MODULATION OF IMMUNE RESPONSES BY GUT RESIDENT MICROBIOTA AND IMPLICATIONS IN TYPE 1 DIABETES ANIMAL MODELS

By

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To my family, Patrick, Patricia, and Joanna Lau
Your support is unending

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LIST OF ABBREVIATIONS

AHR	Aryl Hydrocarbon Receptor
AICD	Antigen Induced Cell Death
APCs	Antigen Presenting Cells
BBDP	Bio Breeding Diabetes Prone
BCG	Bacillus Calmette-Guerin
BMDC	Bone Marrow Derived Dendritic Cells
CD	Cluster of Differentiation
CFA	Complete Freud's Adjuvant
CM	Cow's Milk
CTLA	Cytotoxic T Lymphocyte Antigen
DC	Dendritic Cells
EAE	Experimental Autoimmune Encephalomyelitis
GAD	Glutamic Acid Decarboxylase
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GRAS	Generally Regarded As Safe
IDD	Insulin Dependent Diabetes
IFN	Interferon
IL	Interleukin
IRF	Interferon Regulatory Factor
JAK	Janus Kinase
LjN6.2	*Lactobacillus johnsonii* N6.2
LrTD1	*Lactobacillus reuteri* TD1
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex

MSC	Mesenchymal Stem Cells
NKT	Natural Killer T
NOD	Non Obese Diabetic
NOR	Non Obese Resistant
PLN	Pancreatic Lymph Nodes
ROR	Retinoic Acid Receptor
SCID	Severe Combined Immuno-Deficiency
STAT	Signal Transducer and Activator of Transcription
T1D	Type 1 Diabetes
TCR	T Cell Receptor
TGF	Transforming Growth Factor
TH	T Helper
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
Tregs	Regulatory T cells

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Type 1 Diabetes is an autoimmune disease that destroys insulin producing cells in the pancreas. Genetic factors are clearly important in disease outcome. However, environmental factors, like diet and gut bacteria, have also influenced onset of the disease. Recently, it has been shown that bacteria isolated from diabetes resistant rodents could prevent diabetes incidence in prone animals through oral feedings. We demonstrated that this resistance was correlated with a TH17 bias.

TH17 cells are typically inflammatory, but can be protective in certain gut autoimmune diseases. Furthermore, their role in Type 1 Diabetes remains unclarified. We have shown that *Lactobacillus johnsonii* N6.2 (LjN6.2), a commensal bacterium isolated from diabetes resistant rodents, was particularly adept at promoting a TH17 bias *in vitro*, requiring T cell receptor stimulation and antigen presenting cells (APCs). As TH17 conversion to TH1 cells has been known to occur *in vivo* a few days following transfer, we ascertained whether the same could occur in our model. We isolated bone marrow derived dendritic cells from diabetes prone NOD mice. Following maturation and treatment with LjN6.2, the dendritic cells were footpad injected into 9-week old NOD mice. Even two months later, the lymph nodes of mice receiving LjN6.2-pulsed dendritic

cells possessed a TH17 bias, as evidenced by their high output of IL-17 and IL-6, two vital TH17 cytokines.

We next investigated the natural affinity of NOD and NOR (diabetes resistant) mice to develop TH17 cells. While NOR pancreas contained less infiltrating leukocytes, it housed higher levels of TH17-related factors compared to NOD mice. NOD lymphocytes produced virtually no IL-6 and lower levels of IL-17 compared to NOR lymphocytes upon *in vitro* stimulation. Moreover, NOD APCs are notoriously defective in antigen presentation and maturation. Therefore, we tested whether LjN6.2 could restore function to APCs. We discovered that APCs only responded to LjN6.2 by upregulating MHCs and decreasing DEC205, demonstrating a shift from antigen uptake to presentation. This data demonstrates that a shift in T Helper phenotype, away from the diabetogenic TH1 state, may be enough to mitigate symptoms of the disease, overriding genetic programming for autoimmunity.

CHAPTER 1
INTRODUCTION

Type 1 Diabetes

Currently, in the United States, one million Americans have been diagnosed with Type 1 Diabetes (T1D) and 15,000 children are diagnosed annually (Juvenile Diabetes Research Foundation International, 2010). The average annual cost for a patient with T1D has been estimated at $14,900. Total costs from medical care and lost productivity are estimated at $15 billion for the U. S. annually (Dall, 2010). T1D is a T-cell mediated autoimmune disease that targets β-cells in the pancreas for destruction. β-cells produce insulin, which tells cells to take up glucose from the blood and store it as glycogen. Upon the loss of β-cells and insulin production, several complications arise. Excessive thirst and urination develop because of the high levels of blood sugar (hyperglycemia). Fluid is pulled from the tissue in response. At times, it can even be drawn from the eyes, causing blurred vision. The inability to take up glucose also causes increased hunger and fatigue. Despite the need to eat more, patients may actually lose weight because muscle and fat cells shrink without glucose (Mayo Clinic, 2011). In order to gain an understanding of the mechanisms involved with T1D, researchers have used rodent models. The Non Obese Diabetic mouse is one of the most well understood and studied animal models for T1D.

The NOD Mouse Model

The Non Obese Diabetic (NOD) mouse model was originally discovered by Makino et al. (Makin, 1980; Kikutani, 1992) in Japan following the selection of a cataract-prone strain obtained from the JcI:ICR mouse line. Through repetitive inbreeding, it was found that the NOD strain spontaneously developed Type 1 Diabetes

(T1D). T1D is gender-biased, as females are typically 80-90% diabetic by 24 weeks of age, while the incidence rate of males is roughly 40% by 30 weeks of age (Kikutani, 1992; Bach, 1994). NODs have become the standard in autoimmune diabetes models primarily because of their well-studied genome. Transgenes or mutations are easily introduced into the NOD model, allowing for the analysis of specific diabetogenic factors.

Genetic Factors in Type 1 Diabetes

In an effort to understand which chromosomes and genes are responsible for T1D, the NOD mouse strain has been bred with various diabetes-resistant strains. Several genetic loci have been discovered and termed *Idd* for insulin-dependent diabetes. To this point, over 30 loci on 15 different chromosomes have been isolated. Most loci also consist of multiple genes rather than one isolated gene, further broadening the range of possible underlying factors in this disease. The most prominent and well-studied *Idd* gene loci will be discussed below.

Idd1 is one of the most essential genes for T1D onset in NOD mice. Within *Idd1* are several genes associated with the Major Histocompatibility Complex (MHC). NOD mice possess a collective set of MHC mutations termed $H2^{g7}$. MHC are responsible for presenting peptide to T cells, activating them to carry out their specific function against the antigen. While nearly all nucleated cells express MHC Class I, mainly “professional antigen presenting cells” possess MHC II. MHC I is programmed to display self-peptide to CD8+ cytotoxic T lymphocytes, while MHC II shows extracellular peptides to CD4+ Helper T cells. The NOD mouse completely lacks MHC Class II E allele and contains mutations at two typically conserved sites, positions 56 and 57 of MHC II A^{g7} allele. These residues have been converted from proline and aspartic acid to histidine and

serine, respectively (Acha-Orbea, 1987). This exchange exposed positive residues in the MHC II complex, allowing negatively charged residues with larger side chains to bind. This expands the repertoire of peptides that the NOD MHC II can recognize. In addition, studies have shown that the NOD MHC II A^{g7} binds with less affinity to peptides compared to other MHC II complexes. The importance of the NOD MHC II A^{g7} was demonstrated when transgenic restoration of the MHC II E allele or a non-MHC II A^{g7} prevented onset of T1D in NOD mice (Miyazaki, 1990; Lund, 1990, Slattery, 1990). Interestingly, this residue mutation is also seen in humans suffering from T1D in the DQ beta chain (Todd, 1988).

Idd3 is associated with the cytokines IL-2 and IL-21. In the NOD mouse, it has been reported that there is unusually low levels of IL-2, but high expression of IL-21 (McGuire, 2009; Yamanouchi, 2007; Lyons, 2000). IL-2 is generally associated with promoting regulatory T cells (Tregs), which are cells associated with suppression of autoimmune diseases. Additionally, treating NOD mice with IL-2 prevented onset of disease. While IL-2 may be protective, IL-21 (detailed below) may be pathogenic. Genetic deletion of the IL21 receptor also prevented onset of T1D. Driver et al. have suggested that these two factors may be directly linked, as the lack of IL-2 prevents proper function of Tregs, making it difficult to regulate the proliferative abilities of IL-21 on T effector cells. *Idd5.1* is also affiliated with Tregs. The CTLA-4 gene is located within this loci. CTLA-4 can influence T1D by interacting with CD80 and CD86 on APCs. CTLA-4 is also required for Treg function and has been shown to inhibit function in T effector cells. In both NOD and human T1D, researchers have discovered

polymorphisms in the CTLA-4 gene, which leads to structural variance, affecting its proper function (Ueda, 2003).

Idd9 reportedly promotes the development of natural killer T (NKT) cells, which have been known to be deficient in the NOD model. NKT cells recognize antigen through a CD1d molecule, and *not* through the typical MHC process (Matsuda, 2008). In addition, these cells are unique because of their ability to quickly produce TH1 (inflammatory) and TH2 (anti-inflammatory/humoral response) cytokines when activated. Several studies have shown NKT can be protective in T1D. Mechanisms include the induction of a TH2 response in the islets (Sharif, 2001; Hong, 2001), Tregs (Pillai, 2009), or tolerogenic dendritic cells (DCs) (Chen, 2005; Wang, 2008). The *Idd9* region is also known to add to abnormal development of B cells, causing them to express auto-reactive antibodies. This may also support a group of B cells focused on promoting and activating pathogenic CD4 T cells (Silveira, 2006). *Idd13* also exerts effects on NKT cell numbers (Esteban, 2003). It is also associated with β2-microglobulin, a structural protein associated with MHC I. Using a β2-microglobulin-deficient NOD model, it was shown that reconstitution with an NOD or NOR (Non Obese [Diabetes] Resistant) β2-microglobulin molecule led to diabetes susceptibility and resistance, respectively (Hamilton-Williams, 2001). It has been proposed that the conformation of the H2g7 MHC Class I molecules changes depending on the type of β2-microglobulin molecule associated with it. In this case, the NOD conformation may allow APCs to more easily recognize CD8+ T cells during positive selection (a process that ensures that developing T cells properly recognize host MHC in the thymus).

APCs in T1D

The first signs of T1D in NOD mice can be seen as early as 4 weeks of age when insulitis can be observed in the pancreas (Mathews, 2005). Termed peri-insulitis, infiltrating lymphocytes begin by surrounding the islets. As the mice undergo puberty, increasing amounts of islets will show insulitis, losing roughly 25-90% of their initial β-cell mass. Of the infiltrating lymphocytes, a large percentage of the cells are CD4+ T helper cells. CD8+ cytotoxic T cells, B lymphocytes, macrophages, and DCs have also been found in the invading group (Jarpe, 1990, 1991; Miyazaki, 1985; Faveeuw, 1995). A decrease in pancreatic insulin levels is observed in NOD females by 12 weeks of age. This drop in males is not observed until several weeks later (Gaskins, 1992). By the time 90% of the β-cell mass is obliterated, hallmark symptoms of T1D can be observed, including: hyperglycemia, excess glucose in the blood; glycosuria, glucose in the urine; and polyuria, which is abnormal or excessive amounts of urine passage (Wilson, 1997). As pancreatic β-cells do not express MHC Class II or other costimulatory molecules, it is unlikely T cells are finding their antigen of choice within the pancreas. It is far more likely that APCs, like DCs, sample antigen from the pancreas and carry it to the pancreatic lymph node (PLN) to activate T cells. This is supported by the fact that removal of PLN from young NOD mice prevents onset of T1D (Gagnerault, 2002; Hoglund, 1999; Zhang, 2002).

Although T1D is a T-cell mediated autoimmune disease, APCs are also vital. APCs can mediate the destruction of auto-reactive T cells originating in the thymus and in the periphery (Walker, 2002). This typically occurs when T cells recognize self-antigen presented on the MHC of APCs. Should they bind too strongly, they are eliminated to prevent autoimmunity. As noted earlier, NOD mice possess defective

MHC Class II markers that bind antigenic peptide loosely. It is also known that antigen induced cell death (AICD) requires stronger activation of T cells than normal effector T cell functions (Ucker, 1992). If APCs are unable to properly stimulate T cells, T cells may simply get activated instead of being deleted from a position of dangerous immune surveillance. Accordingly, several defects have been associated with the APCs of NOD mice.

Dendritic Cells in T1D

Dendritic Cells (DCs) are important bridges between the innate and adaptive arms of the immune response. In an immature state, DCs are efficient antigen samplers. Upon maturation, markers for antigen uptake are decreased, while markers for antigen presentation (i.e., MHC I and II) are upregulated to stimulate T cells (Figure 1-1). However, NOD DCs, like other NOD APCs, fail to upregulate markers associated with the costimulation of T cells (CD80, CD86) and MHC markers when properly stimulated with antigen like lipopolysaccharide (Strid, 2001). In addition, NOD bone marrow derived dendritic cells (BMDCs) are notoriously difficult to increase in culture, showing lower proliferative ability (Nikolic, 2004). DCs are also known to promote different T Helper cell phenotypes based on their maturation state. For instance, mature DCs are known to produce less IL-12, which skews T cells to a TH1 phenotype (Feili-Hariri, 1999). The differentiation state of DCs also affects whether they can induce tolerance, anergy, deletion, or activation of T cells (Reis e Sousa, 2006). It has been shown that mature DCs isolated from the pancreatic lymph node of NOD mice can prevent onset of the disease when transferred into young NOD mice (Clare-Salzler, 1992). Many of the tolerogenic functions of DCs map to the *Idd3* and *Idd5* loci (Hamilton-Williams, 2009).

Macrophages in T1D

Macrophages, one of the most adept uptakers of antigen, are also defective in NOD mice. When stimulated with LPS and IL-1, NOD macrophages fail to activate CD8+ cytotoxic T cells in the same manner as non-diabetic strains (Serreze and Gaedeke, 1993; Serreze and Gaskins, 1993). Similar to DCs, macrophages may not be able to properly stimulate T cells to induce tolerance through cell death. In addition, T cells require a stronger set of activation signals to convert to TH2 cells. The level of activation for TH1 cell conversion is much lower, possibly biasing the immune system toward an inflammatory response (Schweitzer, 1998). In addition, NOD macrophages have difficulties properly clearing apoptotic bodies. When the apoptotic bodies are finally cleared, NOD macrophages produce higher levels of IL1β and TNFα, two inflammation-associated cytokines. Macrophages from NOD mice also tend to express elevated levels of granulocyte/macrophage colony stimulating factor (GMCSF). STAT5 is consistently activated in these cells, leading to overproduction of prostaglandin E2 (PGE2), which is associated with developing the chronic inflammatory environment seen in the NOD mouse (Litherland, 2005).

Apoptosis refers to programmed cell death, which occurs in a regulated, controlled manner. Following the proper stimuli (i.e., CD95 stimulation), a series of caspases are activated in cascade fashion. Caspases cleave and disrupt central components of cell function. During apoptosis, the cell membrane is also warped, as the cytoskeleton degrades. In research, the early stages of apoptosis are measured by an Annexin V antibody, which binds phosphatidylserine (PS) (Koopman, 1994). PS is restricted to the inside of the membrane, toward the cytoplasm. Upon apoptosis, the membrane loses its rigidity, and PS begins to get expressed on the surface of the cell, which can then be

bound by Annexin V antibody. Late stages of apoptosis are measured using 7-AAD, a compound that binds DNA (Rabinovitch, 1986). When a cell has neared the end of apoptosis, the entire membrane is disrupted and 7-AAD, a nuclear staining dye, can bind to the cell.

B cells in T1D

B cells are the generators of antibodies. T1D is associated with several autoimmune antibodies, the most well-studied being for insulin, glutamic acid decarboxylase (GAD), protein tyrosine phosphatase IA2, and Zinc Transporter ZnT8 (Lien and Zipris, 2009). Initial studies indicated B cells were essential in T1D, as suppression of B cells with antibodies or eliminating BAFF/BLyS, a B cell survival factor, ameliorated the disease (Hu, 2007; Fiorina, 2008). However, recent studies have shown that B cells alone are not sufficient to cause T1D. The transfer of serum antibodies from diabetic NOD mice into a B-cell deficient NOD model did not increase insulitis, nor did it cause T1D (Serreze, 1998). In addition, the simple addition of NOD diabetic T cells alone into a B-cell deficient model can cause T1D. While autoantibodies undoubtedly aid in disease progression, it is more likely B cells act in an antigen presenting capacity. When MHC II was eliminated from B cells, but not from macrophages and DCs, T1D was suppressed in NOD mice (Noorchashm, 1999). While B cells are not required for the initiation stages of T1D, their presence enhances reactivity against islet β-cells.

T cells in T1D

As stated earlier, T cells are the main mediators of T1D. T cells recognize antigen through their T cell receptor (TCR). MHCs complex with the TCR. If the TCR is specific for the presented antigen, that T cell becomes activated if given the proper costimulation. T cells mature in the thymus after undergoing several checkpoints to

ensure proper MHC recognition and limited reactivity to self. If a T cell fails any of these tests, it is marked for deletion. In the NOD mouse, there are underlying factors that are leading to the false maturation and export of self-reactive T cells into the periphery (Sebzda, 1994). It has been shown that positive selection (MHC self-restriction) requires less T cell activation compared to negative selection (the elimination of self-reactive cells). In other words, these pathogenic T cells bind with just enough strength to the MHC to be positively selected and recognized as MHC-restricted, but weak enough that their self-reactivity is not deemed dangerous, escaping negative selection (Ashton-Rickardt, 1994).

Even in a normal, non-autoimmune model, some T cells will escape proper selection. In the periphery, these cells are typically removed from the body through AICD. However, NOD T cells display elevated levels of c-FLIP, Bclx-L, and TGFβ, all anti-apoptotic factors (Decallone, 2003; Arreaza, 2003). In addition, they display lower levels of IL-2, IL-4, FasL, and Caspase-8, which are pro-apoptotic factors. By 6 weeks of age, NOD mice can express their full complement of T cell receptors. These T cells have been shown to become more pathogenic with age, as they increase in proliferative ability and production of IFNγ while decreasing generation of immunoregulatory factors like IL-4 and IL-10. This may lead the T cells to resist regulation by Tregs. Combined with their penchant for eluding AICD, this may explain why T cells become pathogenic in T1D.

It was discovered that both CD4+ and CD8+ T cells are required for disease progression in the NOD model. These experiments used an NOD.SCID model. SCID mice lack catalytic polypeptide (Prkdc). Prkdc is involved with repairing double-stranded

DNA breaks and recombining the variable (V), diversity (D), and joining (J) regions of immunoglobulin (antibody) and T cell receptor genes. Because of this defect, properly functioning T cells and B cells never develop in the mouse. Crossed onto an NOD background, this allows for the study of a particular T or B cell group's influence in an NOD setting (Shultz, 1995). CD4+ cells can transfer diabetes into an NOD.SCID *if* the donor cells were already diabetic. If CD4+ cells from young, prediabetic mice are transferred to an NOD.SCID mouse, they do not cause T1D. CD8+ cells alone cannot cause T1D in an NOD.SCID as they are unable to home to the islets to start destruction (Christianson, 1993). Both cell types are thought to mediate destruction of β-cells through IFNγ production (Suarez-Pinzon, 1996). CD8+ cells are also thought to cause β-cell death through perforin and granzyme B (Estella, 2006).

The phenotype of CD4+ T helper cells is dependent on the cytokine environment provided by the APC, typically DCs (Figure 1-2). CD4+ helper phenotype has also been implicated as important in T1D, as TH1 cells are typically associated with initiating disease onset, producing abundant amounts of IFNγ (Delovitch, 1997). Conversion of naïve T cells from a TH1 phenotype to a TH2 phenotype is associated with protection from T1D (Falcone, 1999). However, the role of T Helper cells is not as simple as one would expect. IL-12 (TH1 differentiation factor) knockout mice actually show an accelerated disease incidence in NOD mice. In addition, IL-4 (TH2 promoting factor) knockouts develop T1D at a rate identical to control NOD mice (Wang, 1998). Despite some of these paradoxical findings, overwhelming evidence typically indicates TH1 related factors are detrimental in T1D.

Tregs are also vital in T1D, but in a protective role. Their depletion results in a greatly accelerated onset of disease (Salomon, 2000; Bour-Jordan, 2004). Recent studies have proven that Tregs do not decrease during the course of the disease, but they may lose their functionality (Mellanby, 2007; Tritt, 2008). Staining for Foxp3, the Treg transcription factor, in the thymus, peripheral lymphoid organs, and pancreas all indicated that Treg numbers do not decline with age or as insulitis begins to occur. However, as the mice age, NOD Tregs lost suppressive effects. Furthermore, the adoptive transfer of Tregs and conventional T cells from young neonatal NOD mice, but not mature NOD donors, inhibited T1D (You, 2005; Gregori, 2003). The declining abilities of Tregs and increasing abilities of T effector cells with age may be two major factors in T1D disease development.

