

MUCOSAL IMMUNITY AND TYPE 1 DIABETES

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To my Mom and Dad who have always been there

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# TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS.....	4
LIST OF TABLES.....	7
LIST OF FIGURES.....	8
LIST OF ABBREVIATIONS.....	9
ABSTRACT.....	12
CHAPTER	
1 INTRODUCTION.....	14
Diabetes Mellitus .....	14
Type 1 Diabetes.....	14
Epidemiology of T1D.....	15
NOD Mouse Model .....	18
NOD Mouse Disease Progression .....	19
T1D Pathology .....	20
Antigen Presenting Cells .....	20
Macrophages.....	21
Dendritic Cells .....	21
B Cells .....	22
Autoantibodies.....	23
T cells.....	23
Immune Regulation .....	24
Thymic Selection .....	24
Peripheral Regulation.....	25
Environmental Contributions.....	26
Mucosal Immunity.....	27
Intestinal Epithelial Cells.....	28
Intestinal Macrophages .....	29
Intestinal Dendritic Cells .....	29
Regulatory T cells and Mucosal Tolerance.....	31
Th17 Cells.....	32
Mucosal Immunity and T1D .....	35
Diet and T1D .....	35
Evidence of Mucosal Inflammation in T1D .....	35
Gastrointestinal Alterations in T1D .....	36
Hypothesis and Summary .....	38
2 MATERIALS AND METHODS .....	39

Murine Models .....	39
Gastrointestinal Permeability .....	39
Flow Cytometry.....	40
ELISA.....	41
Statistics .....	42
<b>3 RESULTS.....</b>	<b>43</b>
Intestinal Permeability.....	44
Movement of Charged Ion Differs between NOD and B6 Mice.....	45
Preliminary Flux Trials .....	45
70kDa Dextran as a Paracellular Permeability Marker.....	46
Elevated TNF $\alpha$ in Duodenal Tissues of NOD Mice when Compared to B6 .....	47
T cell Population Differences between NOD and B6 Mice.....	48
Th17 and Th1 Differences between NOD and B6 Mice .....	49
NOD MLN and PLN T cell Populations over the Course of the Disease .....	50
Decreased Treg Frequency in the MLN and PLN of NOD Mice.....	51
<b>4 DISCUSSION.....</b>	<b>63</b>
<b>LIST OF REFERENCES.....</b>	<b>68</b>
<b>BIOGRAPHICAL SKETCH.....</b>	<b>90</b>

## LIST OF TABLES

<u>Table</u>	<u>page</u>
3-1 Gastrointestinal Barrier Function Analysis.....	52

## LIST OF FIGURES

<u>Figure</u>	<u>page</u>
3-1 Temporal Evaluation of Intestinal Barrier Function and Inflammation.....	53
3-2 Decreased Gastrointestinal Movement of Charged Ions in NOD mice. ....	54
3-3 Optimization of Gastrointestinal Permeability Assays.....	55
3-4 Evaluation of 70kDa Dextran as a Paracellular Permeability Marker.....	56
3-5 NOD mice exhibit Elevated Duodenal TNF $\alpha$ Levels.. ....	57
3-6 Gating schemes for Flow Cytometric Analysis.....	58
3-7 Frequencies of Polarized T cells in the MLN and PLN.. ....	59
3-8 Frequencies of IL17 expressing T cell populations.....	60
3-9 Distribution of T cell populations within the MLN and PLN of NOD mice over the course of disease.. ....	61
3-10 Decreased Frequencies of Tregs in the MLN and PLN of NOD mice.....	62

## LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
B6	C57Bl/6 mice
BBDP	Bio-Breeding Diabetes Prone Rats
CIA	Collagen-induced Arthritis
CTE	Cortical Thymic Epithelial Cells
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DC	Dendritic Cells
DC	Distal Colon
DI	Distal Ileum
DP	Double Positive Thymocytes
EAE	Experimental Autoimmune Encephalomyelitis
EGFR	Epidermal Growth Factor Receptor
FoxP3	Forkhead Box P3 – Treg transcription factor
GAD65	Glutamate Decarboxylase 65
GDM	Gestational Diabetes Mellitus
GI	Gastrointestinal
$G_T$	Tissue Conductance
HLA	Human Leukocyte Antigen
IA-2	Insulinoma antigen 2
IAA	Insulin Autoantibody
IBD	Inflammatory Bowel Disease
ICA	Islet Cell Antibodies
IDO	Indoleamine 2,3-dioxygenase
IEC	Intestinal Epithelial Cells

