comparison using fold-change of 1.4 or greater and asymptotic unpaired t-test p-value computation of p<0.05.

For real-time RT-PCR validation of microarray expression data, total RNA from samples was reverse transcribed into cDNA using the QuantiTect Reverse transcription Kit according to the manufacturer's protocol (Qiagen). Quantitative PCR was performed in triplicate reactions with the Opticon 2 DNA Engine machine (Biorad) following manufacturer’s instructions. In brief, 12.5 µl FastStart SYBR Green master mix (Roche), 0.2 µM oligonucleotide primers, 12.4 µl of cDNA (5ng) and dH2O in a total volume of 25 µl. PCR primers were the following: 18S rRNA sense 5′-AAACGGCTACCACATCCAAG; 18S rRNA antisense 5′-CCTCCAATGGATCCTCGTTA

Eno1 sense 5′- CACCCTCTTTCTTGCTTTG; Eno1 antisense 5′-CTTTTGCCTTGTACAGATCG

Agtrap sense 5′- GTCTACCACATGCACCCTG; Agtrap antisense 5′-GAGGGTCGAAGAAATCGGG

S100pbp sense 5′- GCCTAAAAGCAATGCCTCATTTC; S100pbp antisense 5′-CAACAAGGAGTCATCCAACCTCAT
Rbbp4 sense 5’- CAGCAGTAGTGAGGACGTG; Rbbp4 antisense 5’-
AGTGGCTTTGGCTTGGAAGTA;
2610305D13Rik sense 5’- GGAATGTCTCAATTTTGTTCAGAG; 2610305D13Rik
antisense 5’- GTCTTGTTCCAAAGACCTTCTCTT
Casp9 sense 5’- TGGACCGTGACAAAATCTTGAG; Casp9 antisense 5’-
ATCTCCATCAAAGCCGTGAC;
Rcan3 sense 5’- TGAGGTTTCTGCTGCTGTG; Rcan3 antisense 5’-
CAAAAGCAAACGCTGCCTCC
Clic4 sense 5’- TCAAGGCGGGAAGTGATGG; Clic4 antisense 5’-
GGTTGTGACACTGAACACGAC
Txlna sense 5’- TTCAGCTGCAGATGGGAACAG; Txlna antisense 5’-
TTGTCGATATGCTCCTCACG;
Rnf19b sense 5’- GCTCAACCCACACGACAC; Rnf19b antisense 5’-
GCAATAACAGCATAAACCACGT;
Syf2 sense 5’- CGGAATGAAGCTCGTAAGCTG Syf2 antisense 5’-
TCCAGACGCGCTTTCTTGG;
Mtor sense 5’- ACCGCGACCATTTGGAAGAAG; Mtor antisense 5’-
CTCGTTGAGGATCAGCAAGG;
Gm13212 sense 5’- TCTTGATCAAGACAGCCAGT; Gm13212 antisense 5’-
GGGATTCAAGAATCACCTTGCT
Fmr1 sense 5’- CAATGGCGCTTTCTACAAGGC; Fmr1 antisense 5’-
TCTGGTTGCCAGTTTTTCA;
Necap2 sense 5’- ATGGAGGAGAGTGAGTACGAGT; Necap2 antisense 5’-CATTCTGAGGCCCTGTAACCA.

PCR specificity was analyzed using a melting curve analysis. The mean Ct was calculated from triplicate samples. Mean fold change in mRNA expression, normalized to 18S rRNA was calculated using the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$ method) (21).

**T cell proliferation**

CD4$^+$ T cells were purified using CD4 (L3T4) microbeads to >95% purity according to the manufacturer’s instructions (Miltenyi Biotech GmbH). Purified CD4+ T cells (5 x 10^4 cells/well) in presence of irradiated (3,200 rad) NOD splenocytes were stimulated with different concentrations of BDC2.5 mimotope p79 (AVRPLWVRME) (22) in complete DMEM supplemented with 10% (v/v) FCS, 100 units/ml penicillin, 100 units/ml streptomycin, 50 µM 2-mercaptoethanol, 10 mM HEPES, 1mM sodium pyruvate, and 1x MEM non-essential amino acids in triplicates of 96-well plates at 37°C for 72 h. During the last 12 h of culture, $[^3]$H-thymidine (0.5 µCi/well) was added to each well and radioactive incorporation was subsequently measured using a MicroBeta Liquid Scintillation counter (PerkinElmer, Santa Clara, CA).

**ELISA**

The concentration of cytokines was determined in culture supernatants of BDC and BDC-Idd9.905 spleen cells stimulated with p79. Cytokine production in 72 h
culture supernatants was assayed by quantitative capture ELISA according to the manufacturer’s guidelines (BD Biosciences, San Diego, CA; R&D Systems, Minneapolis, MN (IL-17)).

**Bioinformatics analysis of microarray data**

Lists of normalized genes that were significantly differentially expressed (FC>1.4, p<0.05) in microarray analysis were subjected to functional annotation cluster analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (23). This program groups genes according to their known biological functions (GO) to determine pathways and processes of major biological significance. In addition, lists of normalized genes were subjected to Ingenuity Pathway Analysis (IPA) 8.6 (Ingenuity Systems, Redwood City, CA) using T cell-specific filters to uncover significant gene networks.

**Adoptive T cell transfer and T1D analysis**

CD4+ T cells were purified from spleens of nondiabetic 6-9 week old BDC or BDC-Id9.905 female mice to >95% purity by using CD4 magnetic beads (Miltenyi), following red blood cell lysis. For *in vivo* analysis of CD4+ T cell proliferation, CFSE-labeled CD4+ T cells (5 x 10^6 cells/mouse) were injected into non-diabetic NOD mice and analyzed 90 h after transfer by flow cytometry as described previously (24). For T1D analysis, 2.5 x 10^6 purified CD4+ T cells from each donor strain were injected (i.v.) into 6-8 week old NOD.scid female mice. Urine glucose concentration of mice was determined using Glucostix (Bayer) at
least twice a week. Animals were considered diabetic when glucose concentration was >250 mg/dl at two consecutive readings. Diabetic mice also exhibited polyuria and weight loss.

**Statistical Analysis**

T cell proliferation, ELISA and real-time RT-PCR data were analyzed using a two-tailed, unpaired Student's t-test. Enrichment of Idd9 genes was calculated using the hypergeometric distribution test (phyper function) in R (version 2.13.2). The Kaplan-Meier analysis with the log-rank test was used to calculate statistical difference of T1D incidence. Values of p<0.05 were considered statistically significant.
3. Results

**Microarray analysis of islet-specific CD4+ T cell from BDC and BDC-Idd9.905 mice**

To identify differentially expressed genes within the Idd9 T1D susceptibility locus of islet-reactive CD4+ T cells, we utilized NOD mice congenic for the C57BL/10SnJ derived Idd9 locus and transgenic for the islet-specific BDC2.5 T cell receptor. We generated these novel mice by mating BDC2.5 TCR transgenic NOD mice (20) with NOD.B10 Idd9 (line 905) mice (17) and subsequent intercrossings to obtain BDC2.5 TCR transgenic litters that were homozygous for the B10 Idd9 region, hereafter referred to as BDC-Idd9.905 mice (see Material and Methods). In contrast to previous studies by others (9, 12-14), CD4+ T cells from BDC and BDC-Idd9.905 mice allowed us to investigate differential expression of Idd9-encoded genes in CD4+ T cells that were predominantly islet-reactive.

Spleen cells from BDC and BDC-Idd9.905 mice were stimulated with BDC2.5 mimotope p79 (22) for 48h or processed immediately without stimulation in three independent experiments. To reduce the chances of within strain variation, we used splenocytes from a pool of two spleens for each experiment. *Ex vivo* or p79-stimulated spleen cells were sorted for BDC2.5 transgenic (CD4+TCRVβ4+) cells by flow cytometry. Total RNA from these samples was then extracted and subjected to genome-wide expression analysis using Illumina MouseWG-6 v2.0 Expression BeadChips with three replicate microarray
hybridizations performed per strain and per condition (12 hybridizations total). We scored differentially expressed genes in the microarray sets as those genes with a fold change (FC) of >1.4 and a value of p <0.05. Based on these criteria, we found 55 genes and 80 genes in ex vivo and p79-stimulated CD4+ T cells, respectively, that were expressed differentially between the two strains (Fig. 1).

Comparable numbers of genes were expressed at higher or lower levels (27 genes and 28 genes, respectively) in ex vivo CD4+ T cells from BDC-Idd9.905 mice (Fig. 1A). In contrast, in p79-stimulated CD4+ T cells there were more genes expressed at lower levels in BDC-Idd9.905 mice (50 genes vs. 30 genes at higher levels, Fig.1B).

Of the 55 genes differentially expressed in ex vivo CD4+ T cells, 29 genes (53%) are located on chromosome 4, and 6 genes (11% ea.) are located in Idd9 or Idd11, which partially overlaps with the Idd9.1 subregion (15). The enrichment of Idd9 genes in ex vivo samples was highly significant (p = 5.3 x 10^-8), as determined by hypergeometric distribution analysis. The remaining genes are distributed among all but four of the other chromosomes at an average density of 0.7 genes per chromosome (Fig. 2A). Of the 80 genes that were differentially expressed in p79-stimulated CD4+ T cells, 35 genes (44%) are located on chromosome 4, including 9 genes (11%) in Idd9 and 7 genes (9%) in Idd11 (Fig. 2B). The enrichment of Idd9 genes in p79-stimulated samples was statistically significant (p = 1.7 x 10^-11). The remaining genes are distributed among all but three of the other chromosomes at an average density of 2.4 genes per chromosome, slightly more than 3-fold higher than the density of differentially
expressed genes in ex vivo CD4+ T cells. Taken together, these findings demonstrate that in both ex vivo and p79-stimulated CD4+ T cells from the two strains the Idd9/11 region and adjacent regions on chromosome 4 were highly enriched for genes that were expressed differentially.

Thirteen genes that were differentially expressed in ex vivo CD4+ T cells were also expressed differentially in p79-stimulated CD4+ T cells. Two of those genes (Agtrap, S100pbp) are located within Idd9 and 10 genes are located on chromosome 4, but outside of Idd9. Thus, the majority of differentially expressed genes in ex vivo CD4+ T cells were not identical to those in p79-stimulated CD4+ T cells, indicating that differential expression of these genes was unique to either condition. These data suggest that differential expression of the shared genes was independent of p79 stimulation.