T Helper 17 Cells

T helper 17 (TH17) cells are a recently discovered subset of CD4+ Helper T cells. Much like other T helper subsets, TH17 cells are identified by their unique cytokine profile and set of transcription factors. Typically, TH17 cells are associated with the production of IL-17A, IL17-F, IL-21, and IL-22. The TH17 lineage specific transcription factors include RORγt, RORα, IRF-4, and AHR. Th17 cells are thought to be intimately involved with host defense against pathogens, particularly extracellular fungi and bacteria (Ouyang, 2008). IL-17A deficient mice suffer from an inability to clear *K. pneumonia* and *T. gondii* infections. However, they do not show increased susceptibility to intracellular bacterial infection (Ouyang, 2008). Although several cell types have been implicated in T1D, the role of TH17 cells in T1D has not been established yet.

TH17 Cell Differentiation

Typical dogma states that TH17 cells are generated from naïve CD4+ cells using Transforming Growth Factor β (TGFβ) and IL-6, a cytokine typically associated with inflammation (Bettelli, 2006; Mangan et al., 2006; Veldhoen et al., 2006). However, as more research is conducted on TH17 differentiation, several variations of TH17 differentiation have been suggested. Several studies have stated that a combination of IL-21 and TGFβ is sufficient to initiate TH17 differentiation (Kom, 2007). This is particularly intriguing because IL-21 drives the production of IL-17, itself, and the expression of IL-23R (Zhou, 2007; Nurieva, 2007; Kom, 2007). In addition, IL-6 may not be absolutely required for TH17 cell development (Elyaman, 2009). IL-$6^{-/-}$ mice have reduced, but not completely absent levels of TH17 cells, suggesting other cytokines may supplement the loss of IL-6 (Bettelli, 2006). Recently, it has been suggested that IL-9 may also replace IL-6 and work with TGFβ to create TH17 cells (Elyaman, 2009). Even TGFβ is no longer sacred in TH17 differentiation, as Ghoreschi et al., has stated that TH17 cells can be made in the absence of TGFβ with a combination of IL-6, IL-23, and IL-1β. Despite the controversy of creating TH17 cells, each proposed mechanism seems to center on various combinations of TH17 associated cytokines.

IL-23 is composed of p40, a subunit also utilized by IL-12, and the p19 subunit. The receptor is composed of IL-12Rβ2 and the unique IL-23R chain. IL-23 is generated by activated dendritic cells and phagocytes (Oppmann, 2000) and was initially believed to be a cytokine required for TH17 differentiation. However, it was demonstrated that IL-23R is not expressed on naïve T cells. Despite not being involved with the initial creation of TH17 cells, IL-23 was discovered to be essential in maintaining a TH17 population and expanding it (Bettelli, 2006; Veldhoen, 2006).

TH17 Transcription Factors

Much like TH1 and TH2 cells, TH17 cells have dedicated, lineage transcription factors responsible for master control of TH17 related genes. A majority of TH17-related cytokines do their signaling via Signal Transducer and Activator of Transcription 3 (STAT3). IL-17, IL-23, IL-6, IL-21, and IL-22 all activate STAT3. STAT3 also regulates expression of IL-21R, IL-21, and IL-23R (Durant, 2010; Ghoreschi, 2010). STAT3 deficiency greatly reduced expression of RORγt and RORα, two TH17-specific transcription factors (Yang, 2007, 2008).

RORγt and RORα both belong to the retinoic acid-related orphan hormone receptor family. RORγt is induced by TGFβ or IL-6 and its overexpression drives TH17 cell differentiation, while simultaneously blocking the development of TH1 and TH2 cells. It was also demonstrated that a deficiency in RORγt led to a lowered ability to create TH17 cells and limited IL-17 production, even in the presence of TGFβ, IL-6, and IL-21 (Ivanov, 2006; Nurieva, 2007). While RORγt deletion limited TH17 cell generation, it was not completely abolished. Mice lacking RORγt still develop Experimental Autoimmune Encephalomyelitis, an animal model of multiple sclerosis where TH17 cells are causative (Yang, 2008). Yang et al., recently discovered TH17 cells also express RORα following induction by TGFβ and IL-6. RORα shares several of the properties of RORγt and the two transcription factors likely share redundant function.

Interferon-Regulatory Factor 4 (IRF4) was also shown to be vital for TH17 cell differentiation (Brustle, 2007), as IRF4 deficiency completely inhibits TH17 differentiation and prevented disease onset in EAE. This was also correlated with a marked decrease in RORγt expression, suggesting that IRF4 may operate upstream of RORγt. The precise functions and abilities of IRF-4 are still being investigated. Finally,

aryl hydrocarbon receptor (AHR) is also believed to play a crucial role in TH17 cell differentiation. While both T regulatory cells and TH17 cells can express AHR, TH17 cells express this transcription factor at significantly higher levels compared to any other T cell subset. AHR-deficient cells do not express IL-22 (Veldhoen, 2008). While less information is available on these two transcription factors, they are nonetheless very important in aiding TH17 cell development.

TH17-Associated Cytokines

Several TH17-associated cytokines are potent in the recruitment, activation, and migration of neutrophils, the most numerous and active members of the innate immune system. This recruitment and subsequent activation of neutrophils is mitigated by the function of IL-17, which stimulates the production of colony-stimulating factors and CXCL8 by macrophages and other tissue resident cells. CXCL8 then induces incoming immune cells to sample antigen through phagocytosis, triggering the activation of certain Toll-like receptors (Lund, 2004).

IL-17 consists of six family members, designated IL17A-F. Among the family members, IL-17A and IL-17F share the most homology and are the most well studied. TH17 cells are capable of producing both IL-17A and IL-17F, while IL17B-E can be produced by non-T cells. IL17A and IL17F can exist as either a IL-17A homodimer, IL-17A and F heterodimer, or IL-17F homodimer, which have been listed in order of decreasing potency/efficacy (Liang, et al., 2007). There are several cell types besides TH17 cells that produce IL17A/F, including: Natural Killer Cells, which are involved in antiviral/anti-tumor responses by destruction of target cells (Bryceson et al., 2011); invariant NKT cells, which recognize a limited repertoire of lipids and glycolipids presented by CD1d rather than the typical MHC-peptide complex (Diana, 2011);

lymphoid tissue inducer(LTi)-like cells, which are involved with lymphoid aggregate formation (Cua and Tato, 2010; Takatori, 2009). Neutrophils, and γδ T cells, which are heavily prevalent in the gut mucosa and associated with gut mucosal barrier maintenance (Cua and Tato, 2010), have also been known to produce IL-17.

The receptor for IL-17 spans IL17RA-IL17RE. IL-17A and IL-17F signal through a heterodimer receptor of IL17RA and IL17RC (Figure 1-3). IL-17RA is found ubiquitously on all cell types, particularly hematopoetic (areas that generate blood cells and leukocytes) tissue, while IL-17RC has low expression in hematopoetic tissue and high expression in the liver, thyroid, joints, and kidney. As IL-17A typically elicits a proinflammatory gene profile similar to those induced by innate immune receptors IL-1R and TLRs, studies were conducted on NF-κB, a classical transcriptional factor associated with inflammation. Accordingly, it has been found that several DNA elements bind NF-κB in the promoter of IL-17A and induce gene expression. Gel shift assays indicated IL-17A activates p50 and p65, two hallmark members of the canonical NF-κB pathway. There has been no confirmation on the use of non-canonical NF-κB pathway as of yet (Ruddy, et al. 2004). Similar to IL-1R and TLRs, IL17 signaling requires TNFR-associated factor 6 (TRAF6), as $Traf6^{-/-}$ mice are cannot activate NF-κB through IL-17. In spite of all the similarity to innate signaling, IL17RA does not contain a typical TRAF-6 binding motif. And, it has been shown that common innate signaling proteins like MYD88, TRIF, IRAK4, and IRAK1 are not necessary for IL-17A signaling. Insight into IL-17 signaling was provided when SEFIR domains were discovered. A bioinformatics study revealed that IL17R all expressed a conserved motif homologous to the Toll/IL-1R domain (termed SEFIR), which provides docking sites for intracellular adaptors like

MYD88. SEFIR then recruits ACT1, which has a TRAF6 binding domain that leads to NF-κB activation.

IL-21 is another major product of TH17 cells. The IL-21R consists of the common γc chain and the unique IL-21R chain and is expressed primarily in the spleen, thymus, peripheral blood, and lymph nodes (Ozaki, 2000; Parrish-Novak, 2000). IL-21R is also constitutively expressed by B cells, dendritic cells, epithelial cells and NK cells. T cells will also express IL21R, but only upon activation (Brandt, 2003; Monteleone, 2006). IL-21 signals through Janus Kinase (JAK) 1 and JAK3, activating Signal Transducer and Activator of Transcription (STAT) 3. It has also been known to activate STAT1 and weakly trigger STAT5 (Asao, 2001). IL-21 has broad immunological effects. It reduces the expression of MHC II, CD80, and CD86 in dendritic cells (Brandt, 2003; Strengell, 2006). It also activates the phagocytic and proteolytic abilities of macrophages and contributes to inflammation and remodeling of extracellular matrix through the action of matrix metalloproteinases (MMPs) (Ruckert, 2008; Monteleone, 2006). IL-21 can induce death, proliferation, and antibody class switching in mature B cells (Mehta, 2003). CD4+ cells are the main producers of IL-21. This aids in TH17 development by inducing upregulation of IL-23R, which is involved with the long term sustainment of a TH17 phenotype (Zhou, 2007). IL-21 can trigger CD8+ T cells to express lower levels of CD44, CD25, granzyme B, interferon gamma (IFNγ), and decreased ability to kill target cells (Hinrichs, 2008). Additionally, IL-21 can induce IL-10 production, which limits CD8+ T cell activation and proliferation (Spolski, 2009).

IL-21 has been implicated in other autoimmune models. In NOD mice, it caused increased T cell turnover (King, 2004). In addition, IL-21 is thought to play a role in the

MRL-*Fas*^{lpr} mouse model, which spontaneously develops lupus (Herber, 2007). As the mice aged and died of severe lupus, increased levels of IL-21 were observed. The CIA mouse model for rheumatoid arthritis showed alleviation of symptoms when IL-21 signaling was blockaded (Young, 2007). As IL-21 is closely associated with B cell proliferation and antibody class switching, it is plausible to believe that the promotion of auto-antibodies may be detrimental in these autoimmune disease models.

While γδ T cells, LTi cells, LTi-like cells, and NK cells have been known to make IL-22, it is another cytokine typically associated with the TH17 cell subset. Like IL-21, IL-22 signals primarily through the STAT3 pathway. The receptor for IL-22 consists of the unique IL-22R chain and the IL-10Rβ chain. IL-22R expression is typically restricted to epithelial cells like keratinocytes and colonic epithelial cells (Lejeune, 2002). Interestingly, while TH17 cells are induced by TGFβ and IL-6, this is not the ideal setup to make IL-22, as TGFβ actually inhibits IL-22 production. IL-22 is generation is dependent on IL-23 and AHR expression, meaning that IL-22 is more heavily involved in the effector phase of this cell type (Siegemund, 2009; Munoz, 2009).

Similar to IL-21, IL-22 has been involved in different models of chronic inflammation like psoriasis and rheumatoid arthritis. However, the increase in IL-22 was a correlation, and not a direct cause, of these diseases (Wolk, 2006; Andoh, 2005). Mice overexpressing IL-22 display psoriasis and an aberrant skin phenotype (Wolk, 2009). In a model using collagen to generate joint autoimmunity, it was shown that an IL-22 deficiency was associated with decreased incidence of arthritis (Geboes, 2009). While IL-22 has been implicated in some inflammatory activity, it has a dual nature. IL-22 has actually shown to be protective in hepatitis (Radaeva, 2004, Zenewicz, 2007).

IL-22 activates anti-apoptotic and pro-survival factors in hepatocytes, leading to their enhanced longevity (Radaeva, 2004). With IL-22, liver tissue has also been shown to regenerate following a partial hepatectomy or alcohol-induced damage (Ki, 2010; Ren, 2010). Patients suffering from inflammatory bowel disease have mutations in genes encoding IL-22 and the IL-10Rβ subunit (Silverberg, 2009; Glocker, 2009). IL-22 has also been protective following the transfer of colitis-inducing CD4+CD45RB^{high} T cells. This protection may be correlated with the ability of IL-22 to induce expression of anti-microbial molecules within the GI tract, which include β-defensins (Wolk, 2004; Zheng, 2008). In addition, IL-22 causes mucin to be produced. Mucin is a heavily glycosolated set of proteins that create a protective barrier lining the GI tract, which limits an immune response by separating both commensal and pathogenic bacteria from the epithelial layer (Sugimoto, 2008). Finally, IL-22 actually plays a role in healing and tissue repair. Mice deficient in IL-22 displayed delayed healing in colonic biopsies compared to controls (Pickert, 2009).

Plasticity of TH17 Cells

T Helper cells are typically classified by their cytokine production profile and the expression of lineage-specific transcription factors. Recent research has shown that TH17 cells demonstrate plasticity, blurring the lines between well-defined T Helper classes. The phenotype of *in vitro* generated TH17 cells was shown to be unstable, as subsequent treatment with TH1 or TH2 polarizing conditions allowed the cells to be converted to TH1-like or TH2-like cells (Lexberg, 2008). And, it has been shown that IL-12 (a cytokine that favors TH1 cell development) and IL-23 are capable of inducing IFNγ production by TH-17 cells. Moreover, IL-12 suppresses TH17-related activities,

upregulating the TH1 lineage transcription factor T-bet. In order to achieve the same type of efficacy, TH17 cells required several treatments of IL-23, demonstrating that IL-12 is a very potent inhibitor of TH17 differentiation and enhancer of the TH1 phenotype (Lee YK, 2009). Upon conversion to TH1 cells, the former TH17 cells also require the activity of STAT4 and T-bet.

The ability of TH17 cells to convert to TH1 cells was investigated by Bending et al. (2011). Bending studied epigenetic control of TH1 and TH17 related factors in Th17 cells created by both *in vitro* and *in vivo* methods. Epigenetics refers to the control of gene expression through factors unrelated to its DNA sequence. Methylation of amino acids is a common method of epigenetic control. For instance, Bending examined H3K4 (Histone 3, Lysine residue 4) and H3K27. The tri-methylation of H3K4 is associated with the actively transcribed genes (Santos-Rosa, 2003), while the tri-methylation of H3K27 is associated with that gene's repression (Ringrose, 2004). Bending demonstrated that both *ex vivo*-isolated TH17 cells and *in vitro* developed Th17 cells display bivalent marks for T-bet, the TH1 transcription factor. Bivalency refers to genes containing epigenetic markers for both repression and activation. In this instance, the bivalency of T-bet in TH17 cells may explain why they can be predisposed to changing to a TH1 phenotype so easily. Interestingly, Bending also showed that *ex vivo* isolated TH17 cells express lower levels of IL-12Rβ2 (one of the chains for the IL-12R) compared to TH17 cells made *in vitro*. This lower expression was due to restrictions created by chromatin modifications, another method of epigenetic control. These restrictions could be lifted upon *in vitro* treatment with IFNγ. In addition, they showed that IL-12 *in vitro* treatment of freshly isolated *ex vivo* TH17 cells removed H3K27 tri-methylation at T-bet.

Epigenetic studies have provided valuable insight as to why *in vitro* generated TH17 cells exhibit plasticity. This ability was highlighted in studies by Martin-Orozco et al. and Bending et al. (2009). Both groups transferred TH17 cells into an NOD.SCID model and noted an onset of T1D. However, they observed that these cells ceased to express TH17-related factors and began pumping out TH1 factors. Furthermore, only antibodies against IFNγ ameliorated disease, while anti-IL-17 antibodies were ineffective at changing disease onset. Both groups provide evidence that TH17 cells first convert into TH1 cells before initiating disease. The NOD.SCID model represents a lymphopenic model, which lacks properly functioning T and B cells. This type of environment may be especially conducive to TH1 conversion. This is particularly important because it can give researchers false indications on the role for a T cell population. For example, many believed TH17 cells could cause T1D upon transfer into a NOD.SCID mouse, but it was later revealed these cells convert to a TH1 phenotype before initiating T1D (Bending, 2009). Pakala et al. also demonstrated that the transfer of TH2 (T helper cells that promote a humoral and allergic response) into NOD.SCIDs still caused T1D. In a lymphopenic environment, there may not be enough regulation from Tregs to prevent conversion to TH1 cells that attack the pancreas.

Sujino et al. demonstrated a similar mechanism in a colitis model, as transferred TH17 cells converted into TH1-like cells before causing onset of the disease. Regulatory T cells stopped this conversion and prevented onset of colitis. There has even been work showing that a transitory Treg/TH17 cell type may exist. Tartar et al. demonstrated that a Foxp3/RORγt population exists naturally in NOD mice and can be expanded upon treatment with an antibody hybrid utilizing GAD protein. Upon transfer

with diabetogenic splenocytes into an NOD.SCID model, Foxp3+/RORγt+ cells prevented onset of disease by trafficking to the pancreas through CD62L expression. The “plastic” nature of TH17 cells allows them to display a wide range of immunological abilities.

The Role of TH17 Cells in Type 1 Diabetes

While TH17 cells have been implicated as causal in other autoimmune models, their role in Type 1 Diabetes (T1D) is far more controversial. IL-21, one of the main products of TH17 cells, was shown to be critical for T1D onset in NOD mice. IL21R knockout mice did *not* display insulitis, with only 1 of 20 animals becoming diabetic (Spolski, 2008). For the one animal that did develop diabetes, it is possible that the infiltrating cells destroyed their target and left the pancreas, an event the histology snapshot could not capture. Emamaullee et al., suggested that TH17 cells may be responsible for causing T1D, as anti-IL17 antibodies and recombinant IL-25 alleviated onset of disease in NOD mice. However, their method of inhibition revolves around a product not unique to TH17 cells. In addition, throughout onset of the disease, the group was unable to identify TH17 cells in the pancreas. Moreover, IL-25 is associated with biasing the immune system toward a TH2 phenotype. It may be more vital, then, to limit the abilities of a TH1 population via conversion of naïve CD4+ T cells to TH2 cells.

Some studies involving TH17 cells and T1D revolved around the use of Complete Freud’s Adjuvant (CFA) or Bacillus Calmette-Guerin (BCG). CFA utilizes killed pieces of Mycobacterium in mineral oil to elicit an immune response. It was shown that a single treatment of diabetic NOD mice with CFA could reverse diabetes onset (Ryu, 2001). Initial reports from Gao et al., implicated a reduction in IL-17 production following CFA

treatment of NOD mice. However, the paper does not specify a particular cell source, only citing that the IL-17 producer was not CD4+, CD8+, CD11b+, CD11c+ or γδ TCR+ cells. The suppressed IL-17 producers were likely an unidentified myeloid population (Gao, 2010). On the other hand, BCG administration to NOD mice has shown a reduction in the proinflammatory cytokines TNFα and IFNγ. Nikoopour et al., also conducted cytokine analysis of NOD mice injected with CFA. Lymphocytes from the CFA-treated mice showed increased production of IL-17, IL-22, IL-10, and IFNγ. They also found that the adoptive transfer of CFA-treated, TH17 polarized cells into an NOD.SCID mouse model delayed onset of T1D (it is likely that CFA treatment is able to maintain the TH17 phenotype and prevent conversion to TH1 cells, which is in contrast to the studies performed by Bending and Martin-Orozco listed above). The addition of anti-IL-17 antibodies restored onset of T1D in these mice. While the production of IFNγ may seem paradoxical, Nikoopour et al., believe the change in balance of IFNγ, IL-17, and anti-inflammatory IL-10 is what proves vital in this model. It is also possible that the time frame could be responsible for the differences seen by Gao and Nikoopour, as they examined cytokine production 18 and 10 days post CFA administration respectively. Yang-Hau et al. demonstrated that using all-trans retinoic acid on NOD mice prevented disease onset via T regulatory cells, limiting IFNγ-producing CD4+ and CD8+ cells, while *not* affecting IL-17 producing CD4+ cells. To this point, the relationship between TH17 cells and T1D in NOD mice is still hotly contested.