IEL	Intraepithelial Lymphocytes
IFN $\gamma$	Interferon Gamma
IGF-1	Insulin-like growth factor 1
IGRP	islet-specific glucose 6 phosphatase catalytic subunit-related protein
IL2	Interleukin-2
IL17	Interleukin-17
I <sub>sc</sub>	Short Circuit Current
J <sub>dex</sub>	Dextran Flux
J <sub>man</sub>	Mannitol Flux
LP	Lamina Propria
LYP	Lymphoid Tyrosine Phosphatase
MAdCAM-1	Mucosal Addressin Cell Adhesion Molecule-1
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Node
NOD	Non-Obese Diabetic Mice
PAMP	Pathogen Associated Molecular Patterns
PAR2	Proteinase-activated Receptor 2
PLN	Pancreatic Lymph Node
PP	Peyer's Patches
PJ	Proximal Jejunum
RA	Retinoic Acid
ROR $\gamma$ t	RAR-related Orphan Receptor Gamma – Th17 transcription factor
T1D	Type 1 Diabetes
TCR	T Cell Receptor
TGF $\beta$	Transforming Growth Factor $\beta$

TJ	Tight Junctions
TLR	Toll-Like Receptors
TNF $\alpha$	Tumor Necrosis Factor Alpha
TREG	Regulatory T cells
VNTR	Variable Number of Tandem Repeats

Abstract of Thesis Presented to the Graduate School  
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The initiating events that lead to autoimmunity which results in the destruction of the insulin producing  $\beta$ -cells in type 1 diabetes (T1D) have yet to be defined. Increasing evidence suggests an involvement of the mucosal immune system in the pathogenesis of T1D. A leaky gut preceding the onset of T1D has been observed in both humans and in rodent models. Additionally, disease-causing T cells have been found in the draining lymph nodes of the gastrointestinal tract in rodent models. Together, this suggests that a breakdown of barrier function and inflammation in the gastrointestinal tract may contribute to the induction of autoimmunity. Thus we hypothesized that a breakdown in intestinal barrier function results in an inflammatory environment that promotes non-tolerizing conditions. It was determined that in a murine model of T1D, the non-obese diabetic (NOD) mice, decreased transcellular ion movement, but no differences in paracellular movement were exhibited. NOD mice also exhibited elevated levels of duodenal TNF $\alpha$ . Similarly there were higher frequencies of Th1, conventional Th17, and pathogenic Th17 CD4 T cells compared to B6 mice along with decreased frequencies of regulatory T cells. Most importantly, these populations changed over the course of the disease within NOD mice in a trend supporting intestinal inflammation

early in disease leading to pancreatic inflammation later in disease. In summary work presented here has added to the evidence that aberrant gastrointestinal barrier function and inflammation contributes to T1D pathogenesis.

## CHAPTER 1 INTRODUCTION

### **Diabetes Mellitus**

Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia due to defects in insulin secretion, insulin action, or both. There are multiple classifications of diabetes mellitus including: type 1 diabetes, type 2 diabetes, and gestational diabetes. Type 1 diabetes (T1D) is a form of diabetes characterized by loss of  $\beta$ -cells, which are the insulin producing cells, and results in hyperglycemia<sup>1</sup>. Type 2 diabetes (T2D) is the most common form of diabetes and is characterized by disorders in insulin action. T2D patients exhibit insulin resistance rather than insulin deficiency<sup>1</sup>. Gestational diabetes mellitus (GDM) is a form of diabetes that occurs during onset of pregnancy characterized by hyperglycemia<sup>2,3</sup>.

#### **Type 1 Diabetes**

Type (T1D) is a chronic inflammatory disease characterized by hyperglycemia due to loss of the insulin producing pancreatic  $\beta$ -cells within the islets of Langerhans. T1D is classified into primarily two forms: type 1A or 1B. Type 1B occurs due to idiopathic loss of  $\beta$ -cells without presentation of autoimmune antibodies or evidence of autoimmunity<sup>4</sup>. The more common form of T1D is type 1A which is characterized by autoimmune destruction of  $\beta$ -cells<sup>5</sup>. Both forms of T1D result in insulin deficiency and hyperglycemia which can cause complications such as retinopathy, diabetic nephropathy, peripheral neuropathy, and cardiovascular disease (reviewed in<sup>6</sup>).