**Cluster and network analysis of differentially expressed genes in islet-specific CD4+ T cells from BDC and BDC-Idd9.905 mice**

To identify biological processes that might be affected by genes expressed differentially in ex vivo or p79-stimulated BDC and BDC-Idd9.905 CD4+ T cells, we analyzed gene-ontology-based gene clustering using the Functional Annotation Clustering (FAC) tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID) (23, 25). FAC analysis of genes whose expression was reduced in ex vivo CD4+ T cells from BDC-Idd9.905 mice revealed a significant (p<0.05) enrichment in Krueppel-associated box (KRAB) genes; genes whose expression was higher in these mice did not cluster into any
enriched annotation group (Fig. 3A and data not shown). KRAB is a transcriptional repressor domain of many eukaryotic Krueppel-type C$_2$H$_2$ zinc finger proteins, which are implicated in cellular development and differentiation (26, 27). Thus, these data suggest that islet-specific CD4+ T cells between the two strains differ in their development or differentiation. Genes that were expressed at reduced levels in p79-stimulated BDC-Iddd9.905 CD4+ T cells clustered into 6 significantly (EASE score >1.3) enriched groups, consisting of regulation of organelle organization, positive regulation of protein modification process, DNA binding, regulation of cellular component biogenesis, leukocyte differentiation and chromosomal proteins (Fig. 3B). As all of these processes are involved in cell proliferation and differentiation, these results suggest that proliferation and/or differentiation of BDC-Iddd9.905 CD4+ T cells in response to p79 stimulation may be reduced as compared to BDC CD4+ T cells. DAVID FAC analysis of genes that were expressed at higher levels in p79-stimulated CD4+ T cells from BDC-Iddd9.905 mice did not identify any gene ontology clusters whose expression differed significantly from that in BDC CD4+ T cell (data not shown).

To analyze the microarray data in a tissue-relevant context, we used Ingenuity Pathway Analysis (IPA) software; a bioinformatics annotation tool that identifies molecular networks among differentially expressed genes in selected tissues. With a T cell-specific filter, the most significant gene network that IP analysis revealed in ex vivo CD4+ T cells from the two mouse strains is associated with cellular development, cellular growth and proliferation, hematological system development and function (Fig. 4A). Four focus genes of
this network were among the genes we identified, and two of them, *Eno1* and *Rbbp4*, are located within the *Idd9* region.

In p79-stimulated CD4+ T cells, the top IPA network of differentially expressed genes between the two strains is associated with cellular development and hematological system development and function (Fig. 4B). Fifteen focus genes of this network were differentially expressed in the microarray data set, four of which were expressed at higher levels in BDC-*Idd9*.905 CD4+ T cells and eleven of which were expressed at lower levels. One of the down-regulated genes and the only one located within the *Idd9* region is *Mtor*, a serine/threonine protein kinase that regulates antigen responsiveness of CD4+ T cells, which in turn directs T helper (Th) effector cell differentiation (28). Consistent with the reduced levels of *Mtor* transcripts in p79-stimulated BDC-*Idd9*.905 CD4+ T cells, expression of *Foxp3*, which promotes the generation of T regulatory cells and is inhibited by *Mtor* (29, 30), was increased (Fig. 4B). Other focus genes of this network that are immunologically relevant are located outside the *Idd9* region (see discussion). In particular, we note that *Cd226* expression in p79-stimulated BDC-*Idd9*.905 CD4+ T cells was reduced. CD226 triggers naive CD4+ T cell differentiation and proliferation via its association with lymphocyte function–associated antigen 1 (LFA-1) (31, 32) and is a candidate gene for T1D susceptibility in humans (33).

Taken together, the T cell-specific IPA and DAVID analyses support the hypothesis that differential expression of *Idd9* genes contribute to differential
proliferation and differentiation of islet-specific CD4+ T cells between BDC-Idd9.905 and BDC mice.

**Evaluation of microarray gene expression by quantitative RT-PCR**

To evaluate our microarray results, we next measured gene expression in BDC and BDC-Idd9.905 CD4+ T cells by quantitative RT-PCR. For the *ex vivo* CD4+ T cell samples, we selected five genes located within *Idd9* and three genes located outside of *Idd9* whose expression in the two strains differed the most. For the p79-stimulated CD4+ T cell samples, we assayed seven genes located within *Idd9* and two located outside of it with the highest expression differences. Consistent with the microarray results, transcripts from the *Idd9* genes *Eno1* (*Idd9.3*) and 2610305D13Rik (*Idd9.2*) were less abundant in *ex vivo* BDC-Idd9.905 CD4+ T cells than in BDC CD4+ T cells, and more *Agtrap* transcripts (*Idd9.2*) were observed in the BDC-Idd9.905 samples (Fig. 5A, Table 1). For genes located outside of the *Idd9* region, *Rcan3* and *Clic4* transcripts were less abundant in *ex vivo* CD4+ T cells from BDC-Idd9.905 than from BDC mice, as predicted by the microarray results.

In p79-stimulated CD4+ T cells, the real-time RT-PCR results were consistent with the differences in RNA expression from the *Idd9* genes *Mtor* (*Idd9.2*), *Agtrap* (*Idd9.2*), *Gm13212* (*Idd9.2*) and *Syf2* (*Idd9.1*) that we observed in the microarray analysis between the two strains (Fig. 5B, Table 2). Expression of *Fmr1* and *Necap2*, both located outside the *Idd9* region, also recapitulated the microarray results.
In contrast to the microarray results, *S100pbp* and *Rbbp4* (both *Idd9.1*) were expressed at lower levels in *ex vivo* BDC-*Idd9.905* CD4+ T cells by qRT-PCR analysis. Interestingly, *S100pbp* expression was reported to be down-regulated in regulatory CD4+ T cells of NOD.B10 *Idd9* (strain 905) mice (15). Levels of *Casp9* RNA, which also appeared to be reduced in BDC-*Idd9.905* CD4+ T cells by microarray, were indistinguishable by real-time RT-PCR analysis. In addition, *Rnf19b* and *Txlna* (both *Idd9.1*) RNA levels were not distinguishable by qRT-PCR, and *S100pbp* (also *Idd9.1*) was expressed at lower levels in p79-stimulated CD4+ T cells from BDC-*Idd9.905* mice, rather than the higher levels seen in the microarray data.

In summary, the results of our real-time RT-PCR analysis showed that combining BDC-*Idd9.905* mice and microarray gene expression analysis was successful in identifying genes that are candidates for mediating *Idd9*-dependent differences in basic cellular activities of islet-specific CD4+ T cells, such as proliferation and differentiation.

**Impaired antigen-specific proliferation and pro-inflammatory cytokine responses by BDC-*Idd9.905* CD4+ T cells**

Bioinformatics analysis indicated that the expression of genes involved in cellular growth and development were down-regulated in p79-stimulated BDC-*Idd9.905* CD4+ T cells. Thus, we hypothesized that BDC-*Idd9.905* CD4+ T cells show reduced antigen-specific T cell proliferation and Th effector cell responses as compared to those from BDC mice. To test this hypothesis, we stimulated
purified CD4+ T cells from BDC or BDC-\textit{l}dd9.905 mice with the BDC2.5 mimotope p79 at different concentrations and determined T cell proliferation and Th cytokine responses by thymidine uptake assay and ELISA, respectively. Indeed, p79-stimulated CD4+ T cells from BDC-\textit{l}dd9.905 mice proliferated significantly less than BDC CD4+ T cells (Fig. 6A). In addition, we measured significantly lower concentrations of pro-inflammatory IFN-\(\gamma\) and IL-17 in supernatants of stimulated BDC-\textit{l}dd9.905 CD4+ T cells than BDC CD4+ T cells at the highest antigen concentrations, indicating that antigen-specific Th1 and Th17 effector responses were impaired in BDC-\textit{l}dd9.905 CD4+ T cells (Fig. 6B). We did not observe detectable differences in the concentration of the Th2 signature cytokine IL-4 in the stimulated spleen cell culture supernatants of either strain (data not shown).

Collectively, these data demonstrate that BDC-\textit{l}dd9.905 CD4+ T cells are hyporesponsive to p79-specific proliferation and Th1 and Th17 cytokine production. Thus, these findings support the results of our bioinformatics analyses and predict that islet-specific BDC-\textit{l}dd9.905 CD4+ T cells may show inferior diabetogenic function as compared to those from BDC mice.

**BDC-\textit{l}dd9.905 CD4+ T cells show impaired proliferation to endogenous autoantigen and reduced capacity to mediate T1D**

Islet-specific CD4+ T cells in NOD mice are primed in PLN where they encounter pancreatic autoantigen (34). To investigate the effect of B10 \textit{l}dd9 on proliferation of islet-specific CD4+ T cells to endogenous autoantigen, we
injected CFSE-labeled CD4+ T cells from BDC or BDC-\textit{Idd}9.905 mice into non-diabetic NOD recipient mice. Four days later, proliferation of transferred CD4+ T cells from PLN of recipients was examined by assessing CFSE dilution by flow cytometry. Notably, the frequency of proliferating BDC-\textit{Idd}9.905 CD4+ T cells was markedly reduced compared to BDC CD4+ T cells (Fig. 7A). As a control, islet-specific CD4+ T cells from both strains failed to substantially proliferate in axillary/brachial lymph nodes, which do not drain the PLN and therefore do not contain pancreatic antigens (Fig. 7B).

To investigate whether inefficient priming/proliferation of BDC-\textit{Idd}9.905 CD4+ T cells to autoantigen results in impaired ability to mediate T1D, we next transferred CD4+ T cells from young non-diabetic BDC-\textit{Idd}9.905 and BDC control mice into NOD SCID mice, which develop neither spontaneous insulitis nor diabetes (20). All (100%) of the recipients (6/6 mice) that received BDC CD4+ T cells developed T1D, whereas only 17% of the recipients (1/6 mice) that had received BDC-\textit{Idd}9.905 CD4+ T cells developed T1D by the end of the experiment (50 days). These results were statistically significant (p=0.009) as determined by Kaplan-Meier analysis and log-rank test (Fig. 8). T1D severity was comparable in diabetic mice of both groups as determined by urine glucose concentration (data not shown). Taken together, reduced p79-specific responses by BDC-\textit{Idd}9.905 CD4+ T cells \textit{in vitro}, correlated with reduced proliferation to endogenous autoantigen and with impaired diabetogenic function of BDC-\textit{Idd}9.905 CD4+ T cells.
4. Discussion

Identification of candidate genes and the molecular mechanisms involved in the development of complex genetic diseases such as T1D have been the focus of intensive research. In this study, we have used BDC and novel BDC-\textit{Idd9.905} mice to determine differences in global gene expression mediated by the \textit{Idd9} from the T1D-susceptible NOD strain or the T1D-resistant C57BL/10 strain in islet-specific CD4+ T cells, respectively.

There were 55 and 80 genes differentially expressed in \textit{ex vivo} or BDC2.5 mimotope (p79)-stimulated CD4+ T cells from BDC-\textit{Idd9.905} mice and BDC mice, respectively, as determined by microarray gene expression analysis (Fig. 1). Notably, differentially expressed genes were greatly enriched (~20%) for those genes located within the \textit{Idd9} and \textit{Idd11} region, overlapping the \textit{Idd9.1} region on chromosome 4 (Fig. 2). The other genes were located outside of the \textit{Idd9/11} region, suggesting that genes within the \textit{Idd9} region altered expression of those genes in both \textit{ex vivo} and p79-stimulated CD4+ T cells. Our results are similar to those by Irie et al., who reported differential expression of genes located both within and outside of the \textit{Idd3/5} region in anti-CD3-stimulated CD4+ T cells between NOD and NOD.\textit{Idd3/5} mice (14).