TH17 Cells, Humans, and T1D

TH17 cells are less established in the human setting. Human TH17 cells do not match their mouse counterpart in cytokines required for differentiation, nor do they carry

the same types of surface markers (Crome, 2009). To this point, a few studies have been conducted on the relationship between T1D, TH17 cells, and humans. Upon treating peripheral blood mononuclear cells with protease-resistant GAD peptide, it was observed that the cells produced less IL-17, IL-6, and TNFα. However, there was also an observed reduction in IFNγ production (Boehm, 2009). This model certainly has its limitations, as it does not represent an *in vivo* method. Honkanen et al. found that recently diagnosed children exhibited higher levels of TH17-related factors compared to healthy sibling controls. Stimulating their peripheral blood mononuclear cells caused an increase in expression of IL-17, IL-22, and RORc. Bradshaw et al. found that human monocytes preferentially secreted IL-1β and IL-6, two cytokines capable of generating human TH17 cells. These monocytes elicited the development of TH17 cells. However, these cells produced IFNγ in addition to IL-17, suggesting a fluid phenotype. A separate study conducted by Chatziegeorgiou et al. demonstrated that patients suffering from T1D express higher levels of TH1-related factors within the first six months of diagnosis (as indicated in protein levels obtained from blood). In patients with disease duration over six months, they noticed an increase in TH17-related factors, suggesting they do not participate in initiation of the disease. Their role as an effector cell, however, is still plausible. While TH17 cells may seem to be pathogenic in an autoimmune diabetic human setting, evidence still points to TH1 cells as the initiators of the disease. The exact role of TH17 cells must still be established.

Environmental Factors in T1D

While genetic factors undoubtedly play a key role in T1D, recent studies have pushed environmental factors into the forefront. This has been supported by studies in humans, as the rate of T1D between monozygotic identical twins is only 30-50%

(Barnett, 1981; Kumar, 1993). In addition, several nations have reported a 3-5% increase in T1D incidence annually over the last few decades (Gale, 2002). There are reports of geographic location influencing T1D development, as regions along the Mediterranean Sea only report 5/100,000 children suffer from T1D (EURODIAB ACE Study Group, 2000). On the other hand, countries like Finland reported 50/100,000 T1D cases in children a ten-fold difference. All of this data points to risk factors located outside of chromosomal hot spots, such as viral infection, diet, and gut microbiota.

Viruses in T1D

Initial interest in the link between T1D and viruses began with studies on rubella. Researchers noticed that infection with rubella was often subsequently followed by T1D onset (Ginsberg-Fellner, 1984). The theory here is that viral infection can either mediate a direct cytolytic effect on pancreatic cells (Yoon, 1991) or induce immunity due to the homology between viral structures and beta-cell antigen. The autopsies of newborn infants that died of Coxsackie virus infection revealed virus-positive islets, while control pancreata did not (Foulis, 1990; Ylipaasto, 2004). In addition, enterovirus RNA was detected in 57% of individuals that developed T1D within 6 months compared to 31% of normal controls (Lonnrot, 2000). The exact role of viruses, however, is still controversial as others have observed no link with T1D development (Graves, 2003; Fuchtenbusch, 2001).

Dietary Factors

One of the biggest focal points in dietary antigen is cow's milk (CM). Initial concern arose when it was discovered that humans produced antibodies against bovine insulin (Vaarala, 1999). Bovine insulin is very homologous to human insulin as they differ at only 3 amino acid residues. Furthermore, studies have demonstrated that children that

ended breastfeeding at an earlier time point and began drinking CM were at an increased risk for T1D (Gerstein, 1994; Norris 1996). The elimination of CM-related proteins in the first 6-8 months of life led to a 40-60% reduction in T1D-associated autoantibodies at 2 years of age (Akerblom, 1999). The infant's gut is far more sensitive to permeability early on in life. As the infant ages, the gut becomes more restricted, tightening junction between epithelial cells (Vaarala, 1999). Therefore, dietary antigens in the infant's early life may influence immune response and development.

Other Environmental Factors

Aside from the items listed above, a few other environmental factors have been considered for their influence in T1D. Vitamin D is among the factors considered to have a negative correlation with T1D onset. A Finnish study observed that infant Vitamin D supplementation was associated with decreased incidence of T1D (EURODIAB Substudy 2 Study Group, 1999). Moreover, T1D onset occurs at a higher rate in winter months than summer months (Knip, 2005). As Vitamin D production is directly associated with exposure to sunlight, this may explain the seasonal difference in diabetes onset and the geographical differences mentioned above.

In order to explain several of the environmental factors detailed above, several theories have been developed. The hygiene hypothesis states that regular, normal infections in childhood may induce tolerance later on in life. As more developed countries become cleaner and cleaner, there has been an observed spike in T1D disease incidence (Bach, 2001; Pundziute-Lycka 2000; Viskari, 2000). A study by EURODIAB Substudy 2 demonstrated that regular preschool attendance and infectious disease exposure was negatively correlated with T1D. The accelerator or beta-cell stress/overload hypothesis has also emerged as a prominent explanation of trends in

diabetes incidence. Essentially, the accelerator hypothesis claims that obesity and excessive weight gain in children leads to an increased demand for insulin and insulin resistance. Recent studies have shown that active beta cells are more susceptible to damage from cytokines than resting cells (Wilkin, 2001). Interestingly, world trends have shown that countries that exhibit an increase in Type 2 Diabetes (caused by insulin-resistance and excessive weight gain) also show a parallel increase in T1D incidence (Onkamo, 1999; Akerblom, 1985;). Environmental factors are gaining credence as a major player in T1D development. In addition to diet and virus infection, bacteria are being indicated as major influencers of the host, modulating immune responses and possibly altering T1D onset.

Possible Treatments for T1D

Over the last few decades, the NOD model has provided great insight into T1D development. However, there are over 200 successful treatments in NOD mice that prevent or delay onset of T1D (Anderson and Bluestone, 2005). A large majority of these treatments have proven unsuccessful in human translational studies. To date, there are several ongoing human trials investigating possible successful interventions and preventions of T1D. Insulin has been a center of focus, as the NOD mouse has shown resistance to T1D if exposed to oral insulin (Atkinson, 1990). The Diabetes Prevention Trial-Type 1 (DPT-1) has attempted to determine whether injected or oral insulin would be effective. While neither treatment entirely prevented onset, a decrease in insulin autoantibodies and a delay in diabetes onset was observed in those who received oral insulin (Kupila, 2003). Further studies are being conducted to determine whether the dosage of insulin plays a role in the treatment (Harrison, 2010). GAD65, another marker the body generates autoantibodies against, is also being tested for

treatment use. The NOD mouse saw prevention of T1D with GAD65 vaccination (Tisch, 1994). In humans, a 20 µg dose of GAD65 maintained levels of C-peptide (a protein that promotes the proper formation of insulin chains). This promising data has led to phase III trials in human patients recently diagnosed with T1D.

In addition to the prevention strategies listed above, several studies are being conducted on intervention methods. The immunosuppression of CD3 cells seems like a plausible treatment, as the inhibition of autoreactive T cells would be beneficial. Anti-CD3 antibodies have been used to target those T cells for destruction. In addition, anti-thymocyte globulin (made by inoculating horses or rabbits with human thymocytes) treatment generates a polyclonal anti-human T cell antibody preparation (Feng, 2008). Following the short-term deletion of these T cells, Tregs may be selectively expanded, stopping any T effector cells that resurface. This treatment requires 6 to 14 day intravenous infusions, which may be a deterrent to patient sign-up. There are also studies focused on stopping B cells, the producers of autoantibodies. Anti-CD20 antibodies like Rituximab have been shown to be effective in limiting the signaling interaction with T cells. In addition to being potent in the NOD model (Xiu, 2008; Fiorina, 2008), Rituximab is a standard part of treatments in several human diseases, like rheumatoid arthritis and non-Hodgkin's lymphoma. Researchers are eagerly awaiting the results of their two-year time point in Rituximab treatments in T1D patients. There are also "cocktail therapy" treatments, primarily using immunosuppressive drugs (Mycofenolate Mofetil (MMF)) and the depletion of activated T cells (Daclizumab (DZB)). To this point, however, neither drug or combination of drugs has been capable of affecting C-peptide levels beneficially (Gottlieb, 2010).

Several NOD mouse studies have demonstrated that the transfer of mature or tolerogenic DCs into young, prediabetic mice was an effective method of preventing onset of T1D (Feili-Hariri, 1999). However, the human rejection of foreign MHCs could prove to be a difficult barrier to overcome. As such, mesenchymal stem cells (MSC) have gained momentum as a possible avenue of treatment. MSCs generate little to no immunogenicity from host patients. In addition, MSCs are thought to suppress inflammation in localized areas. While MSCs do not live for extensive amounts of time in the human body, this could be beneficial as there are concerns about creating an immortal cell-line for *in vivo* use. “Boosters” of MSCs can also be easily injected due to their ability to avoid stimulating the immune system. MSCs have also been shown to stimulate β-cell repair and enhance the production of insulin (Fiorina, 2009; Madec, 2009).

In order to create a proper treatment for T1D, the agents must be considered for efficacy and safety. As research progresses in this field, products are not only being selected simply because they were advantageous in NOD models. Agents are being selected because they have also shown beneficial effects in other autoimmune models like lupus or multiple sclerosis. In addition, these treatments must have limited side effects. In light of this, probiotics may be a new line of treatment for autoimmune disease like T1D. Many probiotic treatments use bacteria that are generally regarded as safe (GRAS) and have shown an enhanced ability to modulate the immune response.

Modulation of the Immune Response by Bacteria

As over 100 trillion microbiota inhabit the human digestive tract (Hooper, 2001), it is important to understand how they can influence the immune response. Gut bacteria can aid the host by absorbing otherwise indigestible nutrients and can even limit the

ability of pathogenic bacteria to adhere to the epithelial cells of the intestine. This is done through the production of antimicrobial compounds, outcompeting pathogenic bacteria for adhesion sites, and modulating the immune system (Borchers, 2009). Typically, dendritic cells sample antigen from the intestinal lumen by reaching through gaps in epithelial cells, or through encounters in M cells. DCs transfer these antigens to Peyer's Patches, which drain to the Mesenteric Lymph Nodes, where they may activate T cells (Figure 1-1). DCs treated with the supernatant of epithelial cell-bacteria cocultures preferentially drove a TH2 or Treg response (Rimoldi, 2005). It has been shown that the influence of gut bacteria on the immune system extends beyond the localized area of the gut. Dobber et al. reported that germ-free mice possessed lower levels of CD4+ T cells in the spleen. These CD4+ cells were also biased to produce IL-4. Therefore, mice lacking gut microbiota are biased to a TH2 phenotype. Commensal bacteria could fix this TH1/TH2 imbalance, as recolonization by *B. fragilis* restored CD4+ T cell numbers and induced expression of IFNγ. Interestingly, this ability was associated with a single product made by *B. fragilis*: polysaccharide A (PSA). Recolonization by *B. fragilis* lacking PSA did not reproduce these results (Mazmanian, 2005).

Probiotics are defined as a class of bacteria capable of surviving the intestinal transit (acid and bile tolerant), adhering to the mucosal surface, and colonizing it for a temporary amount of time. In addition, these bacteria can produce antimicrobial substances, antagonizing pathogenic bacteria. Typically, probiotic bacteria are also associated with well-documented and validated health benefits (Borchers, 2009). Lactobacillus (LB), along with Bifidobacteria, are considered probiotic bacteria. While

the ability to stimulate IL-12 is varied in LB, a majority of the species are able to induce high levels of IL-10 (Fink, 2007). Several LB strains have been noted as potent inducers of APC maturation, which may mediate tolerance through anergy or AICD (Borchers, 2009). Oral feedings of *Lactobacillus casei* prevented T1D in NOD mice, mainly by inducing IL-2 and IL-10 (Matsuzaki, 1997). VSL#3, a probiotic mixture of LB and Bifidobacteria strains, was shown to limit the production of TH1 associated cytokines and induce production of IL-10 from human DCs (Jijon, 2004; Hart, 2004). It also prevented onset of T1D in NOD mice, again through the production of IL-10 (Calcinaro, 2005). LB is also known to improve epithelial cell integrity, limiting the ability of microbes to breach into direct contact with the immune system (Madsen, 2001). Upon LB stimulation, gut DCs have also been shown to be capable of inducing Tregs and IgA production from B cells, which binds up pathogens in the gut (Tezuka, 2007). LB-treated DCs limited inflammation in the gut via a MyD88 (a major protein involved in the recognition of pathogen associated molecular patterns) and TLR2 pathway (Round, 2010). Furthermore, Valladares et al. demonstrated that feeding *Lactobacillus johnsonii* N6.2 (LjN6.2), isolated from diabetes resistant rats, to diabetes prone rats delayed onset of T1D. This delay of T1D was correlated with a TH17 cell bias observable in both the mesenteric lymph nodes and spleen (Lau, 2011). Taken together, this data shows that bacteria have a profound ability to alter the host immune response. In the context of T1D, the probiotic treatment of LjN6.2 may limit the skewing of diabetogenic T cells to a TH1 phenotype, preventing the initiation step of the disease.

Gut Bacteria and T1D

Vaarala et al. have suggested that a series of immunological signs align and contribute to the onset of T1D. The first sign is an altered gut bacteria composition. Gut

bacteria play a major role in this theory, and has been supported by research showing that rodents derived by Caesarian means or kept in germ-free environment develop T1D at an increased pace (Like, 1991). In addition, certain antibiotics decreased the occurrence rate of T1D in NOD mice (Brugman, 2006). Rakoff-Nahoum et al. demonstrated that a low level of activation of MyD88 is beneficial for epithelial cells, rather than detrimental. Activation of the MyD88 pathway by commensal bacteria creates products associated with protection, tissue repair, and angiogenesis (the development of new blood vessels). Among these products, TGFβ and IL-6, a mediator of epithelial cell protection (Tebbutt, 2002), are particularly interesting, as they are involved in TH17 generation. While inflammation may be helpful in small doses, a constant, exacerbated level of gut activation through TLRs could be dangerous. Wen et al. demonstrated that MyD88$^{-/-}$ NOD mice do not develop T1D. However, if these knockouts are kept germ-free, diabetes incidence returns, and is only ameliorated by the addition of bacteria to the digestive tract. Clearly, the interaction between immune cells and gut bacteria can maintain a healthy homeostasis. If environmental factors change the composition of gut bacteria, abnormal immune responses may disrupt the established balance of tolerance and inflammation. Gut leakiness is the second focal point of the “perfect storm” for T1D. Typically, junction proteins, like claudin, keep epithelial cells tight and packed together, sealing off the luminal contents from direct interaction and activation of the immune system. Studies have shown that both rodents and humans display an increased permeability in the gut following recent diagnosis of T1D. Increased exposure to the gut in an uncontrolled manner may set off this autoimmune reaction. An altered intestinal immunity is the final factor in this model. This

may include aberrant immune responses in the gut. For example, lamina propia (the area directly underlying the epithelial cell layer) cells of diabetic patients show elevated levels of MHC Class II molecules and α4β7-integrin, which is necessary to home to the intestines (Savilahti, 1999;Westerholm-Ormio, 2003). In addition, diabetic patients may show decreased levels of Tregs and an increased presence of autoimmune cells in the gut (Tiittanen, 2008). These autoimmune cells, in turn, may release cytokines that promote an inflammatory environment and recruit more immune cells to the affected area. The perfect storm follows the old-friends hypothesis, where the consistent presence of normal commensal bacteria maintains a tolerant environment, possibly stimulating Tregs to produce anti-inflammatory molecules like IL-10 and TGF-β (Rook, 2005). As infiltrating lymphocytes in the islets express the gut homing receptor, α4β7-integrin, it is likely that they circulate from the gut to the pancreas (Yang, 1997; Hanninen, 1996). In addition, DCs carrying intestinal antigen may travel to the pancreatic lymph nodes to stimulate T cells. Therefore, specific interactions or aberrations in the gut can likely influence the development of T1D in the periphery.

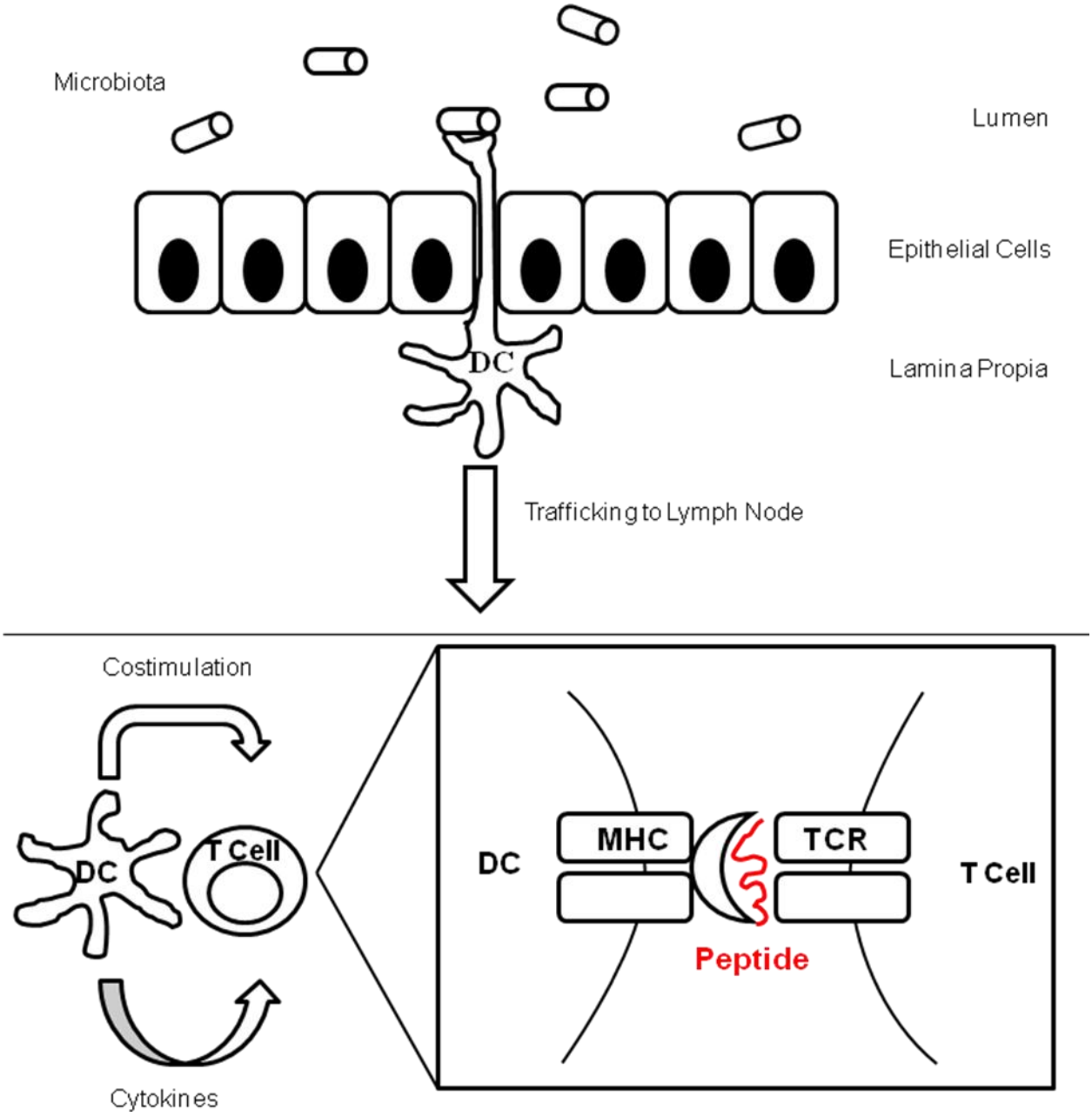

Figure 1-1. Gut antigen sampling and presentation by dendritic cells. Dendritic cells (DC) sample antigen through the epithelial cell layer in the intestines. DC will traffic to various lymph nodes displaying the broken down peptide on its major histocompatability complex. T cells that possess a T cell receptor (TCR) specific for the peptide will become activated while the DC provides costimulation and a cytokine milieu.

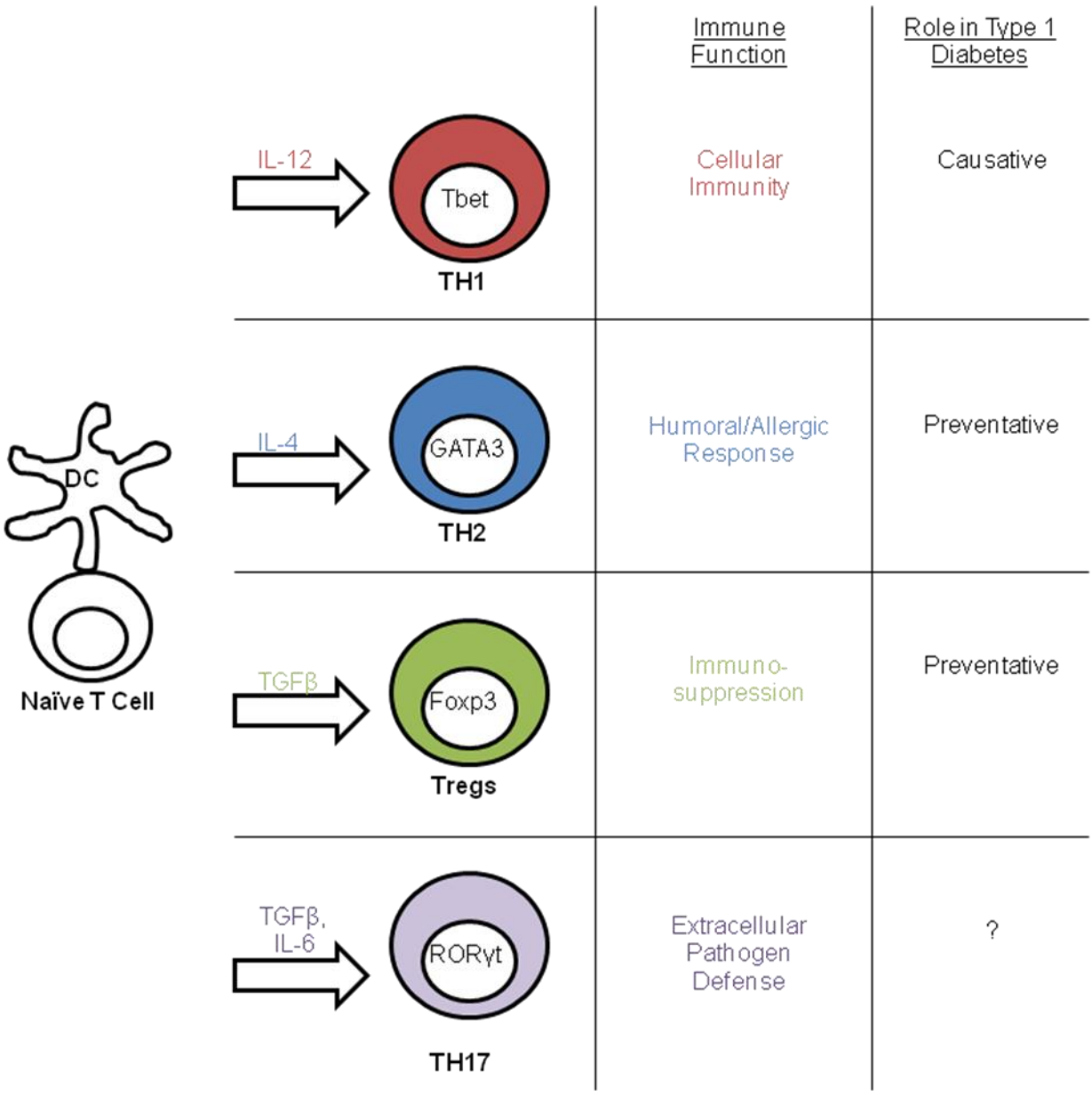

Figure 1-2. T helper cell differentiation. Following activation by DCs, naïve CD4+ T cells will differentiate into different T Helper classes depending on the cytokines presented to it. Each T Helper class is listed with its hallmark cytokines required for differentiation, its main transcription factor, and its function in the immune system.

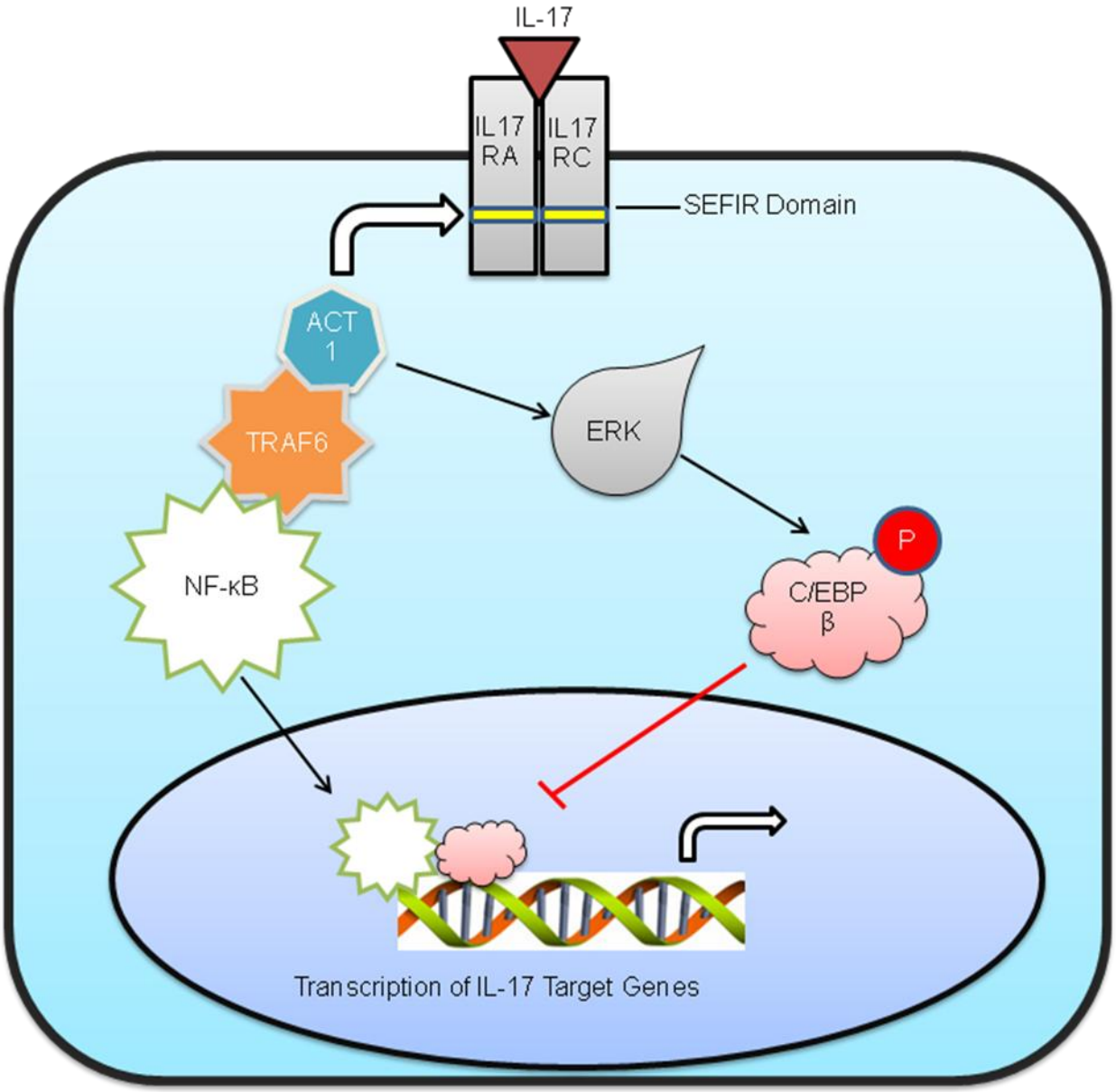

Figure 1-3. IL-17 signaling. The IL-17R consists of IL-17RA and IL-17RC. Upon ligation, ACT1 is recruited to the SEF/IL-17R (SEFIR) domain. ACT1 recruits TNFR-associated factor 6 (TRAF6). TRAF6 leads to the activation of Nuclear Factor κB (NF- κB). Act1 also activates extracellular signal-regulated kinase (ERK), which phosphorylates CCAAT/enhancer binding protein β. When it is phosphorylated, its ability to promote transcription decreases. This is one of the few inhibitory activities IL-17 is known for.

CHAPTER 2
MATERIALS AND METHODS

Characterization of a TH17 Bias Induced by LjN6.2

Animals

C57BL/6 mice (The Jackson Laboratory®, Bar Harbor, ME) were maintained in specific pathogen-free conditions at the Association for Assessment and Accreditation for Laboratory Animal Care–accredited University of Florida under the supervision of the Institutional Animal Care and Use Committee in strict accordance to approved protocols.

Bone Marrow Derived Dendritic Cell Preparation

Bone marrow was removed from the femur and tibia bones of NOD mice and washed. Progenitor cells were subsequently incubated in RPMI 1640 supplemented with 10% FBS, 1% antibiotic/antimycotic, and GM-CSF (20 ng/mL) in 24-well plates (1×10^6 cells/well). Old medium was removed and replaced with 1 ml fresh complete RPMI 1640 medium containing 20 ng/mL GM-CSF every 2 d. On day 8, aggregates were dislodged and transferred with complete RPMI 1640 medium into 100-mm petri dishes at a maximum of 1×10^7 cells/dish. At 24 and 48 h time points following transfer, nonadherent, nonproliferating, maturing bone marrow-derived DCs (BMDCs) were collected from the dish and stored in a sterile flask. 70% of the BMDC preparation was identified as expressing CD11c through flow cytometry. Prior to footpad injection into NOD mice, some BMDCs were incubated with LjN6.2 for 12 h, followed by extensive washing.

Dendritic Cell Vaccinations

Nine-week-old NOD mice received three weekly hind paw injections of either PBS or LjN6.2-pulsed BMDCs (5×10^5). Mice receiving the injections were sacrificed on week 13 of life, followed by isolation of pancreas, spleen, and various lymph nodes.

T Lymphocyte Purification

Lymph node and splenocytes from C57BL/6 mice were stained with biotin-conjugated Abs to mouse B220, CD11c, CD11b, and NK1.1 for 10 min at 4°C, followed by streptavidin-conjugated MACS beads®, followed by passage over a MACS LS column® according to the manufacturer's protocol (Miltenyl Biotec®, Bergisch Gladbach, Germany) (Guay, 2007). The negatively selected fraction contained $CD3^+$ lymphocytes (>95% pure as confirmed by FACS), and the positively selected fraction served as APCs. Of the APC fraction, the populations typically consist of 72% B cells (B220+), 14% macrophages (CD11b+), and 14% dendritic cells (CD11c+). The percentage of NK cells has not been determined by our group.

Proliferation Assays

Lymphocyte proliferation assays were performed, as previously described (Larkin III, 2007), with modifications. Total splenocytes (4×10^5) or 2×10^5 MACS-purified T lymphocytes were incubated with 4 µg/mL anti-CD3 (clone 17A2; eBioscience®, San Diego, CA), 2×10^5 APCs, or 0.5 µg/mL anti-CD28 (clone 37.51; eBioscience®) in the presence of LjN6.2 at various concentrations, as indicated. Cell cultures were incubated in supplemented RPMI 1640 (10-040-CV; Cellgro®, Manassas, VA) containing 10% FBS (10082-147; Life Technologies®, Carlsbad, CA) and 1% antibiotic/antimycotic (30-004-CI; Cellgro) in 96-well round-bottom plates. After 72 h of incubation, cultures were pulsed with 0.5 mCi [^{3}H] thymidine (GE Healthcare®, Arlington Heights, IL) per well and

harvested 16–18 h later. [^{3}H] Thymidine incorporation was measured using a Beckman LS3801 Liquid Scintillation System™.

In Vitro Cytokine Secretion Analysis

Leukocytes from C57BL/6 mouse spleen and lymph nodes were isolated and coincubated with 4 µg/mL anti-CD3, APCs, or 0.5 µg/mL anti-CD28 (BD Biosciences®, San Diego, CA), and LjN6.2 or LrTD1 at various concentrations. To inhibit IL-6 signaling, leukocytes were also coincubated with anti-CD3, APCs, and LjN6.2 in the presence or absence of an IL-6 signal-neutralizing Ab mixture containing the following: 0.2 mg/mL rabbit polyclonal to IL-6 (Abcam®, Cambridge, MA), 2 µg/mL anti-mouse IL-6 (554398; BD Biosciences), and 10 µg/mL anti-mouse CD126 (554459; BD Biosciences) for 48 h. At various time points, 100 µL supernatant was removed from each well and replenished with fresh medium, as previously described (Lerman, 2004). Cytokine ELISAs were subsequently performed on harvested supernatants. IFNγ (555138; BD Biosciences®) and IL-6 (555240) ELISA kits were obtained from BD Biosciences®. Capture mAb (555068) and detection mAb (555067) for IL-17A were also obtained from BD Biosciences®. Cytokine standard for IL-17 was purchased from eBioscience® (14-8171-80).

Role of TH17 Cells in an Autoimmune Diabetes Setting

Animals

NOR/LtJ and NOD/ShiLtJ (The Jackson Laboratory®, Bay Harbor, ME) were maintained in specific pathogen-free conditions at the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC) accredited University of Florida under the supervision of Institutional Animal Care and Use Committee (IACUC). Blood glucose levels of NOD/ShiLtJ mice were monitored using a blood glucose monitoring kit.

NOD mice were defined as diabetic following repeated blood glucose levels over 250 mg/dL for two consecutive days. NOD mice were euthanized following diabetes onset. Axillary lymph nodes, mesenteric lymph nodes, pancreatic lymph nodes, and spleens were extracted from each mouse for *in vitro* or *ex vivo* analysis.

Flow Cytometry

Single cell suspensions of pooled lymph nodes (axillary, inguinal, brachial, mesenteric, and superficial cervical), and spleen were stained with the following mAbs for flow-cytometric analysis: anti-CD4-Pacific Blue (RM4-5;), anti-MHC I-FitC (KH95), anti-CD11c-PE (N418; eBioscience®, San Diego, CA), anti-B220-APC (RA3-6B2, eBioscience®), anti-CD11b-A700 (M1/70), anti-CD11b-FitC (M1/70, eBioscience®), anti-CD11c-FitC (N418, eBioscience®), anti-CD86-A700 (GL1), anti-CD80-PE (16-10A1), DEC 205-APC (205yekta) and anti-MHC II-FC (39-10-8,) mAb. All flow cytometry antibodies were purchased from BD PharMingen® unless otherwise stated. 50,000-100,000 live events were collected on a LSRII (BD PharMingen®) and analyzed using FlowJo™ software (Tree Star, San Carlos, CA). The absolute numbers of cells recovered from various organs was determined by multiplying the total number of cells isolated from various tissues by the percentage of total cells bearing a lineage specific marker denoted by flow cytometry.

Pancreas RNA Isolation/Histology

Pancreas was harvested from NOD and NOR mice and snap frozen in OCT (Fisher®, 14-373-65, Pittsburgh, PA) embedding medium in a dewer of liquid nitrogen and 2-methylbutane (Fisher®, O3551-4). Blocks were sectioned on a Leica CM 1950 cryostat at a thickness of 40 microns. RNA was then isolated using the Arcturus PicoPure™ RNA Isolation Kit (Applied Biosystems®, KIT0204, Carlsbad, CA) and

protocol. Purity of RNA was confirmed using a Nanodrop ND 1000 Spectrophotometer™. 5 micron sections were cut and stained for H&Es. Photos were taken at 20x magnification using the Leica DM 2500 Microscope™ equipped with an Optronics™ color camera and MagnaFire™ software (Optronics®, Goleta, CA).

RNA Isolation and RT-qPCR.

Total RNA was extracted from the lymph nodes and spleens of NOD or NOR mice using the SV Total RNA Isolation System™ (Promega®, Corp., Madison, WI, USA), according to the manufacturer's recommended spin column extraction protocol. The concentrations and purity of the total RNA were determined using a SmartSpec Plus Spectrophotometer™ (BioRad®, Hercules, CA, USA). First-strand cDNA synthesis was performed using ImProm-II Reverse Transcription System™ (Promega®, Corp., Madison, WI, USA) or iScript RT Supermix for RT-qPCR™ (BioRad®, 170-8841). Absolute QPCR SYBR Green Mix™ (ABgene Epsom®, Surrey, UK) or iQ SYBR Green Supermix Sample™ (BioRad, 170-8880S) and gene specific primers (Table 1) at 200nM were used to amplify relative amounts of cDNA on a PTC-200 Peltier Thermal Cycler™ with a CHROMO 4 Continuous Fluorescence Detector™ (BioRad®). Amplification was performed as previously described (Lau, JI 2011). The fold-change in expression was calculated using the double ΔC_T method (i.e. using the equation $2^{-\Delta\Delta CT}$) using BioRad® software.

Bacterial Enumeration and Cell Lysis

E. coli, L. brevis, L. johnsonii N6.2, and *L. reuteri* TD1 strains of bacteria were all provided courtesy of Dr. Graciela Lorca. Briefly, bacterial cells were lysed by treatment with 0.1 mm glass beads in a bead beater for 3 minutes. Cells were then centrifuged at 100,000 g for 15 minutes to separate the membrane (pellet) and cytoplasm

(supernatant) fractions. Concentration of bacteria was determined by performing ten-fold serial dilutions, plating 0.1 mL of the sample on MRS plates. Plates were incubated for 48 hours at 37^0C under anaerobic conditions.

Statistical Calculations

Statistically significant differences were determined using Graph Pad Prism® software using an unpaired, two-tailed student t test. Significance and statistics for studies involving diabetes incidence were determined using the Gehan-Breslow-Wilcoxon test.

CHAPTER 3
RESULTS

Characterization of a LjN6.2 Mediated TH17 Bias

LjN6.2 Induces Apoptosis at High Concentrations

Valladares et al. demonstrated that feeding diabetes prone rats with LjN6.2 was capable of mitigating T1D onset. While the beneficial effects of probiotics have frequently been demonstrated, they have not always been explained mechanistically. And, while there have been studies on the effects of LjN6.2 on onset of T1D in diabetes prone rats (Valladares, 2010), the same has not been done in NOD mice. It is particularly important to use the NOD model as it shares several of the genetic defects found in T1D susceptible humans (see *Idd* genes listed above). Therefore, it is vital to understand how LjN6.2 can affect the onset of T1D, beginning with its *in vitro* effects on mouse immune cells. Several studies have stated that certain strains of Lactobacillus are capable of suppressing cytokine production by immune cells. In accordance, we initially observed decreased IFNγ and IL-6 production with increasing concentrations of LjN6.2. In addition, there was a severe reduction in proliferation in splenocytes treated with higher concentrations of LjN6.2. We next wanted to determine whether the reduced cytokine profile was due to active suppression by LjN6.2 or whether it was causing cell death. Leukocytes were incubated with LjN6.2, *Lactobacillus brevis*, *E. coli* (a gram negative control bacteria), or antiCD3 control. At the 48 hour time point, the cells were stained for Annexin V, an early marker of apoptosis. As seen in Figure 3-1, LjN6.2 induced a higher level of Annexin V expression than any other sample set. In addition, an increasing concentration of LjN6.2 corresponded to increased expression of Annexin V in both CD4+ and CD8+ T cells. As the killing of all immune cells provides little

insight into mechanism and serves as a detriment to the host, future experiments scaled back to LjN6.2 concentrations that yielded immune responses and reflected physiological concentrations, but did not cause overwhelming cell death.

T Cells Require TCR Stimulation to Create a TH17 Response to LjN6.2

Oral feedings of LjN6.2 were capable of preventing onset of T1D in diabetes prone rats. Lau and Benitez et al. demonstrated that these rats had elevated levels of several TH17-related factors in the localized region of the mesenteric lymph nodes. Additionally, this effect was not restricted to the gut, as splenocytes also displayed this increase. Therefore, we characterized what factors are required for generating a TH17 bias. Studies have shown that T cells are capable of directly reacting to bacterial agonists through the use of Toll Like Receptors, which recognize common patterns expressed by bacteria and other microbiota (McAleer, 2010). Therefore, we incubated splenocytes with increasing concentrations of LjN6.2 in the absence of antiCD3, which stimulates T cells through the T cell receptor, mimicking the act of antigen presentation by APCs (Leo, 1987). As seen in Figure 3-2, minimal proliferation was recorded in response to LjN6.2 without antiCD3 stimulation. As APCs are the main producers of IL-6 and would be unaffected by a lack of antiCD3 (Diehl, 2002), IL-6 is generated in increasing amounts as higher doses of LjN6.2 are added (until the CFU/mL crosses a concentration threshold and induces cell death). However, despite the presence of the TH17 differentiation factor, IL-6, T cells did not respond by producing IL-17 in the absence of antiCD3. And, at higher concentrations of LjN6.2, no proliferation or cytokine production was seen, confirming that these doses likely killed all cultured cells, as indicated in Figure 3-1.

To test whether T cells require TCR stimulation to respond to LjN6.2, we cultured splenocytes and lymphocytes with antiCD3 (4 µg/ml). The cells were also incubated with either LjN6.2 or *Lactobacillus reuteri* (LrTD1), the strain that increased T1D incidence in diabetes-prone rats. Over the course of 48 hours, cells treated with antiCD3 and LjN6.2 responded with marked increases in IL-6 beginning as early at 12 hours of incubation. By 48 hours, LjN6.2 treated cells generated over 3 times as much IL-6 as antiCD3 controls (Figure 3-3A). Following IL-6 production, IL-17 protein is induced at 36 hours. By 48 hours, the IL-17 output by LjN6.2-stimulated cells is nearly 4 times the amount made in antiCD3 alone controls. While LrTD1 was capable of creating a small boost in IL-17, it is considerably less than its Lactobacillus counterpart, LjN6.2. In addition, we demonstrated that this IL-17 production was dependent on IL-6 (Serada, 2008). An antibody cocktail against IL-6 caused a 30% decrease in IL-17 production in LjN6.2 stimulated cells.