Real-time RT-PCR analysis validated differential expression of most selected genes located within \textit{Idd9} such as \textit{Eno1}, \textit{Agtrap} and 2610305D13Rik (\textit{ex vivo} CD4+ T cells) and \textit{Mtor}, \textit{Agtrap}, \textit{Syf2} and \textit{Gm13212} (p79-stimulated CD4+ T cells) (Fig. 5). Interestingly, real-time RT-PCR did not validate the microarray expression of some other examined genes, including \textit{Rbbp4}, which
could potentially be due to a number of reasons, including low fold changes in microarray gene expression, or low microarray spot intensities (35, 36). Bioinformatics analyses using DAVID and T cell-specific IPA revealed that genes associated with cellular growth and development, which included ldd9-encoded Eno1, Rbbp4 and Mtor, were most significantly enriched among the downregulated genes in ex vivo and p79-stimulated BDC-lld9.905 CD4+ T cells (Fig. 4 and Fig. 5). Enolase1 (Eno1, ldd9.3), which was expressed at lower levels in ex vivo BDC-lld9.905 CD4+ T cells, encodes a multifunctional enzyme that plays a role in growth control, glycolysis and allergic responses (37). Notably, Eno1 has been implicated in a variety of autoimmune and inflammatory diseases, including systematic lupus erythematosus, rheumatoid arthritis and Hashimoto's encephalopathy because antibodies against this protein have been found in patients affected by these diseases (38, 39). Furthermore, elevated expression of Eno1 in inflammatory cells has been reported to promote migration to inflammatory sites (40, 41). Rbbp4 is a ubiquitously expressed and highly conserved nuclear protein that mediates chromatin assembly in DNA replication (42). The gene encoding retinoblastoma binding protein (Rbbp4, ldd9.1) was down-regulated in ex vivo BDC-lld9.905 CD4+ T cells as determined by real-time RT-PCR. Interestingly, a previous gene expression analysis in CD4+ T cells from NOD and congenic NOD mice did not find any significant differences in Rbbp4 gene expression between the two strains by qRT-PCR analysis (15). It is possible that Rbbp4 expression differences in autoreactive CD4+ T cells may
have been masked by those in non-autoreactive CD4+ T cells present in the study by Hamilton-Williams et al.

Both Eno1 and Rbbp4 are encoded by Idd9 and were focus genes of the most significant gene network in ex vivo CD4+ T cells identified by T cell-specific IPA. Based on the analysis of this network, Rbbp4 interacts directly with chemokine receptor 2 (Ccr2), which is expressed on activated and memory T cells, including Th1 cells (43). Reduced expression of Rbbp4 in BDC-Idd9.905 CD4+ T cells could therefore result in its diminished interaction with CCR2 in activated islet-specific CD4+ T cells and may impair their ability to differentiate into Th1 effector cells.

In addition, this analysis also suggests that reduced expression of Eno1 and/or Rbbp4 in ex vivo BDC-Idd9.905 CD4+ T cells could lead to increased and reduced expression of two other Idd9 genes, Agtrap and 2610305D13Rik, respectively. While 2610305D13Rik encodes a KRAB-containing zinc finger protein with unknown function, Agtrap codes for Type-1 angiotensin II receptor-associated protein, which interacts with the angiotensin II type I receptor and negatively regulates angiotensin II signaling (44). Agtrap is expressed in many tissues and inhibits the calcineurin/nuclear factor of activated T cells (NFAT) pathway, which regulates cell growth and plays an important role in the transcription of cytokine genes and other genes critical for immune responses (45, 46). Interestingly, Agtrap expression was sensitive to TCR activation because its expression was increased in p79-stimulated compared to ex vivo BDC-Idd9.905 CD4+ T cells.
Rcan3 and Clic4, which are both located outside of the Idd9 region were expressed at significantly lower levels in ex vivo CD4+ T cells from BDC-Idd9.905 mice than BDC mice. As both of these genes are NOD-derived in both strains, differential expression of these genes is likely the effect of altered expression of gene(s) within the Idd9 region. The gene product of regulator of calcineurin 3 (Rcan3) belongs to a well-conserved family and inhibits NFAT-dependent expression of cytokine genes such as IL-2 and IFN-γ in activated Jurkat T cells (47). Clic4 encodes chloride intracellular channel 4 protein, which in association with Schnurri-2 translocates to the nucleus, leading to enhanced TGF-β signaling (48). As TGF-β signaling is important for differentiation of thymic T cells and survival of peripheral CD4+ T cells (49), reduced Clic4 expression in islet-specific CD4+ T cells of BDC-Idd9.905 mice could impair TGF-β signaling and thus their ability to develop in the thymus and/or survive in the periphery. Thymic development and selection of islet-specific CD4+ T cells in both strains appeared to be comparable because we did not detect significant differences in the proportions or absolute numbers of their peripheral BDC2.5 transgenic CD4+ T cells (data not shown). However, it remains to be determined whether survival of these autoreactive T cells are different between the two strains.

Mtor was the only focus gene encoded by Idd9 (Idd9.2) that was part of the most significant gene network in p79-stimulated CD4+ T cells identified by T cell-specific IPA. Mtor, which was down-regulated in p79-stimulated BDC-Idd9.905 CD4+ T cells, is a serine/threonine protein kinase that regulates cell growth and proliferation in response of growth factor signals and insulin.
Importantly, *Mtor* is a critical regulator of T helper cell functions and differentiation (28). Our gene network analysis revealed that *Mtor* indirectly interacts with *Foxp3*. *Mtor*-deficient CD4+ T cell differentiate into Foxp3+ Treg cells, while failing to develop into Th1, Th2 or Th17 effector cells (30). Differential expression of *Mtor* between NOD mice and C57BL/6 mice, which share *Idd9* sequence identity with C57BL/10 mice could be due to several SNPs that exist in the intron region of *Mtor* (15). Interestingly, Hamilton-Williams et al. did not detect any differences in Mtor protein expression in naive or activated CD8+ T cells between NOD.B10 *Idd9* and NOD mice (15). Their results combined with ours suggest that *Mtor* expression differs between CD4+ and CD8+ T cells subsets of these strains. Lower expression of *Mtor* as well as 2610305D13Rik was reported in B cells from T1D-protected NR4 mice, which are congenic for the NOR resistance locus 2 that overlaps with the *Idd9.1* region (9). Thus, based on those and our data, lower expression of *Mtor* and 2610305D13Rik does not appear to be restricted to CD4+ T cells from T1D-protected mice.

The IPA network of genes differentially expressed in p79-stimulated CD4+ T cells between BDC-*Idd9.905* and BDC mice contained a number of genes that are relevant to CD4+ T cell responses. *Cd200* and *Cd226*, both of which are immunoglobulin superfamily members, showed reduced expression in BDC-*Idd9.905* CD4+ T cells. *Cd200* (OX-2) is expressed by activated T cells and has been implicated as an important immunosuppressive molecule controlling autoimmunity, inflammation and adaptive immune responses (50). *Cd226* is primarily expressed on T cells and positively regulates naive T cell differentiation.
and proliferation (31). Notably, a T1D GWAS study in humans identified CD226 as a candidate gene (51). Ly6a whose expression was higher in BDC-\textit{Idd9.905} CD4+ T cells than in BDC CD4+ T cells is involved in the negative regulation of antigen-specific CD4+ T cell proliferation and cytokine production (52). \textit{Csf2} whose transcripts level were reduced in BDC-\textit{Idd9.905} CD4+ T cells compared to BDC CD4+ T cells encodes granulocyte-macrophage colony-stimulating factor (GMCSF), which is secreted by CD4+ T cells following antigen stimulation and functions in the initiation autoimmune inflammation in certain models of autoimmune diseases (53, 54).

By demonstrating that BDC-\textit{Idd9.905} CD4+ T cells were hyporesponsive in p79-specific proliferation and Th1 and Th17 cytokine responses \textit{in vitro} as compared to BDC CD4+ T cells, we were able to validate our bioinformatics results, which predicted that islet-specific BDC-\textit{Idd9.905} CD4+ T cells were impaired in their ability to proliferate and to develop effector T helper cell responses. A previous report showed that expression of protective \textit{Idd9} alleles by CD4+ T cells was sufficient to restore CD8+ T cell tolerance in NOD mice, albeit with contribution from non-lymphoid cells (18). We cannot exclude that \textit{Idd9} in non-lymphoid cells could also contribute to differential gene expression between CD4+ T cells from BDC and BDC-\textit{Idd9.905} mice due to potentially different signals received from non-lymphoid cells during T cell development. However, BDC-\textit{Idd9.905} CD4+ T cells proliferated less efficiently to antigen presented by non-lymphoid NOD cells \textit{in vitro} and \textit{in vivo} compared to BDC CD4+ T cells. In addition, BDC-\textit{Idd9.905} CD4+ T cells were less diabetogenic compared to BDC
CD4+ T cells in recipient mice that had identical non-lymphoid NOD cells, suggesting that differential diabetogenic function of CD4+ T cells between BDC-Idd9.905 and BDC mice was likely mediated by T cell-intrinsic effects of Idd9. Thus, our data suggest that Idd9-mediated CD4+ T cell intrinsic differences contributed predominantly to differential Th functions between BDC and BDC-Idd9.905 mice.

In conclusion, by combining microarray gene expression and bioinformatics analyses we have identified Eno1, Rbbp4 and Mtor as Idd9 genes involved in T cell proliferation and/or differentiation that were differentially expressed between BDC-Idd9.905 and BDC CD4+ T cells. We propose that these Idd9 candidate genes contribute to Idd9-dependent differences in the cellular and diabetogenic function of islet-specific CD4+ T cells between BDC-Idd9.905 and BDC mice.
Figure legends

Figure 1. Volcano plot of genes differentially expressed in CD4+ T cells between BDC-Idd9.905 and BDC mice.

Volcano plot showing the relative abundance of transcripts in ex vivo (A) and p79-stimulated CD4+ T cells from BDC-Idd9.905 mice compared to BDC mice. Log2 of fold change (FC) is presented on the x-axis and -log10 of p values is represented on the y-axis. Transcripts that passed the cutoff of p<0.05 and FC>1.4 (log2=0.5) were considered to be differentially expressed and are shown in black. Genes that are significantly up-regulated in BDC-Idd9.905 CD4+ T cells compared to BDC CD4+ T cells are on the right, while down-regulated genes are on the left of FC=0. Grey dots indicate genes, which did not show differential expression between the two strains.

Figure 2. Chromosomal location of differentially expressed genes in CD4+ T cells between BDC-Idd9.905 and BDC mice.

Location of the genes that were differentially expressed in ex vivo (A) and p79-stimulated (B) CD4+ T cells between BDC-Idd9.905 and BDC mice were visualized on chromosomes using chrView tool of the biological database network (bioDBnet) software. Each gene is shown as a horizontal bar on the specific chromosome.
Figure 3: DAVID functional annotation cluster analysis of differentially expressed genes between BDC-\textit{Idd9.905} and BDC CD4+ T cells.

Analysis was performed on the 55 genes and 80 genes that showed differential expression in \textit{ex vivo} (A) and p79-stimulated (B) CD4+ T cells between BDC-\textit{Idd9.905} and BDC mice. Bars describe the gene ontology terms and represent the most significantly enriched functional annotation clusters for down-regulated genes (p<0.05, FC>1.4). Significance of enrichment of gene ontology terms is shown (p<0.05) as EASE score, which is defined as the minus log10 transformation on the geometric mean of p-values (modified Fisher's exact test) in a corresponding annotation term that associates with the gene group's members.