APCs are Required to Create a LjN6.2-Mediated TH17 Bias.

IL-6 is an important factor for TH17 differentiation. While APCs are generally the main producers of IL-6, T cells have also shown to be capable of making IL-6 (Sofi, 2009). In order to determine whether properly stimulated T cells alone could generate a TH17 bias, we separated T cells from APCs on a MACS separation column. T cells cultured alone were incubated with antiCD3 and antiCD28, as proper costimulation in the absence of APCs is required for a T cell response (Clark, 1987). T cells incubated with APCs were given antiCD3 as described before. Both sample sets were co-cultured with LjN6.2 in increasing concentrations. As seen in Figure 3-4, both T cells alone and T cells with APCs were capable of proliferating at equal rates, as shown by trititated thymidine incorporation. Isolated T cells were also competent in IFNγ production.

However, T cells were unable to produce a strong IL-6 response in the absence of APCs. The presence of APCs allowed for a dose-dependent increase in IL-6 production. In addition, without APCs, very low levels of IL-17 were recorded. This data shows that in order to create a favorable TH17 environment, APCs are required for the generation of IL-6, which causes robust IL-17 production by T cells.

BMDCs are Capable of Promoting a Long-Term TH17 Bias

Dendritic cells are the main samplers of gut lumen and potent activators of T cells (Leser, 2009). In addition, several studies have reported that BMDC transfers into NOD mice were capable of preventing onset of T1D. Therefore, we examined whether LjN6.2 could mediate a TH17 bias using DCs as the main APC. BMDC precursors were isolated from bone marrow and matured. BMDCs were co-incubated with pure, CD3+ T cell populations and a dose of LjN6.2 where indicated (Figure 3-5). As we have previously shown, the lack of TCR stimulation did not yield IL-17 production, while the DCs were still able to produce IL-6. With the addition of LjN6.2, BMDCs were provided higher levels of IL-6 and were sufficient to provide a pro-TH17 environment to allow for increased IL-17 production.

As our treatment of BMDCs was sufficient to create a TH17 bias, we transferred LjN6.2 pulsed BMDCs (LJ-BMDC) into 9 week old NOD recipient mice. A set of mice were sacrificed at 13 weeks of age, when all mice were still confirmed euglycemic and non-diabetic. mRNA was isolated from the spleens of the sacrificed mice and tested for TH17 factors. Mice receiving LJ- BMDCs exhibited increased message for both IL-6 and IL-17 compared to control-treated mice (Figure 3-6).

TH17 cells have been reported as notoriously fickle in plasticity. They have been shown to convert into TH1 cells following transfer into NOD.SCID mice (Bending, 2009).

In order to determine the stability of the TH17 environment induced by LJ-BMDCs, we observed long-term LJ-BMDC recipient mice. NOD mice were allowed to live until they were determined diabetic by blood glucose levels. Mice were sacrificed at 20 weeks of age if they were still euglycemic at the end of our time course. Splenocytes were plated *in vitro* and supernatants were analyzed at 48 hours for cytokine production by ELISA. PBS-control splenocytes produced no IL-6 or IL-17 without antiCD3 stimulation. Interestingly, splenocytes of LJ-BMDC mice produced large amounts of IL-6 and IL-17 even without TCR stimulation, in contrast to our *in vitro* work shown above. And, while PBS control mice do produce IL-6 and IL-17 with the addition of antiCD3, LJ-BMDC splenocytes produce significantly higher levels of both cytokines (Figure 3-7). We also investigated whether LJ-BMDC could prevent onset of T1D. While LJ-BMDC slightly delayed onset of T1D, there was no difference in prevention of T1D in the two treatments by 20 weeks of age (Figure 3-8).

The Role of TH17 Cells in an Autoimmune Diabetic Setting

NOD Mice Display a TH17 Deficiency Compared to NOR Mice

While we have previously established a role for TH17 cells in the BBDP rat (Lau, 2011), their role in the NOD mouse is still being investigated. The NOD mouse is the preferred method of T1D study, as it allows for easy genetic manipulation and avails itself to a large range of reagents. The NOR mouse serves as the non-diabetic counterpart to NOD mice. Originally derived from NOD mice backcrossed with C57BL/6 mice, the NOR mouse still shares several defects associated with the NOD mouse, i.e., the prototypical diabetogenic MHC II^{Ag7} and defective Treg populations (Serreze, 1994). Yet despite these deficiencies, the NOR mouse does not develop T1D and serves as a control for the NOD mouse. To begin, we investigated the natural capability of NOD

lymphocytes to create a TH17 bias. We sacrificed prediabetic NOD mice and compared them to NOR mice. Lymph node cells were plated *in vitro* with antiCD3. At 48 hours, the supernatants were analyzed for cytokine secretion. There were no significant differences in proliferation or IFNγ between NOD and NOR lymphocytes (Figure 3-9). However, NOD lymphocytes produced no IL-6 and lower amounts of IL-17 compared to NOR mice. While NOD mice are competent in regards to TH1 cytokine production, they lack the same ability to produce TH17 factors.

In addition to examining the peripheral lymph nodes, we focused on the autoreactive site of the pancreas. Pancreas from 11 week old age-matched mice were snap frozen in liquid nitrogen and Optimal Cutting Temperature (OCT) liquid. This method allows for reliable RNA isolation. In addition, we cut thin sections for hematoxylin and eosin staining, providing a snapshot of possible infiltrating leukocytes. In agreement with published data, Figure 3-10 shows that NOD mice display massive insulitis, with leukocytes encroaching upon islets. While NOR mice also display infiltration, they do not exhibit insulitis. qPCR confirmed that NOD mice tend to display elevated levels of CD3+ cells compared to NOR counterparts. To restrict our qPCR to T cell messages, we used CD3 as a reference gene and examined the pancreas for several TH17-relevant messages (Figure 3-11). IFNγ production was not significantly different, while IL-17 began to trend toward significance. Similar to what was observed in the peripheral lymph nodes, NOD mice display lower levels of IL-17. They also show lower levels of IL-6 and importantly, RORγt, the hallmark transcription factor for TH17 cells. Despite possessing less T cells, the NOR pancreas expresses significantly higher levels of TH17-associated cytokines and transcription factors.

NOD Lymph Nodes Contain Lower Quantity of APCs Compared to NOR

We next determined whether the lack of IL-17 production in the lymph node was due to a lack of T cells in the NOD mouse. Peripheral lymph nodes (axillary, mesenteric, cervical) from the NOD mouse were pooled and compared to NOR lymph node cells with flow cytometry. As seen in Figure 3-12A, there were no statistical differences between either CD4+ or CD8+ populations in the two strains of mice. We then decided to focus on APCs, and measured their percentages by staining for CD11b+ (macrophages), CD11c+ (DCs), and B220+ (B cells). Figure 3-12B shows that the NOR lymph nodes possess 1.6 times as many APCs as the NOD mouse. The NOD lymph nodes display an imbalance in the T cell to APC ratio, which may affect its ability to create TH17 cells.

LjN6.2 Increases NOD APC Numbers and Maturation

Previous work has shown that NOD APCs suffer from several defects. BMDCs reportedly generate lower yields following harvesting from bone marrow. In addition, they can develop macrophage-like qualities. NOD APCs are also less adept at activating T cells through antigen presentation. In addition, even upon LPS stimulation, NOD APCs do not mature properly (as indicated by upregulation of MHC markers and costimulatory molecules) (Strid, 2001). With all of the deficiencies listed in NOD APCs, we sought to determine whether treatment with LjN6.2 could alleviate some of the issues. Splenocytes were incubated with antiCD3 and a concentration of either LjN6.2 or LrTD1 where indicated. Flow cytometry was performed, staining the cells for MHC markers, costimulatory molecules and other maturation markers (DEC 205). Treatment of APCs with LjN6.2 increased CD11b+ expression, increasing from 11.4% to 17.4% at the highest concentration (Figure 3-13). A similar trend was observed in CD11c+

expression, increasing from 12.7% to 18.9%. Our data indicates LjN6.2 treatment may be capable of restoring the lack of APCs found in the NOD lymph nodes.

With the observed increase in APC numbers, we next investigated whether LjN6.2 treatment could restore APC antigen presentation and maturation. Figure 3-14 demonstrates that LjN6.2 treatment increased MHC class I and II expression on both macrophage and dendritic cell types. In both cell types, the percentage of MHC class I and II expressing cells were nearly doubled. LrTD1, which increased diabetes onset in a rat model, was incapable of generating the same type of reaction, as the levels of MHC I and II remained relatively unchanged.

As we observed an increase in MHC II, we next examined DEC 205. DEC 205 is dendritic cell surface receptor associated with the upregulation of MHC II, cell maturation, and receptor-mediated endocytosis (Inaba, 1995). As we increased the concentration of LjN6.2, we noticed a corresponding drop in the expression of DEC 205 (Figure 3-15). Overall, there was an 18% drop in the amount of cells expressing DEC 205. Once again, this effect was not seen with LrTD1 treatment of APCs. DEC 205 was likely being internalized following stimulation with LjN6.2, transferring antigen internally and promoting the upregulation of MHC II.

LjN6.2 Triggers BMDC Immunity Through a Surface Antigen

As we have seen several immunogenic responses to LjN6.2, we next attempted to determine what antigen was stimulating APCs. LjN6.2 was lysed through the use of a bead beater and glass beads. The membrane and cytoplasm fractions were then separated by using an ultracentrifuge at 100,000g for 15 min. The pellet was considered the membrane fraction, while the supernatant was considered the cytoplasm fraction. We then treated NOD BMDCs with either whole bacteria cells, the membrane fraction,

or the cytoplasm fraction. Following 48 hours of incubation, supernatant from the culture was analyzed for IL-6 protein by ELISA. As seen in Figure 3-16A, untreated BMDCs did not respond by making any IL-6. Interestingly, the cytoplasm fraction also elicited very low levels of IL-6 from BMDCs. Only the membrane fraction of LjN6.2 was capable of mediating a high level of IL-6 production comparable to the unlysed bacterial fraction. In addition, we confirmed that the response elicited by the membrane fraction was not because a high concentration of bacteria survived the lysis process. As seen in Fig. 3-16B, membrane fractions of LjN6.2 showed no CFU/mL on the 10^6 dilution on MRS media, whereas whole LjN6.2 had a count of 23. Our data indicates a surface antigen likely triggers the observed immune responses and changes we have listed above. Given all the data we have obtained concerning the interaction between LjN6.2 and immune cells, a preliminary model has been created (Fig. 3-17) and will be discussed later on.

Table 3-1. Primers used and/or discussed in this study.

Primer	Sequence	Annealing Temperature (°C)
Actin	F: 5'-CCT TCC TTC TTG GGT ATG CA-3	55
	R: 5'-GGA GGA GCA ATG ATC TTG AT-3'	55
Actin (Pancreas Only)	F: 5'-CCA CAG CAC TGT AGG GTT TA-3'	55
	R: 5'-ATT GTC TTT CTT CTG CCG TTC TC-3’	55
CD3	F: 5'-GAC CTG ACA GCA GTA GCC AT-3'	55
	R: 5'-CTC CTT GTT TTG CCC TGT GG-3'	55
IFNγ	F: 5'-AAC TAT TTT AAC TCA AGT GGC AT-3'	55
	R: 5'-AGG TGT GAT TCA ATG ACG-3'	55
IL-6	F: 5'-GGA AAT GAG AAA AGA GTT GTG C-3'	57
	R: 5'-CTC CAG AAG ACC AGA GGA AAT-3'	57
IL-23p19	F: 5'-GCT CTC TCG GAA TCT CT-3'	55
	R: 5'-AAG CAG AAC TGG CTG TTG T-3'	55
IL-23R	F: 5'-CAG AAA ATT GGA AGT TGG GAT ATG TT-3'	55
	R: 5'-CAG AAA ATT GGA AGT TGG GAT ATG TT-3'	55
IL-17A	F: 5'-ACT CTC CAC CGC AAT GA-3'	55
	R: 5'-CTC TTC AGG ACC AGG AT-3'	55
RORγt	F: 5'-ACA GCC ACT GCA TTC CCA GTT T-3’	63
	R: 5'-TCT CGG AAG GAC TTG CAG ACA T-3'	63

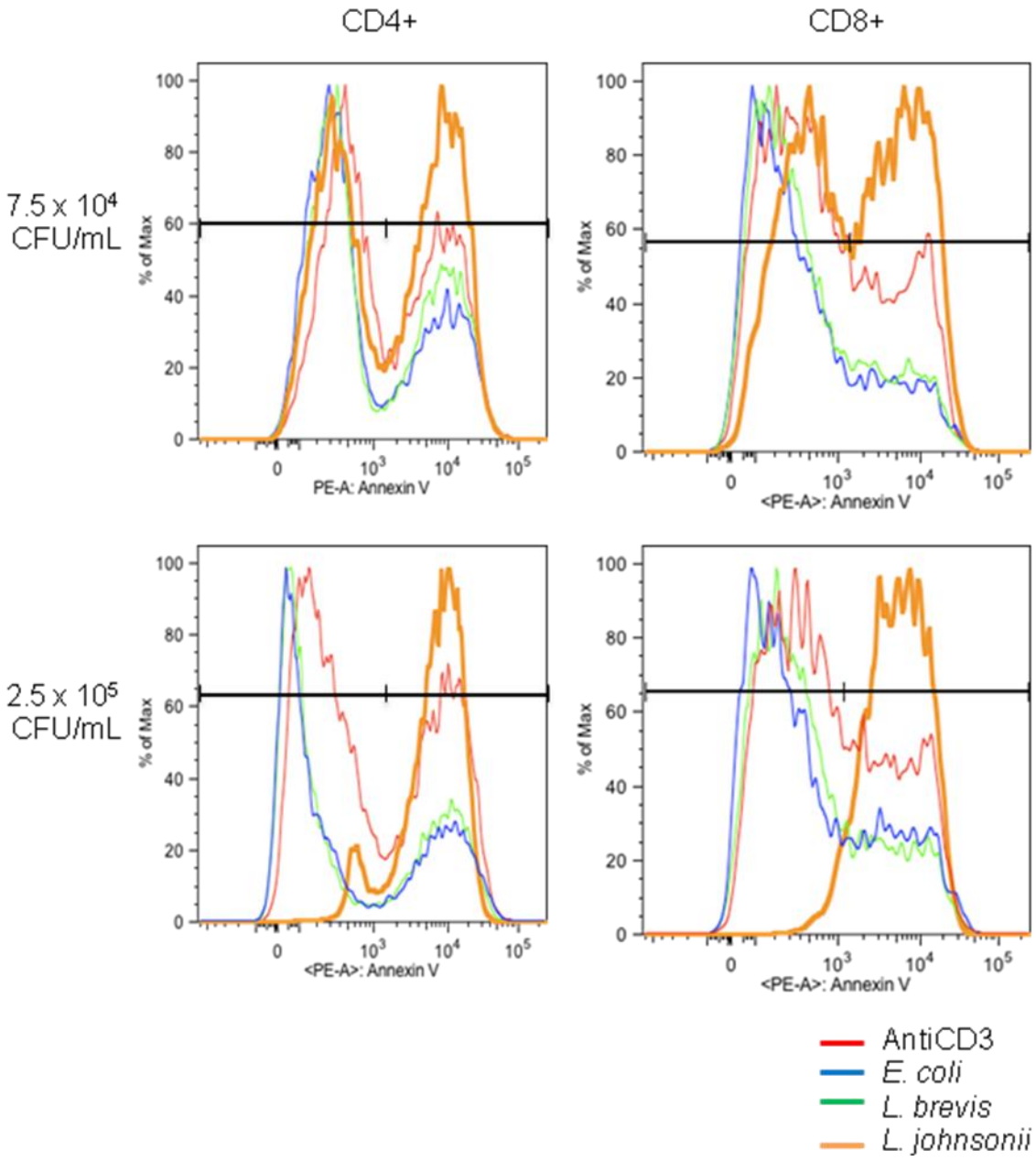

Figure 3-1. *Lactobacillus johnsonii* induces apoptosis at high end concentrations. Splenocytes and lymphocytes were incubated at a concentration of 4 x 10^5 cells/well for 48 hours with 4 µg/mL antiCD3 and an indicated dose of bacteria where indicated. Cells were then stained with CD4+, CD8+, and Annexin V antibodies for FLOW cytometry.

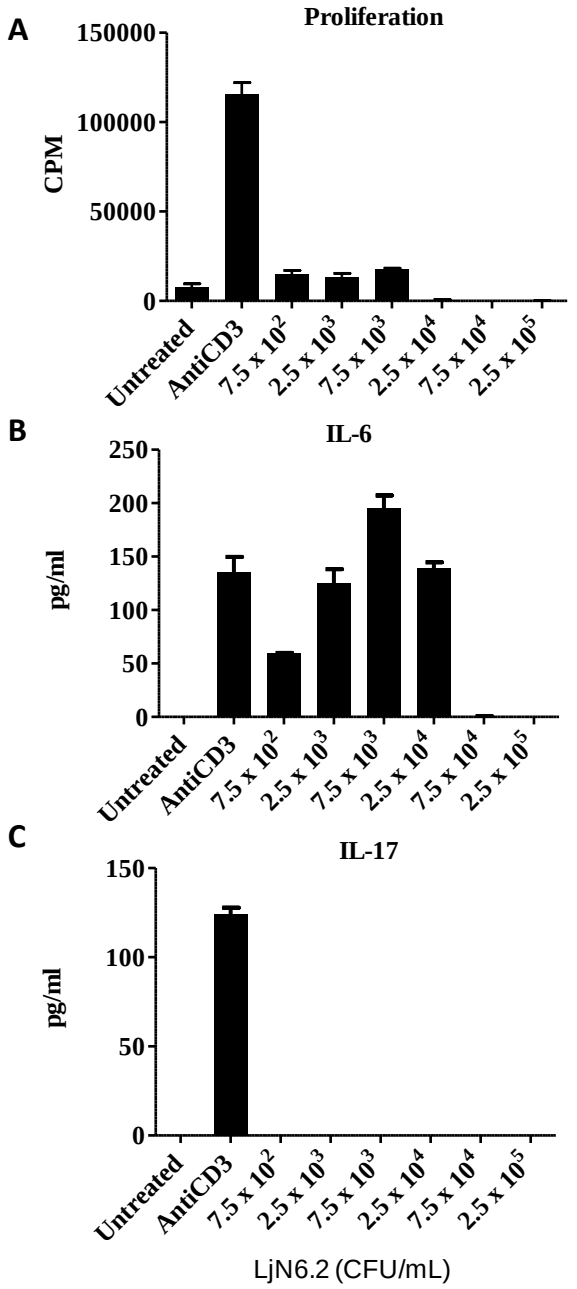

Figure 3-2. LjN6.2 mediation of a TH17 bias requires proper TCR stimulation. Graphs show (A) proliferation, (B) IL-6 production, (C) or IL-17A production by leukocytes (4 x 10^5 cells/well) incubated with antiCD3 (4 µg/mL) or indicated concentrations of LjN6.2 for 48 hours. Supernatants were removed at 48 hours for ELISAs, while tritiated thymidine was administered at 72 hours and incubated for 16-18 hours. Data is representative of 3 separate experiments.

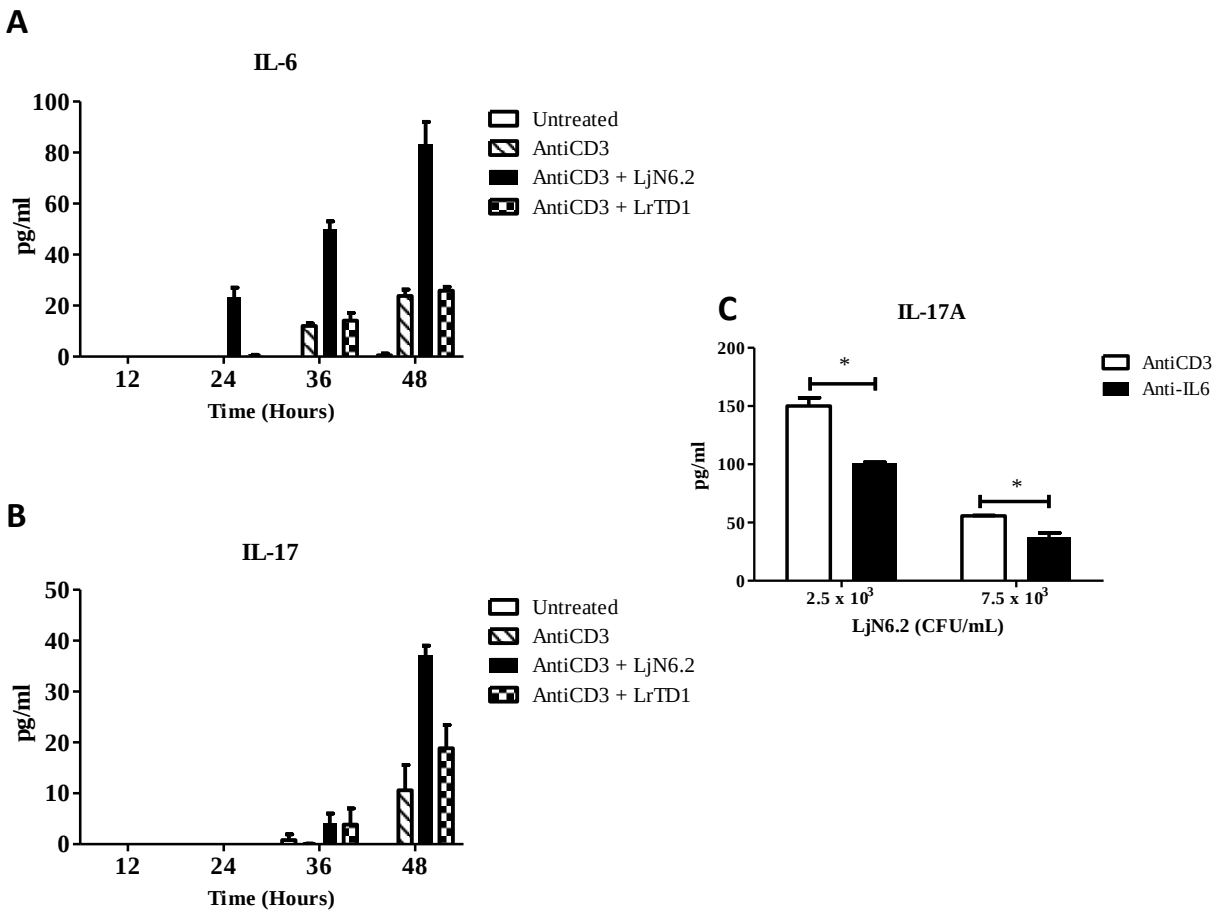

Figure 3-3. LjN6.2 boosts production of TH17 related cytokines in a time dependent manner. Leukocytes were incubated in the presence of anti-CD3 (4 µg/mL), and/or LjN6.2, LrTD1 (7.5×10^3 CFU/mL) for the indicated amounts of time. Graphs show A) IL-6 or B) IL-17 production mediated by the presence of LjN6.2 or LrTD1 over the indicated time periods. C) Splenocytes were incubated at 4×10^5 cells/well for 48 hours with the indicated concentration of LjN6.2 in the presence or absence of an anti-IL6 antibody cocktail. Supernatants were removed at 48 hours for ELISAs. Data is representative of 3 separate experiments. *=$p<0.05$

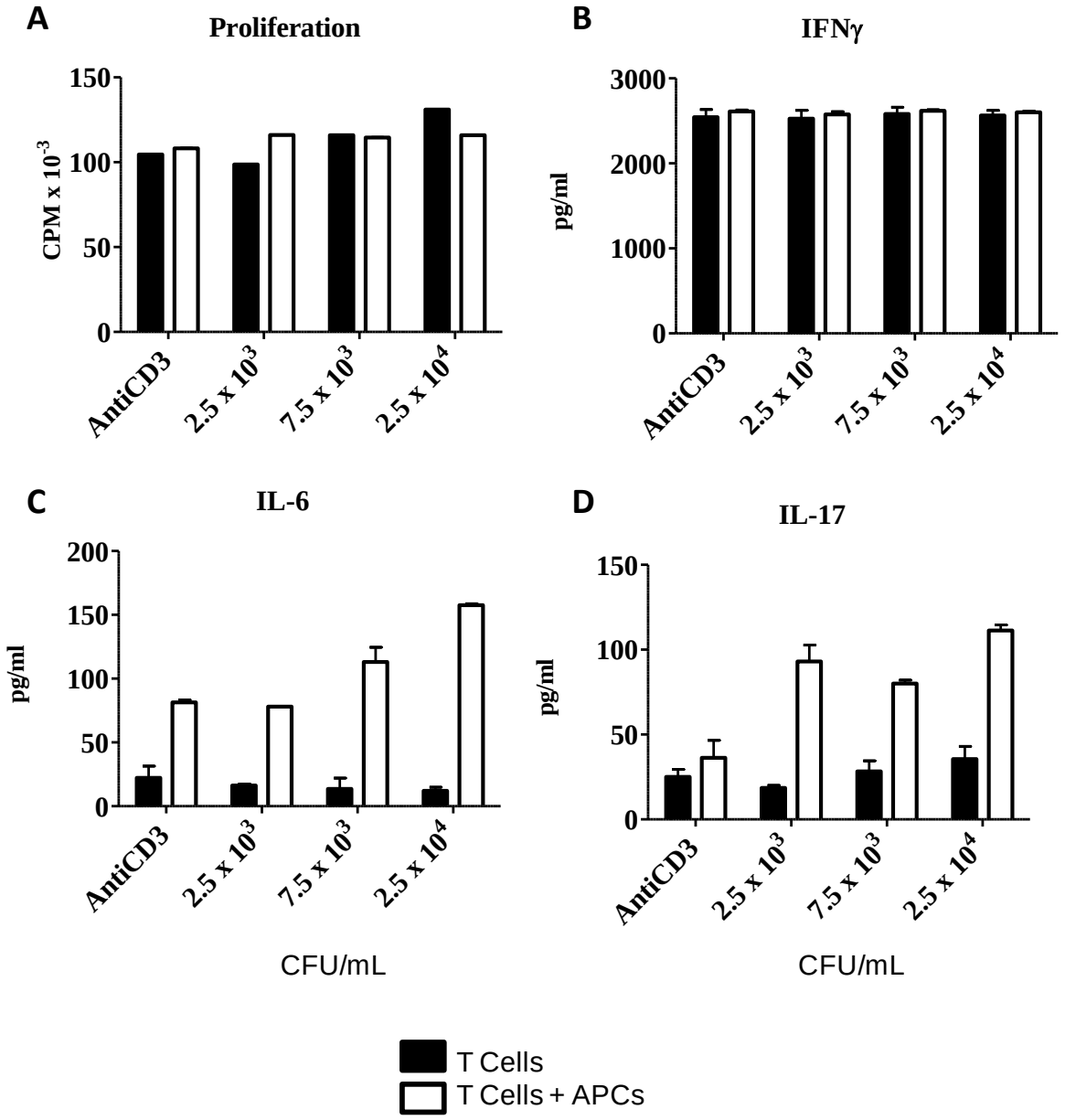

Figure 3-4. LjN6.2-mediated TH17 bias requires the presence of APCs. Leukocytes were sorted on a MACS column. T cells only samples were incubated alone (4×10^5) with 4 µg/mL antiCD3 and 2 µg/mL antiCD28. T cell and APC samples contained 2×10^5 cells each of T cells and APCs in the presence or absence of anti-CD3 (4 µg/mL). A) Tritiated thymidine was administered at 72 hours and incubated for 16-18 hours to analyze proliferation. Supernatants were removed at 48 hours for B) IFNγ, C) IL-6, or D) IL-17 analysis through ELISA. Data is representative of 3 independent experiments.

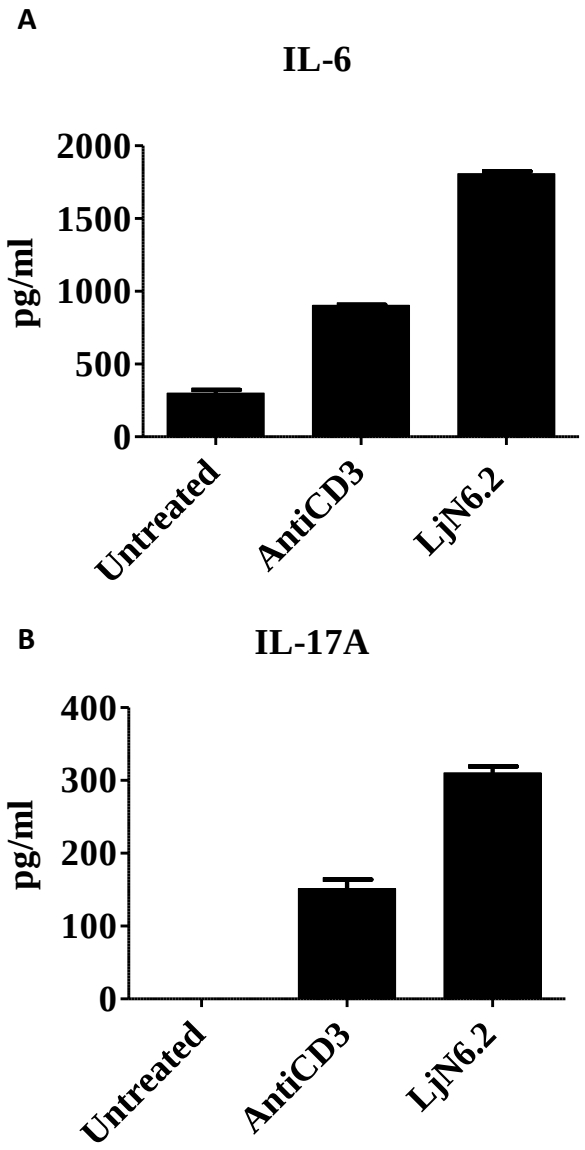

Figure 3-5. BMDCs are sufficient to drive a LjN6.2-mediated TH17 bias. Graph shows A) IL-6 and B) IL-17 production (as measured by ELISA) mediated by the incubation of NOD-derived BMDCs and T cells together with antiCD3 (4 µg/mL) and LjN6.2 (7.5×10^3 CFU/mL) where indicated for 48h. Data is representative of 3 separate experiments.

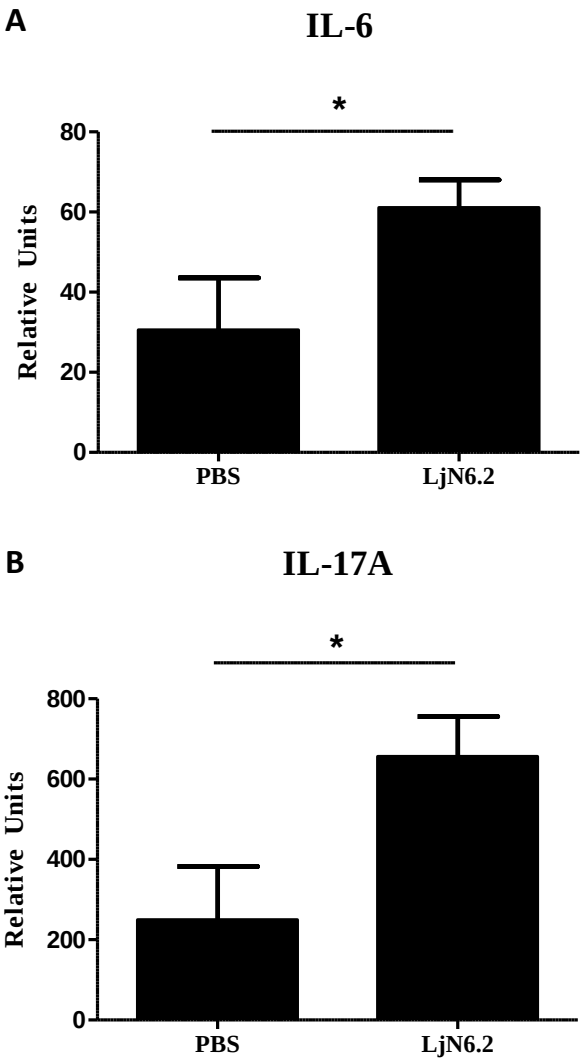

Figure 3-6. LjN6.2-pulsed BMDCs mediate a TH17 bias in recipient NOD mice. 9 week old NOD mice received 3 weekly injections of 5×10^5 LjN6.2 pulsed BMDCs or PBS controls. At 13 weeks of age, the mice were sacrificed and the spleens were obtained for RNA isolation. Graph shows IL-6 and IL-17 message in the spleens of NOD mice receiving LjN6.2–DC vaccination compared with PBS-treated control mice. N =3. *=$p<0.05$

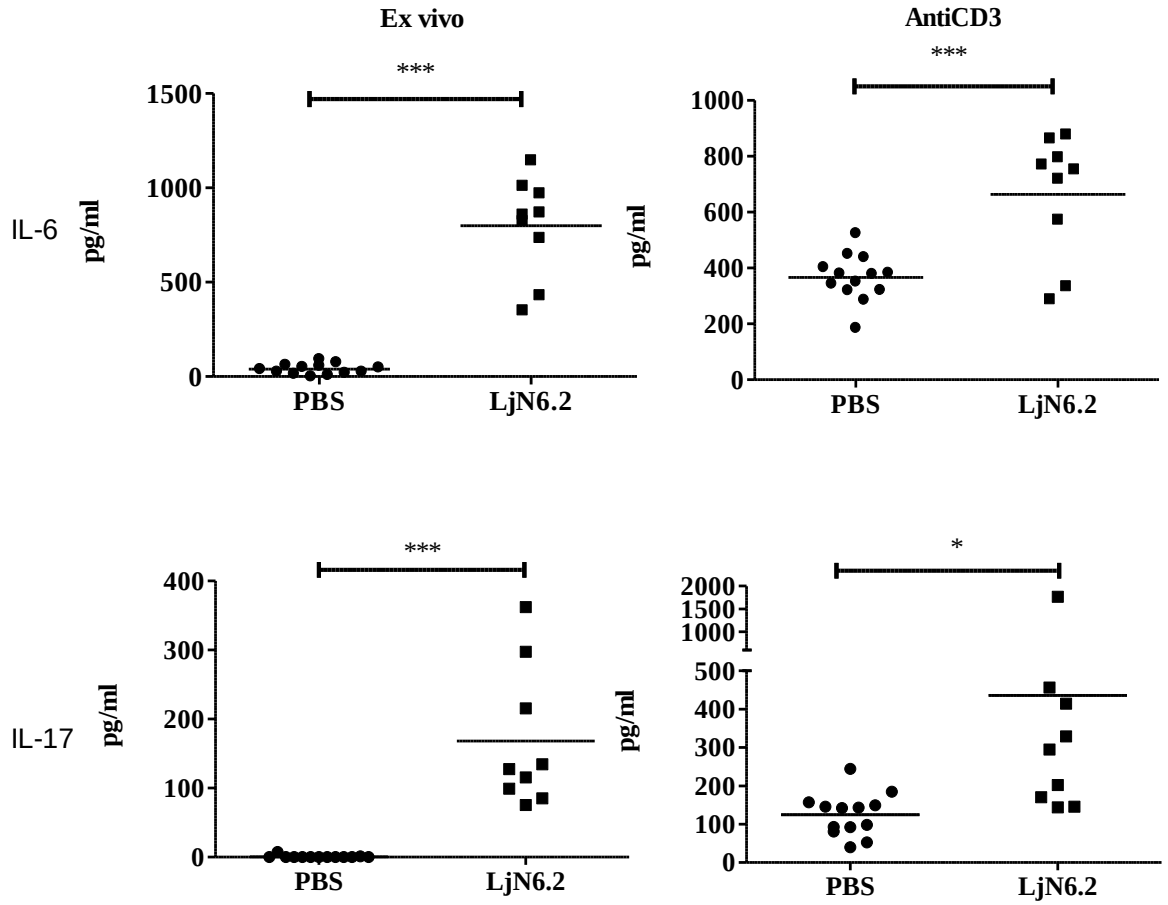

Figure 3-7. LjN6.2-pulsed BMDCs can mediate a longterm TH17 bias in recipient NOD mice. 11 week old NOD mice received 3 weekly injections of 5×10^5 LjN6.2 pulsed BMDCs or PBS control. Mice were sacrificed upon two consecutive days of observed hyperglycemia or allowed to progress to 20 weeks of age. Upon sacrifice, 4×10^5 splenocytes were plated in the presence or absence of antiCD3 (4 µg/mL). Graphs show IL-6 and IL-17 ELISA data for supernatants obtained at 48 hours. 13 control mice and 9 LjN6.2-pulsed BMDC mice were used. *=p<0.05, ***=p<0.005

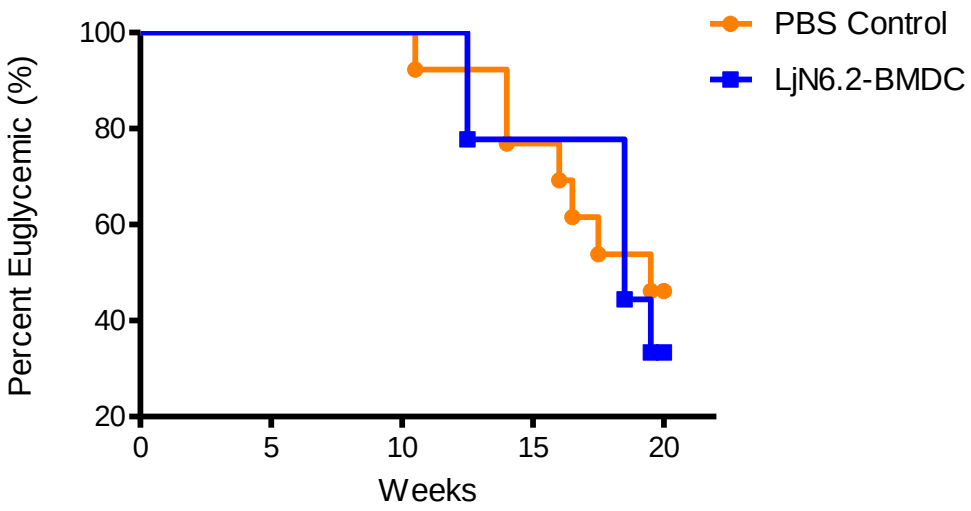

Figure 3-8. Transfer of LjN6.2 pulsed BMDC does not prevent onset of T1D in NOD mice. NOD mice were injected weekly at 9 weeks of age for a total of three treatments with either PBS or BMDC pulsed with LjN6.2. Mice possessing blood glucose levels over 250 mg/dL for two consecutive days were considered diabetic. p= 0.8905. Figure was provided courtesy of Erin Collins.