Figure 4: Gene network of differentially expressed genes in CD4+ T cells between BDC-\textit{Idd9.905} and BDC mice.

The 55 genes and 80 genes that were differentially expressed in \textit{ex vivo} (A) and p79-stimulated (B) CD4+ T cells between BDC-\textit{Idd9.905} and BDC mice, respectively were subjected to Ingenuity Pathway analysis. The most significant molecular network for each gene set is shown. Nodes represent gene products and biological relationships between two nodes are represented as a line (direct interaction: solid line; indirect interaction: broken line). Green and red nodes indicate elevated and decreased expression levels, respectively.
**Figure 5. Validation of differential expression of selected genes by quantitative RT-PCR.**

Selected genes that showed differential expression (FC $>1.4$, p<0.05) in microarray analysis of ex vivo (A) and p79-stimulated (B) CD4+ T cells between BDC-Idd9.905 and BDC mice were validated by real-time RT-PCR. Data were normalized to 18S rRNA expression and shown as mean fold change in BDC-Idd9.905 samples relative to the mean fold change in BDC samples ± S.D. (error bars). *p<0.05, **p<0.01, ***p<0.001 (Student's t test).

**Figure 6: Reduced proliferation and Th1 and Th17 responses in BDC-Idd9.905 CD4+ T cells following stimulation with BDC2.5 mimotope.**

Purified CD4+ T cells from BDC-Idd9.905 or BDC mice were stimulated with indicated concentrations of BDC2.5 mimotope p79 in presence of irradiated NOD spleen cells as APCs for 3 days. (A) CD4+ T cell proliferation was determined by $[^3]H$thymidine incorporation assay and shown as mean counts per minute (CPM) of triplicate cultures. (B) Concentrations of indicated Th cytokines in supernatants of p79-stimulated CD4+ T cell cultures were assayed in duplicate by ELISA. One of three independent experiments each with similar data is shown. Error bars represent SD. * p < 0.03 (A) * p < 0.02 (B) (Student's t test).

**Figure 7: Impaired proliferation of BDC-Idd9.905 CD4+ T cells to endogenous autoantigen.**

BDC-Idd9.905 and BDC CD4+ T cells were CFSE-labeled and transferred (5 x
10^6, i.v.) into non-diabetic NOD mice. After 90 hours, proliferation of transferred CD4+ T cells from pancreatic lymph nodes (A) and from axillary/brachial lymph nodes (B) as control was determined by assessing CFSE dilution by flow cytometry. One of three independent experiments each with similar data is shown. Numbers in histograms represent percentages of CFSE+ CD4-gated T cells that underwent cell divisions.

Figure 8: Impaired diabetogenic function of BDC-Idd9.905 CD4+ T cells.
Kaplan-Meier analysis of T1D in NOD SCID mice following injection (i.v.) of purified CD4+ T cells (2.5 x 10^6 cells/mouse) from non-diabetic BDC or BDC-Idd9.905 mice. Recipient mice (n=6 mice/group) were examined for T1D for indicated time by measuring urine glucose concentrations at least twice a week. Mice with glucose concentration ≥250 mg/dl at two consecutive time points were diagnosed as diabetic. Mean data from one experiment are shown. p = 0.009 (log-rank test).
Fig. 1

A.

B.
Fig. 2

A.

B.
Fig. 3

A. - log10 (p-value) vs. Krueppel-associated box

B. - log10 (p-value) for various biological processes:
- regulation of organelle organization
- positive regulation of protein modification process
- DNA binding
- regulation of cellular component biogenesis
- leukocyte differentiation
- chromosomal protein
Fig. 4

A.

B.
Fig. 5

A.

B.

- Eno1
- Agtrap
- S100pbp
- Rbbp4
- 2610305D13Rik
- Casp9
- Rcan3
- Clic4

- Txlna
- Agtrap
- S100pbp
- Rnf19b
- Syf2
- Mtor
- Gm13212
- Fmr1
- Necap2

Fold change

BDC-Idd9.905 vs BDC
Fig. 6

A. CD4+ T cell proliferation [CPM]

B. IFN-γ [pg/ml] and IL-17 [pg/ml] production

- * indicates significant difference compared to control.

- CPM: Counts Per Minute

- IFN-γ: Interferon-γ

- IL-17: Interleukin-17
Fig. 7

**A.**
Pancreatic lymph nodes

**B.**
Axillary/brachial lymph nodes

- **BDC CD4+ T cells**
  - Pancreatic lymph nodes: 72.3%
  - Axillary/brachial lymph nodes: 5.5%

- **BDC-Idd9.905 CD4+ T cells**
  - Pancreatic lymph nodes: 55.6%
  - Axillary/brachial lymph nodes: 4.0%
Incidence of T1D [%]

Weeks after CD4+ T cell transfer

- BDC (n=6)
- BDC-Idd9.905 (n=6)

p=0.009
**Supplemental Table 1.** Differentially expressed transcripts between ex-vivo BDC-Idd9.905 vs. BDC CD4+ T cells detected by Illumina microarray probes.

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* Fold change in expression of BDC-Idd9.905/BDC samples.
* Gene ID: Entrez database
ACKNOWLEDGMENTS

We thank Dr. Willard Freeman and Robert Brucklacher at the PSU College of Medicine Genome Sciences Facility for their assistance with microarrays. We thank Drs. David Spector and Laura Carrel for critical review of the manuscript.

DISCLOSURES

The authors have no financial and commercial conflicts of interest.
REFERENCES


diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. *J Immunol* 166: 908-917.


Table 1: Expression of selected genes in *ex vivo* BDC-*Idd9.905* vs. BDC CD4+ T cells by quantitative RT-PCR and microarray analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Entrez gene ID</th>
<th>Location</th>
<th>FC Microarray</th>
<th>p-value</th>
<th>FC qRT-PCR</th>
<th>p value</th>
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RNA from *ex vivo* BDC or BDC-*Idd9.905* CD4+ T cells was processed for microarray or qRT-PCR analysis as described in Material and Methods. Data show fold changes (FC) of indicated gene transcripts (BDC-*Idd9.905*/BDC) as determined by microarray hybridization using Illumina MouseWG-6 v2.0 R3 Expression BeadChips or by qRT-PCR in triplicate using SYBR green and gene-specific primers. N.S., not significant.
Table 2: Expression of selected genes in p79-stimulated BDC-Idd9.905 vs. BDC CD4+ T cells by quantitative RT-PCR and microarray analyses

<table>
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<tr>
<th>Gene</th>
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<th>p-value</th>
<th>FC qRT-PCR</th>
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RNA from p79-stimulated BDC or BDC-Idd9.905 CD4+ T cells was processed for microarray or qRT-PCR analysis as described in Material and Methods. Data show fold changes (FC) of indicated gene transcripts (BDC-Idd9.905/BDC) as determined by microarray hybridization using Illumina MouseWG-6 v2.0 R3 Expression BeadChips or by qRT-PCR in triplicate using SYBR green and gene-specific primers. N.S., not significant.
Chapter VI: Constitutive expression of miR-34a using a Retroviral Vector Delivery System

I. Background and hypothesis

Constitutive expression of microRNA-34a (miR-34a) has previously been shown to have a profound effect on B cell development through its binding of Foxp1 mRNA, resulting in repression of protein expression. Foxp1 is a critical transcription factor in B cell development (187). In a mouse model of type 1 diabetes, it has also been previously shown that while nonobese diabetic (NOD) mice spontaneously develop T1D at an incidence of ~90%, a congeneric strain of NOD mice containing the 19 gene Idd9.3 region from T1D-resistant C57BL/10 mouse strain, NOD.B10 Idd9.3 mice, show 40% protection from T1D development (102).

We have begun to investigate the mechanism(s) underlying the reported Idd9.3-mediated protection in these mice and have shown that NOD.B10 Idd9.3 mice have a profound B cell developmental defect, starting at the pro- to Pre-B cell stage in the bone marrow. This B cell developmental defect leads to a vast reduction in the number of peripheral B cells, including in the pancreatic lymph node (PLN), the site of diabetogenic T cell priming. Furthermore, we have shown that these mice have a significant increase in miR-34a expression and a concomitant decrease in Foxp1 levels in these same cell populations when compared to NOD mice (see data chapter 6). Based on these findings, we hypothesize that peripheral B cell paucity
contributes to T1D protection of NOD.B10 ldd9.3 mice, and that this B cell reduction is mediated by increased miR-34a expression and subsequent Foxp1 repression.

We hypothesize that elevating miR-34a expression in NOD bone marrow should lead to reduced B cells in the periphery, including the PLN, and should protect NOD mice from T1D development.

II. Materials and Methods

Mice

BDC2.5 TCR transgenic NOD (BDC) mice were originally obtained from D. Mathis and C. Benoist (Joslin Diabetes Center, Boston, MA) and were maintained in our facility. The CD4+ T cells in these mice are predominantly monospecific, islet-specific CD4+ T cells (21). To maintain colony, BDC mice are bred to NOD mice and the peripheral blood lymphocytes of litters are stained with anti-TCR Vβ4 and anti-CD4 for 20 minutes at 4°C and are then analyzed for transgenic expression of TCR Vβ4 on CD4+ T cells using a a Canto II cytometer (Becton Dickinson) and the data were analyzed using FlowJo software (Tree Star). This analysis identifies transgenic BDC littermates expressing the BDC2.5 TCR. All mice were housed at the Pennsylvania State College of Medicine specific pathogen-free (SPF) facility in accordance with Pennsylvania State Institutional Animal Care and Use Committee guidelines.
## Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
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<td><strong>Buffers, Solutions, and Media</strong></td>
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<tr>
<td>Ammonium-chloride-potassium (ACK) buffer</td>
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<td>cDMEM</td>
<td>DMEM supplemented with 10% (v/v) FCS, 100 units/ml penicillin, 100 units/ml streptomycin, 50 μM 2-mercaptoethanol, 10 mM HEPES, 1mM sodium pyruvate, and 1X MEM non-essential amino acids</td>
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<tr>
<td>1X YT media</td>
<td>8 g Bacto-tryprone, 5 g Bacto-yeast extract, 5 g NaCl, pH 7.2 adjusted to 1 L in ddH₂O</td>
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<tr>
<td>1X YT plates with 50 μg/ml ampicillin</td>
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<td>BDC2.5 mimotope peptide p79 (AVRPLWVRME) (242)</td>
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<td><strong>Plasmids</strong></td>
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<td>MGP (243)</td>
<td>Modified Mouse Stem Cell Virus with puromycin resistance marker (pMSCV) retroviral vector with Green fluorescent protein (GFP) placed downstream of 5’ LTR and miR cassette cloned downstream of GFP stop codon (O’connell PNAS 2009).</td>
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<td>miR-34a (187)</td>
<td>MGP vector with miR-34a sequence expression (Rao Immunity 2010).</td>
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<td>miR-34a-155</td>
<td>Unpublished, Gift from Dr. Dinesh S. Rao (University of California, Los Angeles).</td>
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<tr>
<td>Platinum-E (Plat-E) retroviral packaging cell line (244)</td>
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<td>GeneJammer Transfection Reagent</td>
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<td>Polybrene</td>
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**Splenocytes Used in Transduction**

Spleen cells from BDC mice were processed into single-cell suspensions using the end of a sterile 10 ml syringe plunger to gently process spleens through a 70 µm cell strainer into a 50 ml conical tube. Cells were then transferred to a 15 ml falcon tube. Red blood cells were lysed using 3 ml ACK buffer on ice for 7 minutes. Following lysis, 10 ml DMEM was added to the tube and cells were pelleted by
spinning for 6 minutes at 1490 RPM. Cells were then re-suspended in 10 ml cDMEM, counted using a phase contrast microscope with trypan blue exclusion and a hemocytometer, and kept on ice until use. Splenocytes used in experiments were from BDC mice and were stimulated with BDC2.5 mimotope p79.