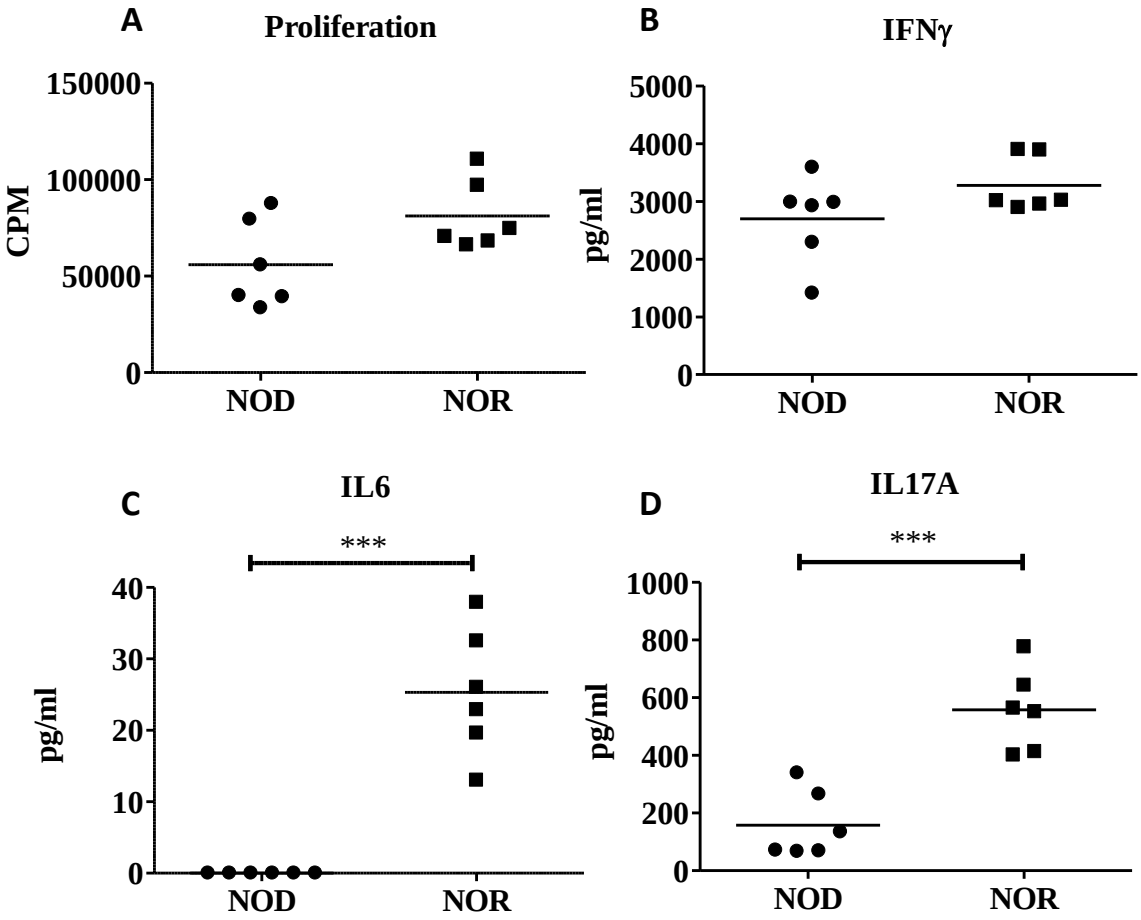

Figure 3-9. NOD lymphocytes display a reduced IL-6 and IL-17A production. Lymph nodes were isolated from NOD and NOR mice 4-12 weeks old and were plated at a concentration of 4 x 10^5 cells/well in the presence of antiCD3 (4 µg/mL) and incubated at 37^0C for 72 hours. A) Graph showing proliferation after a 16-18 hour incubation with tritiated thymidine following the 72 hour mark. Supernatants were removed for ELISA analysis of B) IFNγ, C) IL-6, and D) IL-17 at 48 hours and replenished with fresh media. 6 mice were used per species. ***=$p < 0.005$

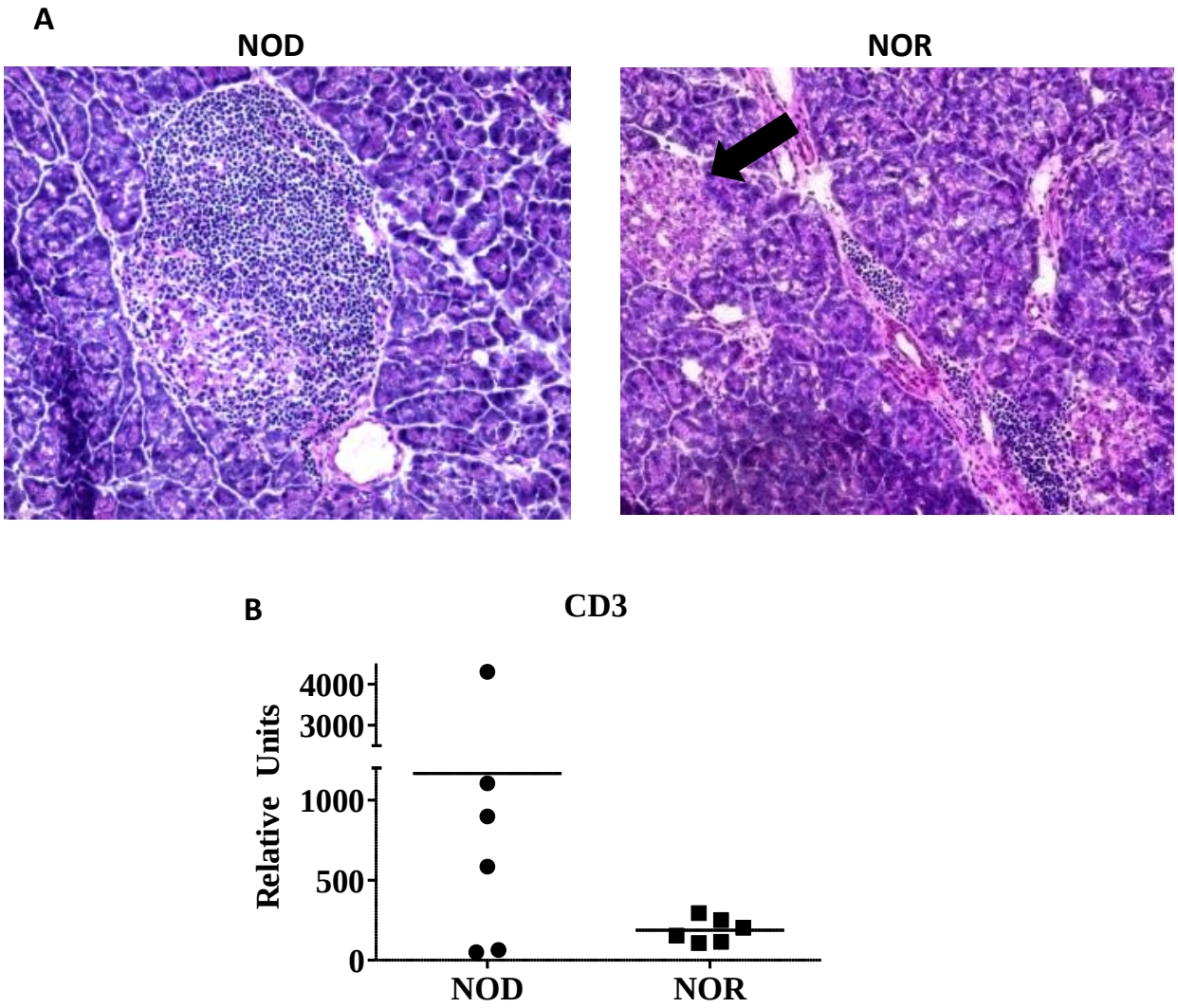

Figure 3-10. NOD Pancreas display insulitis and higher infiltrating levels of T cells. Pancreas was isolated from NOD and NOR mice and snap frozen in OCT medium using 2-methylbutane and liquid nitrogen. Sections were isolated with a cryostat machine. A) H&E stains performed on 5 micron thick pancreas sections isolated from NOD or NOR mice. Photos are at 20x magnification. Arrow indicates location of an islet. B) Graph showing CD3 qPCR results for 40 micron sections of pancreas relative to actin. A total of 6 mice per category were examined.

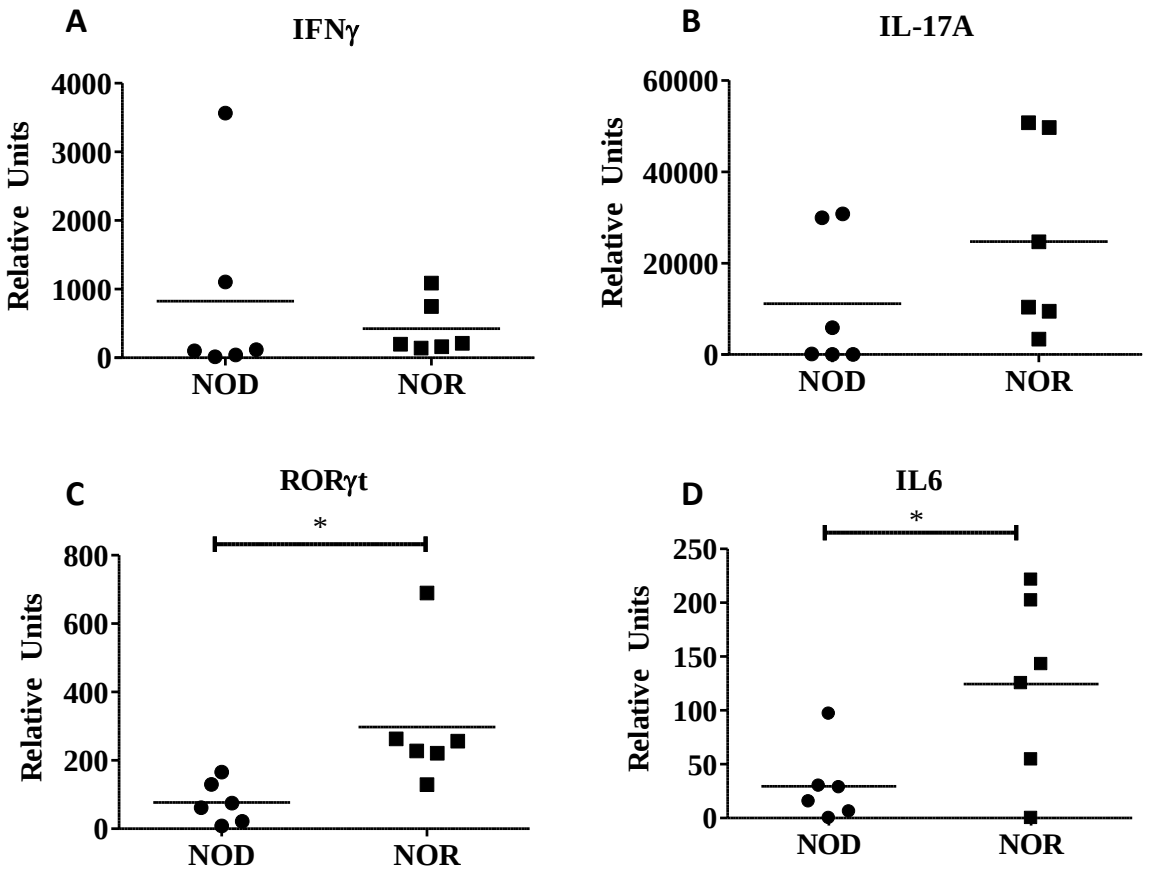

Figure 3-11. NOD Pancreas display reduced levels of TH17-related factors. Pancreas was isolated from NOD and NOR mice and snap frozen in OCT medium using 2-methylbutane and liquid nitrogen. 40 µm sections were isolated with a cryostat machine. Graphs show qPCR results for A) IFNγ, B) IL-17A, C) RORγt, and D) IL-6 expression. Results are relative to CD3. A total of 6 mice per category were examined. *=p <0.05

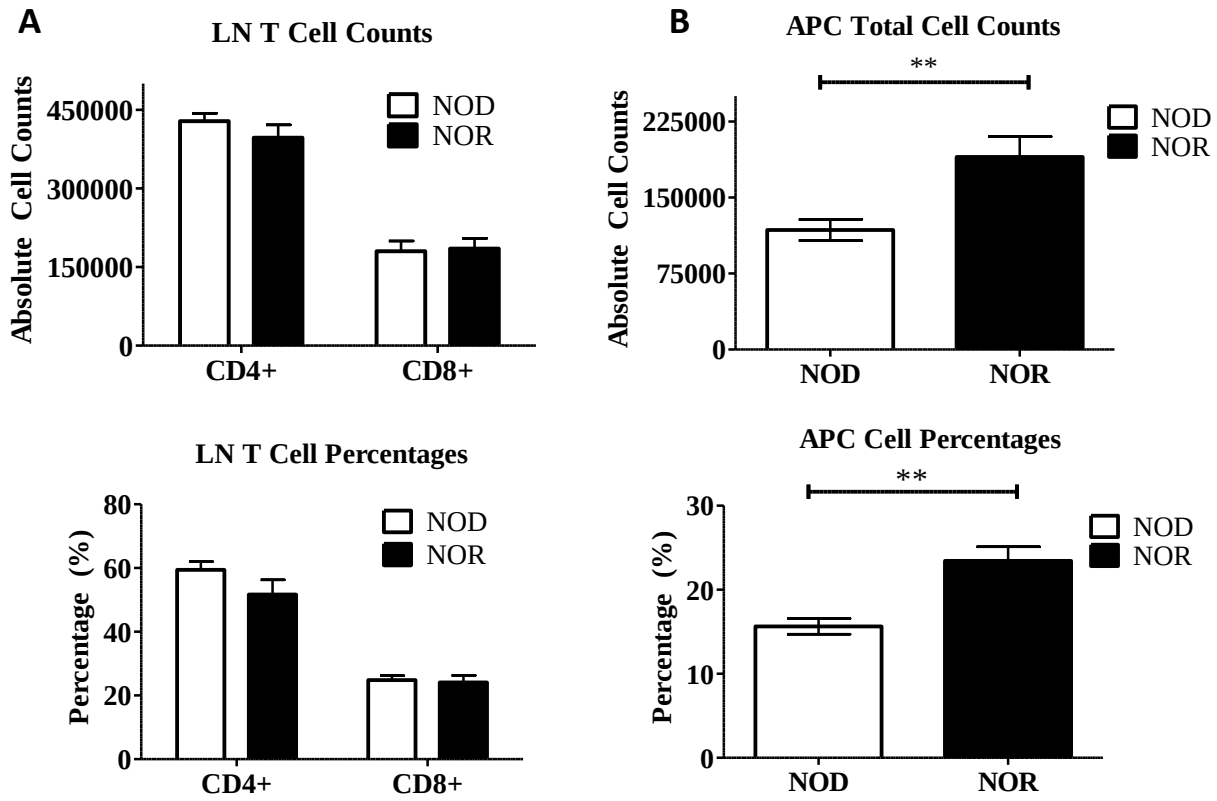

Figure 3-12. NOD lymph nodes display reduced levels of APCs. Cells isolated from Axillary, Mesentery, and Cervical Lymph Nodes were examined *ex vivo* using flow cytometry. A) T cells were stained for CD8 and CD4 markers. B) APCs stained for CD3, B220, CD11b, and CD11c. Absolute cell number counts for CD11b+, CD11c+, and B220+ populations were obtained by gating first for the CD3- population. **=p< 0.01. Data is averaged for 3 mice per set.

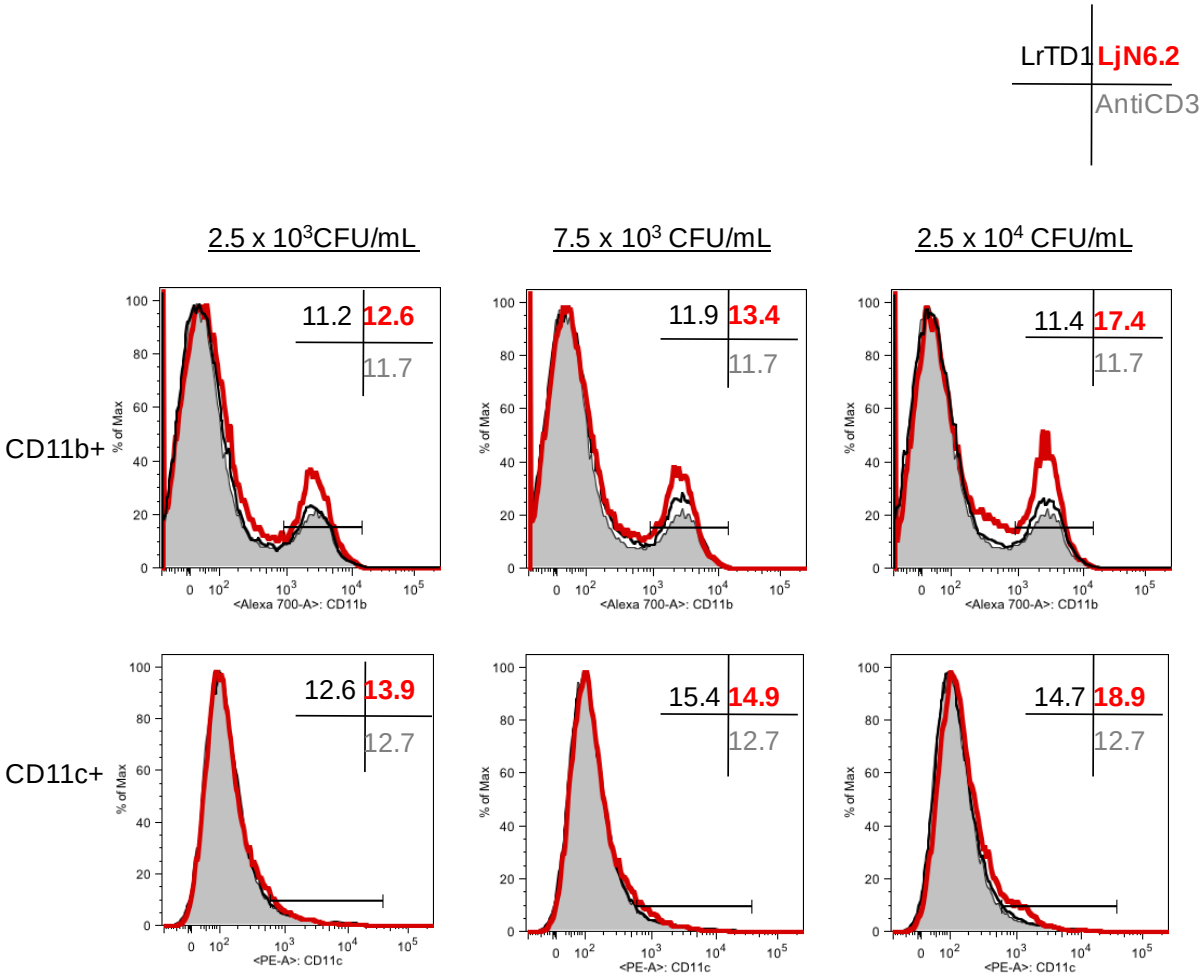

Figure 3-13. LjN6.2 increases CD11b+ and CD11c+ expression. Splenocytes were harvested from NOD mice and plated at a concentration of 4 x 10^5 cells/well with 4 µg/mL antiCD3 and a dose of bacteria where indicated (CFU/mL). Following incubation at 37°C for 48 hours, samples were analyzed through flow cytometry. Graphs show changes in CD11b or CD11c expression. Numbers represent percentage of the indicated cell population. Data is representative of 3 mice per set.

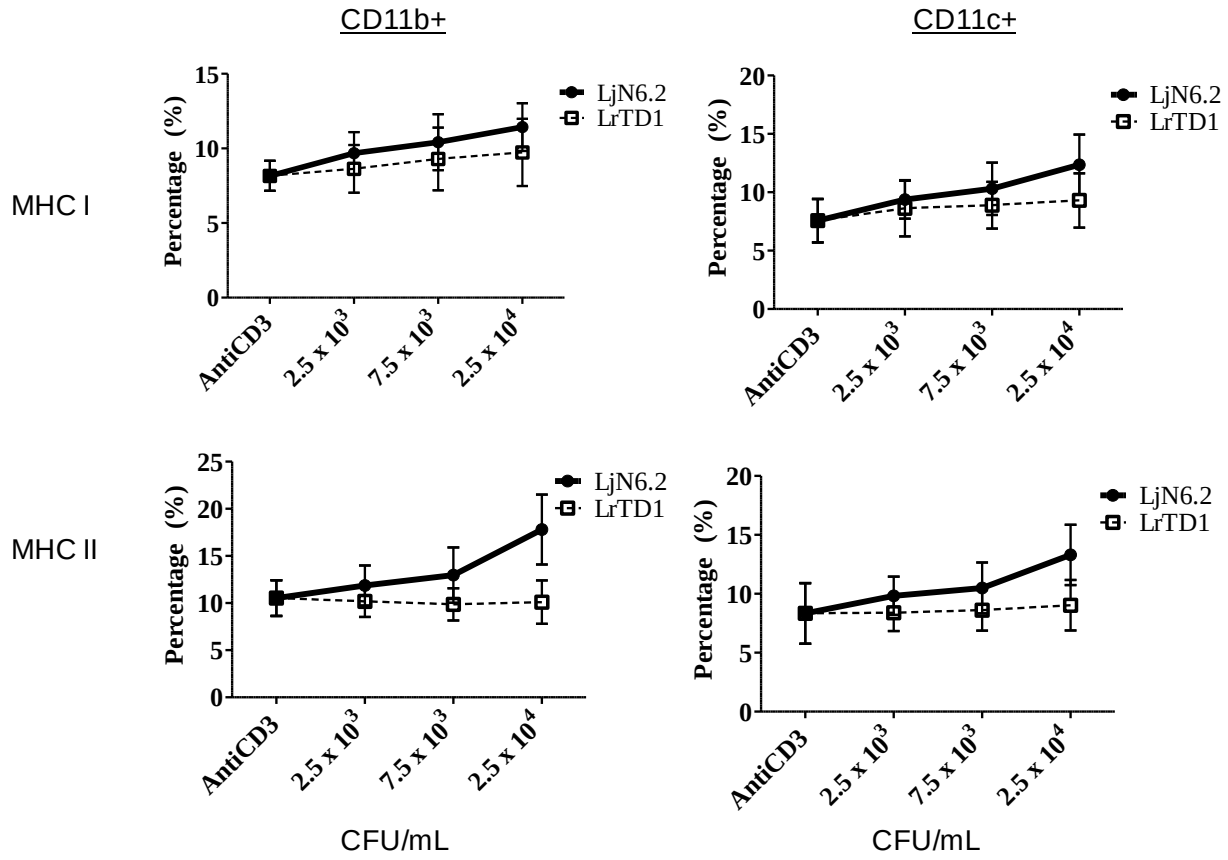

Figure 3-14. LjN6.2. enhances CD11b and CD11c expression of MHC I and II markers. Splenocytes were harvested from NOD mice and plated at a concentration of 4 x 10^5 cells/well with 4 µg/mL antiCD3 and a dose of bacteria where indicated (CFU/mL). Following incubation at 37°C for 48 hours, samples were analyzed through flow cytometry. Graphs showing percentages of cells stained for CD11b, CD11c, MHC I, and MHC II for flow cytometry. Data is averaged on 3 mice per set. The p values for these figures are not statistically significant with the current sample size.