DNA constructs

MGP (empty vector), miR-34a, and miR-34a-155 constructs were kind gifts from Dr. Dinesh S. Rao (University of California, Los Angeles). Plasmids were amplified by transducing Subcloning Efficiency DH5α Competent Cells, a commercially available competent E. coli cell line, exactly following the transformation protocol provided (Part No. 18265017.pps Rev 17 Jan 2006). Transformed E. coli were then grown overnight on 1X YT plates with 50 μg/ml ampicillin for selection overnight at 37°C and a single colony was picked for growth. Colony was then grown up in 5ml of 1X YT media with 50 μg/ml ampicillin for selection for 5 hours at 37°C shaking at 250 revolutions per minute (RPM). 5 ml culture was then added to 245 ml 1X YT media with 50 μg/ml ampicillin for selection and grown for 16 hours at 37°C shaking at 250 RPM. Following growth, plasmids were purified from transformed cells using a Qiagen Plasmid Maxi Kit following Qiagen Plasmid Maxi Kit protocol.

Transfection of bacteria
Transfection was performed using retroviral vector packaging cell line platinum-E (Plat-E) (244). Plat-E cells were originally generated from 293T cells and utilize the EF1α promotor. These cells can stably produce high retroviral titers as reported by Morita et al. 2000. 16 hours prior to transfection, 2 ml of 1.5 x 10^6 cells/ml of Plat-E cells in cDMEM were seeded onto 6-well cell culture plates (corning). MGP, miR-34a, and miR-34a-155 plasmids were transfected into cells following GeneJammer transfection reagent Instruction Manual Revision A.02 (Agilent Technologies, La Jolla, CA). Transfected Plat-E cells were then grown for 48 hours at 37°C and 5% CO₂ and 2 ml supernatants were drawn off using a pipette. Drawn off supernatants laden with retrovirus were immediately filtrated for purity using a 0.45 μm syringe filter prior to transduction step. 2 ml cDMEM was then added back to the same Plat-E cells and cells were again incubated at at 37°C and 5% CO₂ for an additional 24 hours, at which time supernatants were drawn off again and filtered again as above, and filtered virus laden supernatants were again used in second transduction step.

**Transduction of spleen cells**

Spleen Cells from BDC mice were seeded onto a 24-well plate (Corning) one day before transduction at a density of 1 x 10^6 cells/well in cDMEM and were stimulated with 0.1 μg/ml p79. Just prior to addition of virus supernatants, 1 ml of media was drawn off splenocytes and 1 ml virus supernatants supplemented with 4 μg/ml polybrene were added to splenocytes. Plate was then spun at 1400 RPM at
32°C for 1 hour for infection. Following this spin, the plate was incubated overnight at 32°C and 5% CO₂ and moved to 37°C and 5% CO₂ in the morning. This process was then repeated 24 hours later starting with the addition of the second set fresh of virus supernatants supplemented with 4 µg/ml polybrene for a second round of transduction. After the second transduction and ON incubation at 32°C and 5% CO₂, 1 ml viral supernatants still on cells were drawn off cells using a pipette and 1 ml cDMEM was added to cells. Cells were then split 1:2 to avoid overgrowth and grown at 37°C and 5% CO₂ in cDMEM for 3 more days to allow for miR-34a expression.

**Fluorescence-Activated Cell Sorting (FACS) Analysis and cell staining**

The transfected Plat-E cells were analyzed for GFP expression using flow cytometry to determine transfection efficiency. For transduction efficiency in individual cell populations, single-cell suspensions of transduced splenocytes (1 x 10⁶ cells/sample) were stained (20 min, 4°C) with CD4 and CD45R/B220 at a 1:100 dilution and were analyzed for GFP expression. Fluorescent staining and GFP expression was assessed on a Canto II cytometer (Becton Dickinson) and the data were analyzed using FlowJo software (Tree Star).

III. Results

i. **Plat-E cells can be efficiently transfected with miR-34a-expressing Plasmids**
We first established that we were able to effectively transfect a retroviral vector packaging cell line to produce viable retrovirus with bicistronic expression of a green fluorescent protein (GFP) reporter label and miR-34a. 48 hours following transfection of the GFP-expressing plasmids into the Plat-E retroviral packaging cell line. After 48 hours, we checked the transfection efficiency of the MGP, miR-34a, and miR-34a-155 plasmids in the transfected Plat-E cells by FACS analysis. GFP expression showed a transfection efficiency of ~50% for each of the plasmids (Table 6.1). In contrast, our no plasmid transfection control showed <1.5% GFP+ cells, verifying that our cell line was not expressing anything else that could be misinterpreted as GFP+ background staining (Table 6.1). These data show that Plat-E cells were efficiently transfected with our miR-34a-expressing vectors, which were functional as judged by GFP expression of the transfected cells.

ii. Lymphocytes can be Effectively Transduced with miR-34a.

BDC2.5 TCR transgenic splenocytes were used for transduction because they can easily be stimulated using p79 peptide to activate transgenic CD4+ T cells present in the culture. We also analyzed B220+ B cells because of the potential ability of activated CD4+ T cells to activate B cells. Splenocytes were analyzed 3 days after last transduction by FACS analysis to determine GFP expression in CD4+ T cells and B cells. Our analysis showed that both CD4+ T cells (Figure 6.2) and B220+ B
cells (Figure 6.3) could be efficiently transduced to express MGP, miR-34a, and miR-34a-155 based on the our GFP expression marker. Live-gated CD4+ T cells showed from 38-50% GFP expression (Figure 6.2), while B cells showed 18-28% GFP expression (Figure 6.3). Overall, these data demonstrate that miR-34a can be effectively expressed in both CD4+ T cells and B220+ B cells using the Plat-E retroviral packaging system with all constructs showing a >50% transduction.
Table 6.1: Plat-E cells can be efficiently transfected with miR-34a plasmids.

The transfection efficiency of the following GFP-labeled plasmids was FACS-analyzed 48 hours after transfection: MGP (empty vector), miR-34a, and miR-34a-155. A mock transfection with no plasmid added was also done as a control. Percentages representing the proportion of total GFP+ cells at 48 hours are shown. Data is representative of two independent experiments.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plat-E Cells Expressing GFP [%]</th>
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<tr>
<td>No plasmid</td>
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<tr>
<td>miR-34a-155</td>
<td>51.2</td>
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Figure 6.1: CD4+ T cells can be effectively transduced to express bicistronic mir34a-GFP plasmids using a retroviral vector delivery system.

FACS analysis was done to determine the transduction efficiency of each of the following GFP-labeled retrovirally-transduced genes in BDC CD4+ T cells: MGP, miR-34a, miR-34a-155. A no virus control was also included. Gating of live cells (1st column), CD4+ T cell gating (2nd column), and percentage of GFP-expressing CD4+ T cells (3rd column) is shown. Data is representative of two independent experiments.
Figure 6.2: B220+ B cells can be effectively transduced to express bicistronic mir34a-GFP plasmids using a retroviral vector delivery system.

FACS analysis was done to determine the transduction efficiency of each of the following GFP-labeled retrovirally-transduced genes in BDC B220+ B cells: MGP, miR-34a, miR-34a-155. A no virus control was also done. Gating of live cells (1st column), B220+ B cell gating (2nd column), and percentage of GFP-expressing B220+ B cells (3rd column) is shown.
IV. Discussion

These data demonstrate that the Plat-E system can be used to efficiently express GFP indicative of miR-34a expression in the CD4+ T cells and B cells of BDC mice. This is a first step towards our goal of expressing miR-34a in the bone marrow cells of these mice and NOD mice to see if we can test our hypothesis that constitutive miR-34a expression results in Foxp1 repression, B cell paucity, and T1D protection.

Since retroviruses only infect cycling cells, it was not unexpected to see that p79-activated CD4+ T cells could be transduced. Unexpectedly, B220+ B cells that do not recognize p79 were also effectively transduced in these experiments. B cell transduction in p79-stimulated BDC spleen cell cultures could be due to co-stimulation through their CD40-CD40L interactions with activated CD4+ T cells upregulating CD40L. The ability to transduce B cells with miR-34a is intriguing because B cell precursors will be our ultimate target population in the bone marrow.
Chapter VII: Overall Discussion and Future Directions

There are greater than 25 different Insulin-dependent diabetes (Idd) regions throughout the genome that contribute to T1D protection or induction (99). In many cases, the mechanism(s) that underlie how these regions confer protection and which genes on these regions are responsible have yet to be elucidated. Functional genomics with congenic NOD mice containing the T1D-resistant Idd regions from T1D-resistant strains of mice have been used to determine how these genetic regions are contributing to T1D protection. This approach has been successful in studying congenic NOD mice containing the C57BL/6 or C57BL/10-derived Idd3 or Idd10, respectively. For Idd3, functional analyses have revealed that differential expression of interleukin (IL)-2 and its effect on Treg function was responsible for disease (245). For Idd10, functional analyses revealed CD101 as a candidate gene on Idd10 likely responsible for T1D protection in congenic mice (246). We have examined the effect(s) of Idd9 and its subregion Idd9.3 on T1D protection in BDC-Idd9.905 mice and NOD.B10 Idd9.3 mice, respectively.

Although Idd9 and sublocus Idd9.3 from the T1D-resistant C57BL/10 mice confer significant protection from T1D in congenic NOD mice (102), the cellular and molecular mechanisms of protection remain unknown.

The overall goal of this thesis was to address gaps in our knowledge concerning the role of Idd9 and sublocus Idd9.3 in T1D protection and to identify candidate gene(s) within these loci that contribute to this protective effect. Specifically, we investigated the role of Idd9 on diabetogenic CD4+ T cell function
and the role of Idd9.3 in T1D protection. The following sections address the implications of our findings and how they have added to the understanding of T1D pathogenesis as shown in our working models (Figure 8.1 and 8.2). Plans for our further investigation and our gaps in knowledge are also discussed.

*Idd9.3-encoded MicroRNA-34a as a T1D candidate gene*

*Idd9.3 and B cell development*

Congenic NOD mice containing the Idd9.3 locus from T1D-resistant C57BL/10 mice (referred to as NOD.B10 Idd9.3 mice) show a 40% reduction in T1D incidence compared to NOD mice (102). Our goal was to determine the mechanisms by which B10 Idd9.3 conferred protection in these mice. Our initial studies included characterization of NOD.B10 Idd9.3 mouse immune cell populations and we found a profound reduction in splenic and pancreatic lymph node (PLN) peripheral B cells as compared to NOD mice. This B cell paucity was of primary interest because B cells are necessary for T1D development in NOD mice through their function as antigen-presenting cells (APCs)(188). This finding focused our investigation on early steps in B cell development in the bone marrow, where we found a partial B cell developmental block at the pro- to pre-B cell transition and a reduction in every subsequent B cell developmental stage, including those in the periphery: Transitional (TR), B1, Marginal Zone (MZ), and Follicular (FO) B cells. In addition, bone marrow chimeras revealed that the partial B cell developmental block was B
cell intrinsic, allowing us to further investigate what gene(s) on *Idd9.3* mediated this partial developmental block.

Our observations of reduced B cell numbers was a novel finding in NOD.B10 *Idd9.3* mice and was an important focal point for studies on T1D regulation because an *Idd* region had not been previously shown to deplete B cells.

**Idd9.3-encoded miR-34a and it's repression of Foxp1**

MiR-34a is one of the 19 genes encoded by *Idd9.3*. In 2010, David Baltimore and colleagues showed that retroviral over-expression of miR-34a led to a defect in B cell development at the pro- to pre-B cell transition due to its binding and repression of the critical B cell transcription factor Foxp1 (182, 187). Since we discovered a partial B cell developmental block at the pro- to pre-B cell transition in NOD.B10 *Idd9.3* mice, and miR-34a is encoded by *Idd9.3*, we hypothesized that increased expression of miR-34a and its repression of Foxp1 may be responsible for the partial B cell developmental block in T1D-resistant NOD.B10 *Idd9.3* mice. Our studies in data chapter V revealed that miR-34a is indeed significantly upregulated and Foxp1 levels inversely correlate with miR-34a expression and are subsequently decreased in NOD.B10 *Idd9.3* pro- and pre-B cells. Interestingly, we also showed that miR-34a was significantly upregulated in NOD.B10 *Idd9.3* follicular (FO) B cells, which are a more mature peripheral population of B cells. This suggests that while miR-34a is critical for B cell development at earlier stages, it may play another unique role in mature B cell function.
The increase in miR-34a expression and subsequent reduction in Foxp1 levels in NOD.B10 ldd9.3 pro- and pre-B cells is a novel finding and is the likely mechanism behind the partial B cell developmental block seen in these mice. This finding is significant because we were able to show that increased expression of ldd9.3-encoded miR-34a correlated with a paucity of mature B cells in NOD.B10 ldd9.3 mice. This subsequent lack of B cells as APCs may contribute to protection from T1D in NOD.B10 ldd9.3 mice. Previous studies of ldd9.3 did not reveal miR-34a as a candidate gene in T1D development T1D (102, 128, 130), making this discovery a novel addition to our understanding of how ldd9.3 contributes to T1D.

**Our findings and previous reports**

ldd9.3 also contains Tnfrsf9, which encodes the inducible T cell co-stimulatory molecule CD137 and has 3 coding variants between B10 and NOD mice (102). CD137 has been the subject of several T1D candidate gene studies. One such study reported that B10 ldd9.3 is responsible for increased anti-Smith (Sm) autoantibody production in NOD mice with the authors concluding that this is likely due to CD137 (130). Alternatively, or in addition to CD137, increased Sm autoantibody production in NOD.B10 ldd9.3 mice could be due to the miR-34a-mediated paucity of B cells in these mice. In fact, B cell lymphopenic A/WySn mice also show increased Sm autoantibody production (247). B-cell lymphocytopenia has also been observed in patients with active SLE and is considered a potential prognostic marker in active disease (248). While a recent study reported that B10-derived ldd9.3 promotes the accumulation of CD137+ regulatory T cells (Tregs) in
aged NOD.B10 ldd9.3 mice as compared to NOD mice (128), it is possible that the drastic reduction of peripheral B cells in these mice could be playing a role in Treg expansion. This explanation is reasonable since our findings suggest that there are less diabetogenic B cells to function as APCs in the periphery of NOD.B10 ldd9.3 mice, which may have an effect on Treg expansion as compared to NOD mice. Also, in a mouse model of rheumatoid arthritis, it has been shown that B cell depletion enhances Treg numbers and function (249), suggesting that a reduction in B cells could increase Treg numbers similar to what is seen in NOD.B10 ldd9.3 mice. Therefore, the previous findings in NOD.B10 ldd9.3 mice would fit with the B cell deficient phenotype we have found.

**A reduction in B cell numbers and function and its effect on T1D**

B cells are critical APCs for T1D development in NOD mice (188). In addition, B cell ablation in NOD mice leads to T1D prevention and reversal (29). We therefore investigated whether this B cell paucity in NOD.B10 ldd9.3 mice led to protection from T1D development. *In vitro* assays were done to determine whether equal numbers of CD19+ B cells from T1D-resistant NOD.B10 ldd9.3 mice were less effective APCs than their NOD B cell counterparts. The results showed that NOD.B10 ldd9.3 CD19+ B cells were less effective at stimulating diabetogenic CD4+ T cells as compared to NOD. We also found that BDC CD4+ T cells proliferated almost 4-fold less in the PLN of NOD.B10 ldd9.3 mice as compared to NOD PLNs, presumably due to the ~9-fold reduction in the frequency of NOD.B10 ldd9.3 B cells. The novelty of this finding is that miR-34a may regulate T1D development by its role in the
development of B cells, due to their role as APCs in priming of islet-specific CD4+ T cells, revealing a likely mechanism behind T1D protection in NOD.B10 Idd9.3 mice. This finding has added to the field of T1D research because it has pointed out a previously unknown mechanism in which Idd9.3-encoded miR34a may contribute to T1D protection. Figure 8.1 lays our working model for this project.

Future directions

Our hypothesis is that NOD.B10 Idd9.3 mice are protected from T1D because of their profound paucity of peripheral B cells. This B cell paucity is directly influenced by increased expression of miR-34a in NOD.B10 Idd9.3 pro- and pre-B cells and subsequent downregulation of Foxp1 as compared to NOD mice. To test this hypothesis, we have started using a retroviral vector gene delivery system to increase miR-34a expression in pro- and pre-B cells in NOD mice. The studies describing the system for this project are presented in data chapter VII. We predict that if we increase expression of miR-34a in NOD bone marrow, mature NOD B cells contributing to T1D pathogenesis will be significantly reduced, resulting in significant T1D protection. We would specifically look at miR-34a expression and Foxp1 levels in pro- and pre-B cells to confirm that increased miR-34a expression leads to decreased Foxp1 levels in these early B cell progenitors, leading to a B cell developmental block. We could then look at the incidence of T1D in these same NOD mice to see if this reduction in B cells leads to a significant drop in the incidence of T1D.
If we increase miR-34a expression in NOD pro- and pre-B cells, but we do not see a reduction in Foxp1 levels, it is possible that another gene on B10 Idd9.3 is responsible for the reduction of Foxp1 levels seen in NOD.B10 Idd9.3 pro- and pre-B cells. Also, if we find that an increase in miR-34a does decrease Foxp1 levels, but this does not lead to B cell paucity like that observed in NOD.B10 Idd9.3 mice, then it is likely that another gene on B10 Idd9.3 besides miR-34a is regulating B cell development. Lastly, if upon increased miR-34a expression, we do see a reduction in Foxp1 levels and a subsequent reduction in peripheral B cells, but this does not lead to a drop in the incidence of T1D, this would indicate that B10 Idd9.3 is mediating T1D protection via another mechanism independent of B cell regulation. All of these results would re-direct our focus towards other potential candidate genes on B10 Idd9.3.

Figure 8.1 shows our working model for how increased miR-34a expression likely leads to T1D protection in NOD.B10 Idd9.3 mice. The experiments proposed above would prove that increased miR-34a levels can indeed lead to T1D protection through their influence in Foxp1 levels and subsequent B cell development.

An important question that remains to be answered is whether B cell subset differences between NOD.B10 Idd9.3 and NOD mice are playing a role in T1D susceptibility. Further characterization of the APC function of B cell populations that reach the periphery could support our findings that B cells in NOD.B10 Idd9.3 mice are less efficient APCs in the stimulation of diabetogenic CD4+ T cells. For example, if one particular B cell subset that is drastically reduced in NOD.B10 Idd9.3 mice, (e.g. MZ B cells which were ~33 fold reduced compared to NOD mice), is
experimentally shown to be more effective APCs than another B cell subset, this would reveal how the preferential reduction of one B cell subset leads to T1D protection in NOD.B10 Idd9.3 mice. It is possible that the reduction of specific subpopulations of B cells more capable of activating these T cells, such as MZ B cells which have been shown to have a significant role in T cell activation (250), could be responsible for the observed APC difference.

Another interesting observation to investigate would be why miR-34a levels were significantly downregulated and Foxp1 expression was upregulated in NOD.B10 Idd9.3 FO B cells as compared to NOD FO B cells. This was the opposite trend of what was seen in the pro-and pre-B cell populations of NOD.B10 Idd9.3 mice. Since FO B cells are in the periphery and well-beyond the developmental stages of pro- and pre-B cells, the role of miR-34a and Foxp1 is likely completely different in this cell population. It would be interesting to see whether these miR-34a and Foxp1 differences influence the function of FO B cells as APCs in NOD.B10 Idd9.3 mice as compared to NOD mice. It is possible that significantly decreased miR-34a and increased Foxp1 in NOD.B10 Idd9.3 FO B cells leads to impaired APC function by these B cells. If this were the case, it would be another plausible explanation as to why NOD.B10 Idd9.3 B cells are less efficient APCs as compared to NOD B cells. These possibilities could be explored by sorting each individual B cell subpopulation and then testing their APC function in stimulation of diabetogenic CD4+ T cells in vitro. This could be done by purifying each B cell subset from NOD.B10 Idd9.3 mice and NOD mice and then using them as APCs to present antigen to diabetogenic BDC CD4+ T cells. These experiments would reveal whether the
protection in NOD.B10 Idd9.3 mice is due to differences in B cell subset numbers, or whether the B cell subsets themselves are less efficient APCs, even when they are normalized for numbers.

If we identify one particular subset of B cells that is functioning as diabetogenic APCs from the in vitro studies, we could potentially do adoptive transfer experiments with the identified B cell subset and diabetogenic CD4+ T cells to see how the purified B cell subset influences T1D development in vivo. We could do this by purifying the B cell subset of interest, such as MZ B cells, from NOD.B10 Idd9.3 and NOD mice and then transferring this purified population with diabetogenic BDC CD4+ T cells into NOD SCID recipients and then monitoring them for T1D development. In the case of MZ B cells, if the reduction in numbers of this B cell subset in NOD.B10 Idd9.3 mice is responsible for T1D protection in these mice, when we normalize for numbers of MZ B cells, we should see equivalent T1D incidences in NOD SCID mice receiving BDC CD4+ T cells and either NOD.B10 Idd9.3 or NOD MZ B cells. Conversely, if the MZ B cells themselves are impaired in their function as APCs, this same experiment should reveal that NOD.B10 Idd9.3 MZ B cells are inefficient APCs as compared to NOD MZ B cells, even when they are provided as APCs in equal numbers. This same approach could be done with any of the B cell subsets of interest.