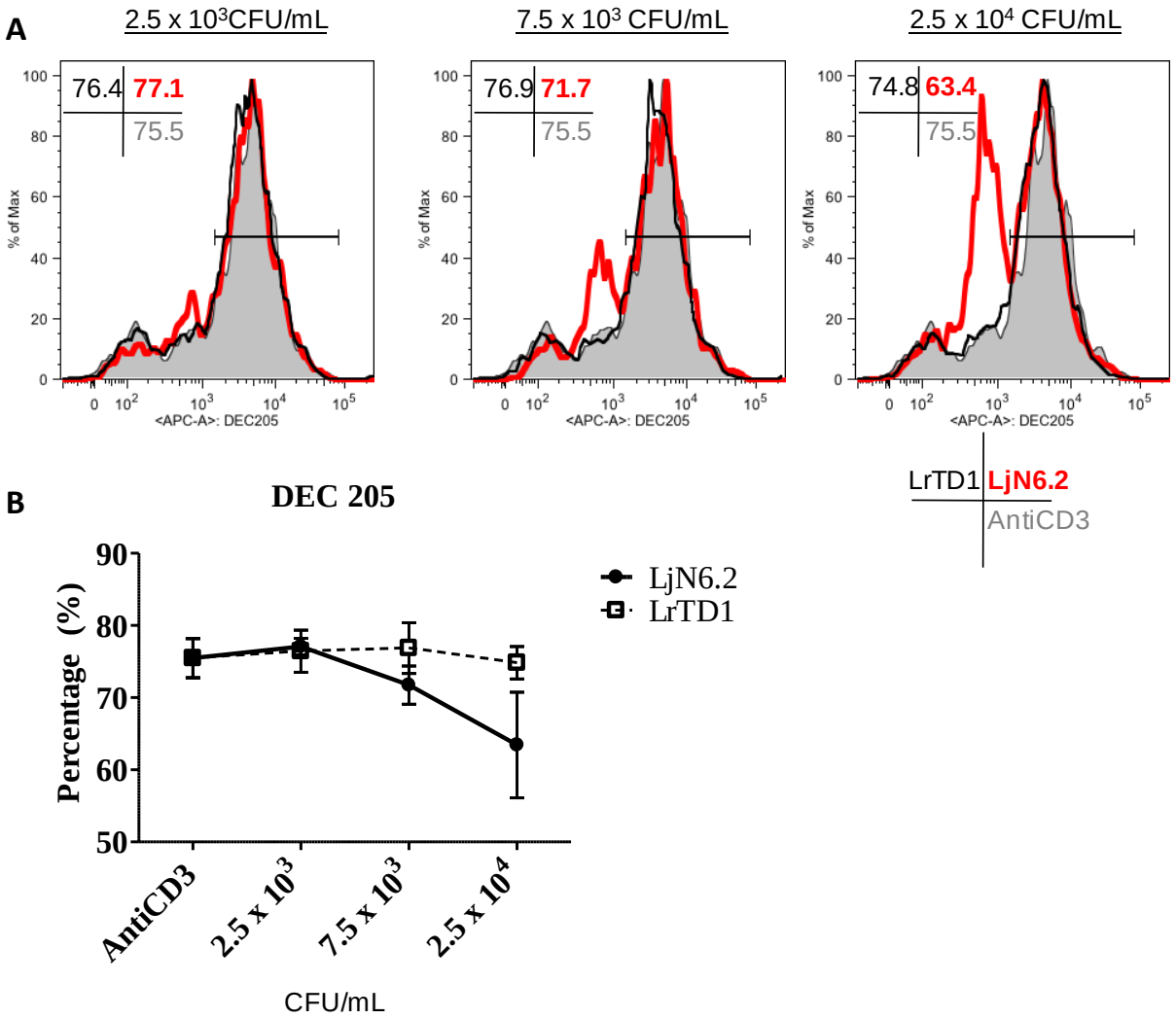

Figure 3-15. CD11c+ cells reduce expression of DEC205 in the presence of LjN6.2. Splenocytes were harvested from NOD mice and plated at a concentration of 4 x 10^5 cells/well with 4 µg/mL antiCD3 and a dose of bacteria where indicated (CFU/mL). Following incubation at 37°C for 48 hours, samples were analyzed through flow cytometry. A) Graphs showing DEC 205 expression by CD11c+ cells. Numbers represent percentage of CD11c+ cells expressing DEC 205. B) Chart displaying percentage of CD11c+ cells positive for DEC 205.

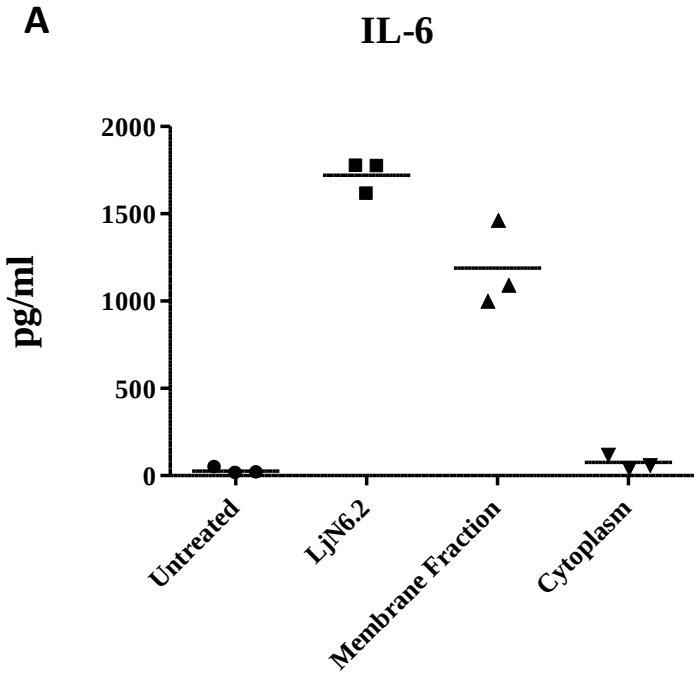

Figure 3-16. LjN6.2 influences APCs via a membrane bound antigen. 4 x 10^5 NOD BMDC were plated per well and treated with whole 2.5 x 10^4 CFU/mL LjN6.2 or the fractions of an equivalent amount of cells. Membrane and Cytoplasm fractions were created by lysing cells with glass beads and a Bead Beater machine. The sample was then centrifuged for 15 min at 100,000 x g. The pellet was considered the membrane fraction, while the supernatant was the cytoplasm fraction. Supernatants were removed at 48 hours and A) analyzed for IL-6 production through ELISA. B) Serial dilutions were done and plated on MRS media and incubated anaerobically. Plates shown are for a 10^6 dilution to determine CFU/mL for whole (left) versus membrane fractions (right) of LjN6.2. 3 mice were used in this experiment.

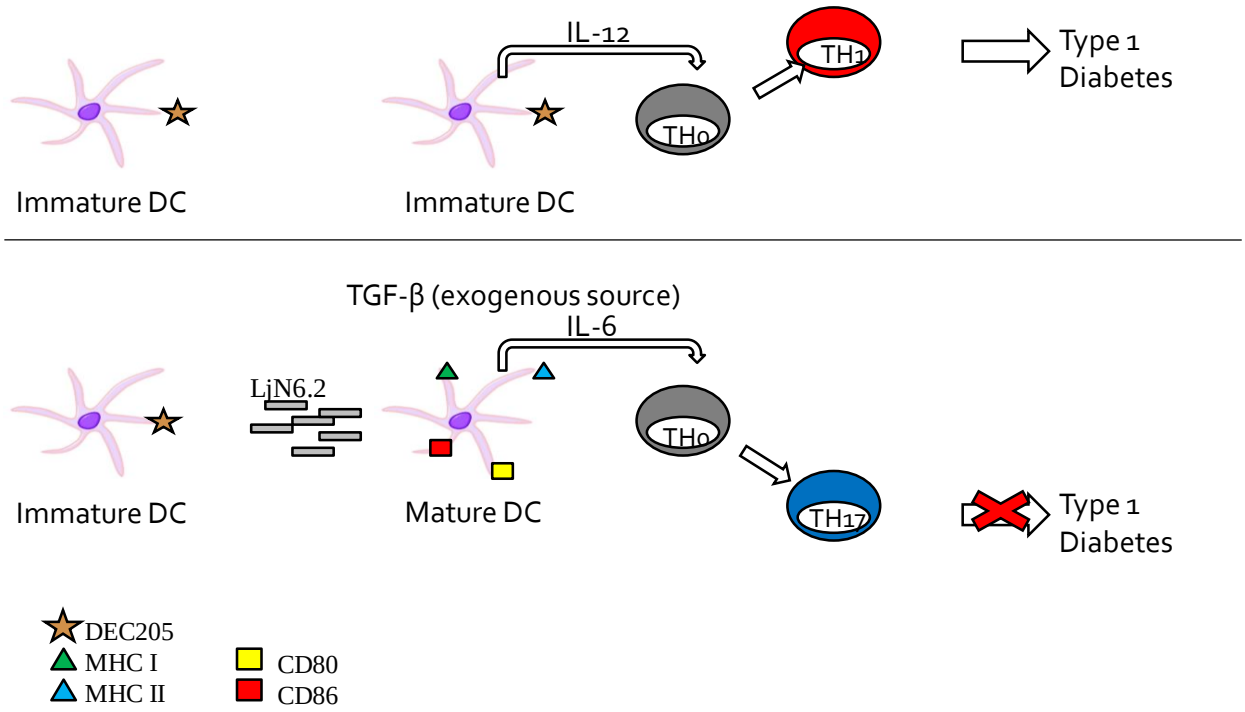

Figure 3-17. A proposed mechanism for the action of LjN6.2 in a diabetes model. In the NOD model, APCs are defective with respects to maturation and proper antigen presentation. The immature state of DCs may lead to a default TH1 bias. However, LjN6.2 may be capable of inducing maturation of DCs, altering the cytokine milieu, and preventing the onset of a TH1 phenotype and T1D.

CHAPTER 4
DISCUSSION

While genetic factors in T1D are being explored, the role of environmental factors has trailed behind. More and more, factors like diet and habitat are being discussed as viable options influencing the onset of T1D. Recently, the use of probiotics has yielded interesting results in animal models, preventing disease onset in both rats and mice. And, while the promise of therapeutic bacteria is encouraging, the mechanism behind its effects is poorly understood.

Animal models have allowed for detailed analysis for the differences between diabetes prone and resistant rodents. Roesch et al. demonstrated that different types of bacteria resided in T1D prone and resistant animals. The stool of the latter contained elevated levels of Lactobacillus and Bacteroides, two genera typically made up of bacteria associated with providing beneficial health effects for its hosts. Furthermore, Valladares et al. demonstrated that feeding *Lactobacillus johnsonii* N6.2 from diabetes resistant rats to diabetes prone (DP) rats could delay onset of the disease. This trend was also common to NOD mice, as *Lactobacillus casei* Shirota strain feedings also prevented onset of the disease (Matsuzaki, 1997). In order to understand how these probiotics treatments mediated its effects in T1D models, we conducted extensive research into the direct effects of LjN6.2 on immune cells, both *in vitro* and *in vivo*.

Our lab has showed that the DP-rats treated with LjN6.2 that did not develop T1D displayed elevated TH17 factors locally in the mesenteric lymph nodes and in more distant sites like the spleen (Lau, 2011). Previous work has shown that gut bacteria can be potent inducers of a TH17 phenotype originating in the gut (Atarashi, 2010). And, as other work has suggested a potentially beneficial role for TH17 cells in T1D, we sought

to investigate how LjN6.2 mediates this TH17 phenotype. Initial *in vitro* work required we determine the proper concentration of LjN6.2 that elicited an immune response but did not induce apoptosis in our mouse splenocytes.

Characterization of LjN6.2's effects began with the analysis of TCR stimulation, as dogma typically states that T cells must receive proper activation through its TCR in order to properly differentiate (Nakayama, 2010). In accordance with this, we demonstrated LjN6.2 was capable of causing an IL-6 response in the absence of TCR stimulation (typically associated with APCs), but not IL-17. With that knowledge, antiCD3 was used to stimulate our splenocytes and we observed that LjN6.2 was particularly adept at inducing IL-6 and IL-17 production in comparison with antiCD3 alone controls or LrTD1-treated splenocytes. Furthermore, we observed a 33% reduction in IL-17 production when we used an antibody cocktail against IL-6, confirming the contributions of this TH17 inducing cytokine. While we would have predicted a larger decrease in IL-17 production with this cocktail treatment, it is possible that other cytokines may serve in a redundant capacity to provide for TH17 differentiation, as detailed above. Future experiments could use additional antibodies against TH17-related cytokines, like IL-23 and TGF-β, to enhance our understanding of the LjN6.2-mediated bias.

We also demonstrated that APCs are the main providers of IL-6 for TH17 differentiation in our LjN6.2 *in vitro* assays, as T cells alone generate minimal levels of IL-6. In turn, we have shown that APCs are required in the presence of TCR stimulation and LjN6.2 to promote TH17 development. Given the importance of APCs in our assay and the antigen sampling role of DCs in the gut, we showed that DCs alone are capable

of creating a TH17 bias. Because several papers have reported that DC transfers are capable of preventing T1D onset (Feili-Hariri, 1999; Clare-Salzler, 1992), we tested the ability of our LjN6.2-pulsed DCs to mediate an immune response in recipient NOD mice. Interestingly, we have demonstrated that NOD mice receiving LjN6.2-pulsed DCs displayed an elevated TH17 response. This response was seen longterm, even in mice sacrificed 11 weeks later. This effect is particularly important, as several papers stated that their attempt to transfer TH17 cells into NOD.SCID mice resulted in their near-immediate conversion to TH1 cells. A longterm TH17 bias may prevent conversion into diabetogenic TH1 cells. Although we were able to observe this effect, we were unable to create a change in the onset of T1D between our sample sets. It is likely that our DC transfers occurred too late in the diabetes timeline. Reports indicate that typically there are two waves of β-cell apoptosis in the pancreas in NOD mice, first at 2-3 weeks of age, and again at 9-10 weeks of age (Turley, 2003). Labs that have reported successful BMDC treatments that delayed or stopped onset of T1D were performed in the time window *between* apoptosis events. Even though our treatment mediated a changed immune response, it occurred toward the end of the time window, and may have not been sufficient to reverse the compounding autoimmune damage. The knowledge of this treatment is very important, as it may only be effective in the initiation stages of this disease and not during the effector phase. These experiments will likely be conducted again, using earlier time points in BMDC treatments.

In addition to the data listed above, we demonstrated a role for TH17s in the autoimmune setting of the NOD mouse. Compared to NOR mice, prediabetic NOD mice display lower levels of TH17-related factors within the mesenteric lymph node and

directly in the pancreas. Additionally, NOD lymph nodes lack the ability to create a TH17 bias upon TCR stimulation. As this data came mostly from 11 week old mice, it would be interesting to monitor TH17 levels over the course of the disease. Preliminary data shows that NOD mice have a peak in lymph node IL-17 production at 8 weeks of age and subsequently drops off 4 weeks later. The use of anti-IL-17 antibodies could elaborate on the effects of TH17 cells during the progression of T1D.

In order to understand how LjN6.2 and its TH17-promoting effects would function in an autoimmune setting, we investigated the effects LjN6.2 had on NOD APCs. Here, we showed that LjN6.2 treatment could increase APC numbers, which is particularly relevant because NOD lymph nodes hold lower percentages of APCs overall. Furthermore, LjN6.2 induced upregulation of both MHC class markers and costimulatory molecules, suggesting the cells undergo maturation. This maturation is important because NOD APCs have several difficulties associated with proper maturation. Proper maturation of APCs was listed as one of the main hypothesis as to how BMDC transfers prevented T1D onset in NOD.SCID models. Mature APCs allows for antigen-induced cell death in improperly activated T cells, as a stronger signal, provided by costimulatory molecules, is required to cause cell death instead of activation. Along with AICD, cross antigen tolerance may also play a role in the restoration of APC function. We observed a decreased expression of surface DEC 205 on dendritic cells, a marker associated with endocytosis and the process of cross-antigen presentation. Cross antigen presentation involves the uptake of exogenous antigen and its presentation on MHC Class I. This theory states that self-antigen may be presented to CD8+ T cells and induce cross-tolerance through apoptosis. The decrease in DEC 205 may seem contradictory at first,

but it is likely that DEC 205 is internalized during endocytosis. It would be interesting to observe intracellular staining through flow cytometry for DEC 205 or use immunofluorescence to monitor internally trafficking DEC 205.

Finally, we have determined that LjN6.2 is likely stimulating APCs through a surface-expressed antigen. This was determined by lysing LjN6.2 cells and separating membrane and cytoplasm fractions via ultra centrifugation. Cytoplasmic fractions were unable to stimulate IL-6 production from BMDCs, while membrane fractions were capable of inducing robust IL-6. We have also shown that membrane stimulation of BMDCs was not due to a high concentration of LjN6.2 surviving the lysing process. Ardissone and Triplett et al. are currently examining differences in gene expression between LjN6.2 and LrTD1 and initial results are promising, demonstrating that LjN6.2 expresses higher levels of pili genes.

While the LjN6.2's promotion of TH17 cells over TH1 cells may seem like exchanging one type of inflammation for another, other immune pathways must be considered. TH17 cells may recruit inflammatory cells during the effector phase of the disease, but TH1 cells have been indicated as the initiators of T1D. Manirarora et al. (2011) demonstrated that certain Lactobacillus strains promote IL-10 production from DCs instead of IL-12. This was correlated with a decreased incidence of disease onset and again, points to the causative role of TH1s in T1D. As previously stated, CFA treatment of NOD mice prevented onset of T1D. This observation correlated with an increase in TH17 factors (Nikoopour, 2010). Manirarora et al. (2008) also stated that CFA treatment of APCs increased their ability to promote Regulatory T cells. They believe this was a function of increasing expression of B7-1, or CD80. Interestingly, we

have also shown that LjN6.2 treatment of APCs induced CD80 upregulation (data not shown). Fig. 3-17 demonstrates a preliminary model of what we believe could be occurring with LjN6.2 and APCs. Without any treatment, NOD APCs suffer from several of the APC defects listed above, including poor maturation. Due to these problems, NOD APCs naturally default to promoting a TH1 phenotype. With LjN6.2 treatments, NOD APCs upregulate MHC and costimulatory molecules, while decreasing DEC 205, indicating a shift from antigen uptake to antigen presentation. This may also change the cytokine production by APCs, preventing T cells from shifting to dangerous TH1 cells. This model still requires further research to confirm the downregulation of TH1-related factors (IL-12, T-bet) upon LjN6.2 treatment. In addition, we have yet to determine whether LjN6.2 induces an antigen specific response or whether the changes observed are broad and non-specific. While we still have a few issues to address with this model, we believe this could be a strong representation of how LjN6.2 influences the host immune response.

While Treg numbers have been shown not to decline during the course of disease in NOD mice, their function becomes impaired (Tritt, 2008). This loss of function was correlated with a lack of Foxp3, the Treg transcription factor. Manirarora demonstrated that CFA treatment in NOD mice increased Foxp3 expression in Tregs. Treg development in the gut remains an unexplored, yet important part of our LjN6.2 treatments. It is possible that proper presentation of LjN6.2 to DCs allows them to not only promote a TH17 environment, but enhance the function and numbers of Tregs. The Tregs could then tolerize the host to the gut microbiota, even if bacteria are promoting an autoimmune response.

Probiotics have encouraging potential as a therapeutic agent. Several publications state the benefits of oral probiotic treatment. Probiotics also suggest that certain sets of gut microbiota may be correlated with a healthy or dysfunctional immune system. If a collection of microbiota can be associated with Type 1 Diabetes, this may lead to a screening process that analyzes individuals at risk for the disease. Furthermore, probiotics may be prescribed to help the patient maintain a healthy gut environment. Probiotics are also relatively cheap (compared to some treatment options) and can be produced by the liters in short amounts of time. Of course, translational studies must be conducted to determine the efficacy of probiotics in T1D patients. And, even though most probiotic strains are Generally Regarded as Safe (GRAS), they should be studied for their longterm effects. It would also be helpful to understand the immune profile generated by the strains of bacteria to be used, as a panel of T Helper cell differentiation could determine which strains could be most effective. Our studies have focused on illuminating the mechanisms behind probiotic effect and function in the autoimmune model of T1D. With each bacterial strain that becomes characterized immunologically, we gain more insight into the interplay between environment and host, providing groundwork for future clinical success.

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BIOGRAPHICAL SKETCH

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