In addition to further defining B cell subsets, we would also like to determine the antigen presentation abilities and co-stimulatory abilities of NOD.B10 Idd9.3 peripheral B cells as compared to NOD B cells. It is possible that the differences in APC function between these two strains is due to differences in MHC II (I-A<sup>$^8$</sup>)
expression. Defining MHC II expression is currently one limitation in this system because while there are antibodies that cross-react with I-A^q7, an adequate antibody to specifically identify I-A^q7 has remained difficult to generate. If NOD.B10 ldd9.3 B cells express less MHC II, this could contribute to their lack of efficient function as APCs. Differences in co-stimulatory molecule expression, such as CD40, between NOD.B10 ldd9.3 and NOD B cells could also be responsible for the lack of NOD.B10 ldd9.3 B cell APC functionality. If NOD.B10 ldd9.3 B cells express less of the T cell co-stimulatory molecule CD40, this could be another reason why these B cells are less able to activate diabetogenic CD4+ T cells.

**Implications of our findings**

*What our findings have added to the field of T1D research*

While B cell knockouts and depletions have been shown to ameliorate T1D (29, 188), we present the first reported case where a mouse spontaneously eliminates most of its B cells as a protective mechanism against T1D development. We have also discovered that this unique regulatory mechanism is likely via the upregulation of miR-34a on ldd9.3. In addition to there being a paucity of B cells in these T1D-protected mice, the B cells that do escape to the periphery operate as less functional APCs in the stimulation of diabetogenic CD4+ T cells. These findings suggest that the B cells depleted by increased miR-34a expression may be the diabetogenic B cells mediating T1D.

*Therapeutic potential of our findings*
To date, the only therapeutic option for T1D treatment has been exogenous insulin. In addition, the overall incidence of disease is on the rise for unknown reasons, further necessitating new treatment options. New knowledge of disease processes is critical for the development of new and novel therapies. B cell depletion therapy may be one such new and viable option to be considered for T1D treatment. Therapies such as rituximab seem to have some effect in T1D clinical trials (33). While rituximab is useful, the B cells it targets may not be the most therapeutically relevant in T1D. For example, MZ B cells play an important role in T1D, but this population is not effectively depleted by rituximab (251).

Instead, or in addition to rituximab, miR-34a targeted therapies may be a viable alternative for B cell depletion. As gene therapy becomes more commonplace, many therapies that specifically target and regulate aberrant gene expression to ameliorate disease may likely replace current less nuanced methods of treatment. Other systems for delivery of miR-34a could include liposome-based delivery of miR-34a to the bone marrow. miR-126 (an angiogenesis promoter) has been successfully delivered to mice for the treatment of hind limb ischemia (252). In fact, miRNA-based therapies are being considered for many disorders ranging from antiviral interventions to chronic inflammatory diseases (253). It is also likely that a miR-34a-based B cell depleting therapy would be effective in B cell-mediated diseases such as SLE.
FIGURE 8.1: The effect of B10 Idd9.3 on B cell development and T1D.

Increased expression of miR-34a in Pro B cells from NOD.B10 Idd9.3 mice as compared to NOD mice leads to a decrease in Foxp1 expression. This decrease in Foxp1 expression leads to a decrease starting at the pro- to Pre-B cell transition. Pre-B cells continue through development and leave the bone marrow and go to the periphery, including the pancreatic lymph nodes (PLN), where they can function as APCs to prime diabetogenic CD4+ T cells. Since NOD.B10 Idd9.3 mice have a significant reduction in B cells that can function as APCs in activating diabetogenic CD4+ T cells and impaired B cell function as APCs, NOD.B10 Idd9.3 mice show a significant reduction in T1D.
NOD

Pro-B cells → Pre B cells

NOD/Ldd9.3

Ch. 4

miR-34a

NOD

B10/Ldd9.3

Pre B cells

miR-34a

Foxp1

BONE MARROW

PLN

Mature B cells

Continued Development

Diabetogenic CD4+ T cell

Substantiation

T1D

NOD.B10 Ldd9.3

Pre B cells

Continued Development

Less/NO T1D
Idd9 contains novel candidate genes Eno1, Rbbp4, and Mtor that are significantly down-regulated in less diabetogenic BDC-Idd9.905 CD4+ T cells.

The genetic expression of Idd9-encoded genes in islet-specific CD4+ T cells

While it was previously shown that Idd9 from T1D-resistant C57BL10SnJ mice is able to confer almost complete protection from T1D in congeneric NOD mice (102), the mechanisms and genes responsible for this protection are currently poorly defined. Since CD4+ T cells are known to play an integral role in T1D development (23, 64), the overall goal of this study was to identify genes and molecular pathways influencing the function of islet-specific CD4+ T cells. To do this, we generated a novel strain of mice BDC-Idd9.905 mice, which are BDC2.5 TCR transgenic NOD (BDC) mice homozygous for the Idd region from C57BL/10SnJ mice. These mice almost exclusively contain CD4+ T cells transgenically expressing the BDC2.5 TCR that also express the T1D-resistant B10 Idd9 locus, allowing us to investigate how the expression of B10 Idd9 in these CD4+ T cells influences their function as compared to the same cells from BDC control mice that contain the NOD-derived Idd9 region. This comparison allowed us to compare the NOD Idd9 (T1D-susceptible) vs. B10 Idd9 (T1D-resistant) genotype to the CD4+ T cell and T1D phenotype.

Microarray analyses of purified BDC and BDC-Idd9.905 CD4+ T cells identified differentially expressed genes between the two strains in the unstimulated (ex-vivo) and BDC2.5 TCR mimotope peptide (p79)-stimulated
conditions, which we then analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) analysis to identify gene clusters of differentially expressed genes, and Ingenuity Pathway (IP) analysis to identify gene networks, in T cells. Both analysis methods identified clusters and networks of differentially expressed genes involved in cellular proliferation, growth and differentiation. In p79-stimulated cells, IP analysis revealed Mtor as the only differentially expressed gene within the ldd9 region, exhibiting a decreased expression in BDC-lldd9.905 as compared to BDC CD4+ T cells. This observation is significant because it has been previously shown that treatment of NOD mice with mTOR protein inhibitor rapamycin leads to protection from T1D (254). This latter finding agrees with our data because activated BDC-lldd9.905 CD4+ T cells expressing less Mtor than BDC CD4+ T cells are also less diabetogenic. Interestingly, a microarray analysis of B cells from T1D-resistant NR4 mice as compared to NOD mice also revealed that Mtor is downregulated in NR4 B cells, potentially revealing a more global role for Mtor in T1D protection (255).

In ex-vivo studies, IP analysis of cells revealed gene networks involved with cellular growth and proliferation as well as hematological system development and function. These studies identified two genes in the ex vivo group on the ldd9 region, Eno1 and Rbbp4. Eno1 was also identified as differentially expressed in CD4+ T cells from parental NOD.B10 ldd9.3 mice, verifying our findings (104). IP analysis also suggested that Eno1 and Rbbp4 were responsible for the observed significant upregulation of Agtrap and a downregulation of 2610305D13Rik, respectively, through their gene network interactions. Both Agtrap and 2610305D13Rik are
located outside of *Idd9*, suggesting that differentially expressed genes on the *Idd9* affect several genes and gene pathways outside of the *Idd9*. Stolp et al. also showed *2610305D13Rik* is highly downregulated in B cells from T1D-protected NR4 mice as compared to NOD mice. While the function of *2610305D13Rik* is currently not known, the differential expression seen in both B cells (125) and CD4+ T cells from T1D-protected NOD congenic mice versus T1D-proned NOD mice strongly suggests a role *2610305D13Rik* in T1D development. This observation was also found in p79-stimulated cells where IP analysis identified differentially expressed genes associated with cellular development and hematological system development and function located outside *Idd9*. Differentially regulated genes outside *Idd9* included Cd200, Cd226 and *Csf2* which were downregulated, and *Ly6a*, which was upregulated in BDC-*Idd9.905* CD4+ T cells. Overall, these analyses showed that genes involved in cellular growth and development, function, and proliferation in *ex vivo* and p79-stimulated BDC-*Idd9.905* CD4+ T cells were less abundantly expressed as compared to BDC CD4+ T cells.

**Linking B10 Idd9 to CD4+ T cell diabetogenicity**

In vitro functional assays revealed that BDC-*Idd9.905* CD4+ T cells proliferated less in response to p79 stimulation. Furthermore, stimulation led to a reduced pro-inflammatory response with less IFN-gamma and IL-17 production in BDC-*Idd9.905* CD4+ T cells as compared to BDC T cells. This reduction in proliferation was also seen in vivo when BDC-*Idd9.905* CD4+ T cells proliferated less in response to endogenous antigen in the PLN compared to BDC CD4+ T cells.
Finally, the ability of BDC-Idd9.905 CD4+ T cells to transfer T1D was greatly reduced when compared to BDC CD4+ T cells, indicating that expression of differentially expressed genes on B10 Idd9, such as Eno1, Rbpb4, and Mtor contributed to lack of CD4+ T cell diabetogenicity.

In summary, these data led us to our overall model that BDC-Idd9.905 CD4+ T cells harboring the T1D-resistant B10 Idd9 show downregulation of Id9 candidate genes Eno1, Rbpb4, and Mtor, which directly leads to less growth and development and less diabetogenic potential in response to autoantigen when compared to BDC CD4+ T cells. See figure 8.2 for our working model.

**Future Directions**

Studies in this thesis suggest that the newly identified differentially expressed Id9 T1D candidate genes Eno1, Rbpb4, and Mtor are responsible for T1D in NOD mice. In support of this hypothesis, we plan to conduct a series of knockdown experiments to decrease levels of Eno1, Rbpb4 and Mtor in BDC CD4+ T cells and compare outcomes to levels seen in BDC-Idd9.905 CD4+ T cells. One strategy would be to use short hairpin RNAs that bind and silence the Eno1, Rbpb4, and Mtor transcripts, which would be done for each individual gene and for each gene combination. If Eno1 and Rbpb4 expression are the genes responsible for the differences seen between BDC and BDC-Idd9.905 CD4+ T cell function, we would predict that BDC CD4+ T cells with Eno1 and Rbpb4 downregulated would be less diabetogenic, would produce less pro-inflammatory IFN-gamma and IL-17, and would cause less disease when transferred into NOD SCID mice. In activated CD4+ T
cells, we could also knock down \textit{Mtor} expression \textit{in vitro} using \textit{Mtor} inhibitors such as rapalogs, immediately following activation and see whether this reduction in expression in BDC CD4+ T cells decreased their pro-inflammatory phenotype. Follow-up experiments would include \textit{in vivo} studies to investigate the impact of knocking down \textit{Mtor} on overall BDC CD4+ T cell diabetogenicity.

Alternatively, we could over-express \textit{Eno1} and \textit{Rbbp4} in BDC-\textit{Idd9}.905 CD4+ T cells and determine whether their pro-inflammatory cytokine production and diabetogenicity increases to the levels seen in BDC CD4+ T cells. Presumably, if \textit{Eno1} and/or \textit{Rbbp4} are the genes responsible for the diabetogenic effect in BDC CD4+ T cells, then upregulation of one or both of these genes should make BDC-\textit{Idd9}.905 CD4+ T cells function more like BDC CD4+ T cells. We would also predict that if decreased \textit{Eno1} is responsible for the upregulation of \textit{Agtrap}, then overexpression of \textit{Eno1} in BDC-\textit{Idd9}.905 CD4+ T cells should decrease \textit{Agtrap} expression in these same cells. Likewise, if decreased \textit{Rbbp4} expression is responsible for reduced expression of \textit{2610305D13Rik}, then overexpression of \textit{Rbbp4} in BDC-\textit{Idd9}.905 CD4+ T cells should increase levels of \textit{2610305D13Rik}. In fact, \textit{2610305D13Rik} would be an extremely sensitive indicator to test the regulatory function of \textit{Rbbp4}, because if the relationship we have hypothesized between \textit{Rbbp4} and \textit{2610305D13Rik} based on IP gene network analysis is true, then a very small increase in expression of \textit{Rbbp4} should lead to significant expression changes in \textit{2610305D13Rik}, since a 2-fold expression difference in \textit{Rbbp4} corresponds with a 1250-fold difference in \textit{2610305D13Rik}. 

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Mtor could also be over-expressed in activated BDC-idd9.905 CD4+ T cells to determine whether over-expression in this state can alter the diabetogenicity of BDC-idd9.905 CD4+ T cells. The over-expression of Eno1 and/or Rbbp4 in ex vivo CD4+ T cells or Mtor in activated CD4+ T cells could be accomplished using a retroviral vector delivery system similar to the system that we have used in data chapter VI. These experiments would be needed to validate Eno1, Rbbp4, and Mtor as candidate genes in T1D.

An additional experimental strategy to validate identified candidate genes would include the development of a transgenic mouse or a knockout mouse strain to determine the effect of candidate gene over-expression or deletion. This approach could be used with each one of the individual gene candidates, or with combinations of the three identified candidate genes. These experiments would follow up the initial over-expression and knockdown studies using short hairpin RNAs and retroviral vector delivery systems done in BDC and BDC-idd9.905 CD4+ T cells. Transgenic and knockout mouse experimental models would be most informative if they were tissue-specific and the candidate gene(s) being studied were only modified in CD4+ T cells. Tissue-specific expression solely in CD4+ T cells would limit the possibility that gene expression in APC populations, rather than CD4+ T cells, is influencing the diabetogenic potential of the transgenic CD4+ T cell population being studied. If modulation of any of our candidate genes leads to developmental defects in our mice, we could alternatively use a conditional knockout, such as the cre-lox system. Candidate gene over-expression using a transgenic model would be most interesting in BDC-idd9.905 CD4+ T cells since these
T cells showed significant decreases in expression of identified candidate genes. Conversely, CD4+ T cell-specific gene knockout experiments would likely be most informative in the BDC group since these T cells showed a significant increase in candidate gene expression.

If *Eno1, Rbbp4*, and *Mtor* are indeed responsible for CD4+ T cell diabetogenicity, we would expect that over-expression of these genes in BDC-Idd9.905 CD4+ T cells would lead to increased diabetogenicity. This would lead to a greater pro-inflammatory response and greater proliferation in response to autoantigen, as well as increased T1D incidence in NOD SCID mice upon CD4+ T cell transfer. Conversely, knockouts of these genes in diabetogenic BDC CD4+ T cells should ameliorate their diabetogenic potential. It is possible that each gene is solely able to influence T cell diabetogenicity, or that all three candidate genes need to be over-expressed or knocked out together to have an effect on disease. The diabetogenic potential of each gene would need to be tested individually, which would be followed up using models looking at combinations of *Eno1, Rbbp4*, and *Mtor* on diabetogenic potential. Using these methods, we could validate identified candidate gene by directly linking their individual expression in CD4+ T cells to diabetogenicity.

Figure 8.2 shows our working model for how decreased expression of *Eno1, Rbbp4*, and *Mtor* in BDC-Idd9.905 CD4+ T cells likely leads to less growth and development, less proliferation, and less diabetogenic potential. The experiments planned above would definitively address whether the expression of these
candidate genes are directly linked to less diabetogenic phenotype seen in BDC-
Idd9.905 CD4+ T cells.

While we have identified that BDC-Idd9.905 CD4+ T cells (containing the B10
Idd9) show a significant decrease in Eno1 and Rbbp4 in the ex vivo group and Mtor in
the activated group as compared to BDC CD4+ T cells (containing the NOD Idd9), we
still do not know what regulates these gene expression differences. It would be of
great interest to know if there are regulatory elements such as promoters or
enhancers on the NOD Idd9 that are leading to an increase in the expression of these
candidate genes as compared to the B10 Idd9. While this region has been sequenced,
the high degree of sequence variation between between the NOD and C57BL/10
Idd9 has made identifying specific variations having an influence on gene expression
difficult [104]. More work needs to be done to identify specific genetic elements
contributing to gene regulation.

Other interesting experiments would include further use of a bioinformatics
approach to link each of the differentially expressed non-Idd9 genes back to their
interaction with Idd9. Since the BDC and BDC-Idd9.905 mice differ only by their
source of Idd9, we can assume that all the other genes differentially expressed must
be regulated, directly or indirectly by Idd9. Knowing more about these interactions
could shed light on additional disease pathways other than those identified by IP
analysis.

**Implications of our findings**

*What our findings have added to the field of T1D research*
We are the first group to report on the influence of Idd9 in islet-specific CD4+ T cells and have found that the expression of T1D-resistant B10 Idd9 influences the global expression of a relatively small number of genes. Our data have uncovered several novel differentially regulated genes likely influencing the function and diabetogenic potential of islet-specific CD4+ T cells from BDC-Idd9.905 mice as compared to BDC mice. On the Idd9 region, we have identified 3 new novel candidate genes: Enol1 and Rbbp4 in ex vivo CD4+ T cells and Mtor in activated CD4+ T cells that likely regulate diabetogenic CD4+ T cell function.

Our identification of the downregulation of Enol1 in less diabetogenic BDC-Idd9.905 CD4+ T cells is intriguing because of its previously identified role in autoimmunity (see chapter V discussion). Our data now links Enol1 to the function of diabetogenic CD4+ T cells in T1D. We have also identified Rbbp4 as a candidate gene on Idd9 due to its downregulation in BDC-Idd9.905 CD4+ T cells as determined by Real-time RT-PCR. We also linked Rbbp4 expression via IP analysis to chemokine receptor 2 (Ccr2), an important gene in T helper cell (Th)1 differentiation (see chapter V discussion for additional details). Therefore, our analysis reveals a previously unreported role for Rbbp4 in T1D. The identification of the downregulation of Mtor in BDC-Idd9.905 CD4+ T cells upon activation was also a novel finding revealed in our analyses. This finding was also intriguing because Mtor deficiency leads to preferential CD4+ T cell differentiation into Tregs instead of Th1, Th2, or Th17 effector cells. Our data shows less differentiation of stimulated BDC-Idd9.905 CD4+ T cells into Th1, Th2, or Th17 cells based on IFN-gamma, IL-4, and IL-17, respectively.
While we have identified 3 new candidate genes on \textit{Idd9}, the expression of B10 \textit{Idd9} in BDC CD4+ T cells also leads to far-reaching regulatory changes outside the \textit{Idd9} region. In fact, only 6 of the 55 differentially regulated genes identified in the \textit{ex vivo} group and 9 out of 80 genes in the p79-activated group were located on \textit{Idd9}, indicating that the expression of B10 \textit{Idd9} in BDC CD4+ T cells has profound genome-wide effects (see chapter V discussion for details).

The findings presented in this thesis have far-reaching implications in the understanding of T1D pathogenesis and islet-specific CD4+ T cell functions because the genetic profile of BDC-\textit{Idd9.905} as compared to BDC CD4+ T cells shown by our microarray analysis is a genetic blueprint revealing the genetic regulation that can make CD4+ T cells less diabetogenic. Now that these genes have been identified, we can also investigate whether they also play role in human T1D. It will also be interesting to determine if they play a more global role in CD4+ T cell dysregulation. We have identified novel pathways such as the link between \textit{Eno1} and \textit{Agtrap} that could be playing a significant role in the diabetogenicity of CD4+ T cells. We have also identified the downregulation of genes on \textit{Idd9} known to be important CD4+ T cell regulation, such as \textit{Mtor}. These findings move the T1D field forward by adding new knowledge about how a known T1D-contributing locus, \textit{Idd9}, regulates one of the most important cell types in T1D pathogenesis, the diabetogenic CD4+ T cell. The study also validates our approach to find candidate genes in other polygenic disorders.

\textit{Therapeutic Implications}
We identified novel genes implicated in the development of, or protection from T1D. Our studies revealed that B10-mediated downregulation of *Eno1*, *Rbbp4*, and *Mtor* correlates with a less diabetogenic phenotype in BDC-*Idd9.905* CD4+ T cells. These observations clearly lead to studies on whether these genes are upregulated in the CD4+ T cell populations of human T1D patients as compared to those without T1D. One non-*Idd9* gene identified in our analysis as upregulated in diabetogenic BDC CD4+ T cells, *Cd226*, has also been identified by GWAS as a human T1D candidate gene (256). Since human GWAS studies were not done on CD4+ T cells, there might be additional genes in common between human and mouse studies that have not yet been identified. If the candidate genes we have identified also play a role in human T1D, then their efficacy as targets in gene therapy could be conducted in our mouse T1D models.
FIGURE 8.2: The effect of *Idd9* on diabetogenic CD4+ T cell function and T1D.

Decreased expression of candidate genes *Rbbp4* and *Eno1* in ex vivo and *Mtor* in activated BDC-*Idd9.905* as compared to BDC CD4+ T cells leads to decreased growth and development and decreased diabetogenicity (pink) in activated BDC-*Idd9.905* CD4+ T cells as compared to activated BC CD4+ T cells (red). This decreased diabetogenicity in CD4+ T cells leads to less diabetogenic BDC-*Idd9.905* CD4+ T cells and a significantly reduced ability to cause T1D.
**BDC (100% NOD background)**

**CD4+ T cell**

**BDC-Idd9 (Idd9 from T1D-resistant C57BL/10 background)**

**CD4+ T cell**

= More Diabetogenic

= Less Diabetogenic
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CURRICULUM VITA
GREGORY J. BERRY

EDUCATION/TRAINING
2009-2014 **Ph.D. in Microbiology and Immunology**, Pennsylvania State College of Medicine, Hershey, PA
2004-2007 **B.S. in Biology**, East Stroudsburg University, East Stroudsburg, PA
*Summa Cum Laude*, Graduate of ESU Honors Program

AWARDS, HONORS, AND SCHOLARSHIPS
AAI Young Investigator Award, Upstate New York Immunology Conference, 2013
Sigma Xi Award, Sigma Xi Research Society of ESU, 2007
Presidential Status, ESU Society for Leadership and Success, 2007
China Study Abroad Scholarship, Office of the Provost, 2006
Honors Program Scholarship, ESU Honors Program, 2006

PUBLICATIONS
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SELECTED ABSTRACTS