Well controlled glycemic levels can reduce these disease associated complications (reviewed in<sup>6</sup>). The focus here will be on type 1A autoimmune diabetes

and the role of the mucosal immune system in development and progression of this disease.

### **Epidemiology of T1D**

T1D is the major type of diabetes effecting youth causing 85% of all diabetes cases in individuals less than 20 years old worldwide <sup>7-9</sup>. Incidence of T1D peaks at 10-14 years of age, starts to decline after puberty, and stabilizes during young adulthood <sup>10-13</sup>. The DIAMOND project initiated by the World Health Organization (WHO) demonstrated that worldwide distribution of T1D varies greatly <sup>14</sup>. The lowest incidence rate was reported for populations in China and South America (<1/100,000 per year) while the highest incidence rate was reported for populations in Sardinia, Finland, Sweden, Norway, Portugal, UK, Canada, and New Zealand (>20/100,000) <sup>14</sup>. The difference in incidence was suggested to be due to differences in genetic admixture or a result of environmental and/or behavioral differences <sup>15</sup>. For instance, effects of birth season on incidence were observed in the SEARCH study. The ratio of observed to expected incidence in births during the winter months (November-February) was low while the same ratio was higher in summer months (April-July). This seasonal pattern was observed in northern locations such as Colorado, Washington, and Ohio, but not southern locations such as South Carolina, Southern California, or Hawaii <sup>16</sup>. It has been hypothesized that these variations in incidence are due to seasonal variations in the maternal vitamin D levels due to geographical location <sup>15</sup>. These epidemiological observations suggest that along with genetic susceptibility there are environmental factors which contribute to the development of the disease that can vary worldwide.

## T1D Inheritance

T1D is a complex multifactorial disease where genetic factors contribute to susceptibility<sup>5</sup>. The highest risk factor for T1D is having an identical (monozygotic) twin with the disease. Interestingly, the concordance rate among monozygotic twins was reported around 30% when looking at a single time point, suggesting that there are other non-genetic factors contributing to disease<sup>17-19</sup>. It has now come to light after following twins who were initially discordant for diabetes that by 60 years of age the cumulative incidence rate is 65%, which still suggests a role for non-genetic factors in contributing to rates of onset of clinical disease<sup>20</sup>.

Studies evaluating the relationships between genetic relatedness within families, between siblings as well as offspring, suggest that T1D development is due to multiple genes combining in an additive fashion to confer risk<sup>21</sup>. The strongest genetic risk is linked to the human leukocyte antigen (HLA) region encoding HLA DR and HLA DQ molecules<sup>22</sup>. Specifically the haplotypes DQA1\*0501-DQB1\*0201 (DR3) and DQA1\*0301-DQB1\*0302 (DR4) are associated with the genetic risk. The presence of both these haplotypes is found in ~40% of T1D patients<sup>22</sup>. On the other hand, the haplotype HLA-DQA1\*0102-DQB1\*0602 confers protection<sup>23</sup>. In a mouse model for T1D, the non-obese diabetic mouse (NOD), susceptibility is also conferred by a major histocompatibility complex (MHC) class II molecule I-A, termed H-2<sup>g7</sup><sup>5, 24</sup>. Here both the beta chain of MHC II I-A and the HLA-DQ exhibit the same non-aspartic acid substitution at position 57<sup>25, 26</sup>. The non-aspartic acid substitution significantly alters the binding partners able to be presented by the MHC molecule<sup>27</sup>.

In addition to the HLA region, 40 additional loci have been described as contributing to disease susceptibility<sup>28</sup>. Many of the genes associated with these loci have not yet been identified. Some of the few non-MHC genes that have been defined include a single-nucleotide polymorphism in the lymphoid tyrosine phosphatase (LYP) encoded by the *PTPN2* gene, allelic variations in cytotoxic T lymphocyte antigen 4 (CTLA-4), a polymorphic region - variable number of tandem repeats (VNTR) - located immediately 5' the insulin gene, as well as variations in the *IL2* and *IL2Rra* gene region<sup>26-36</sup>.

LYP plays a role in T cell receptor (TCR) signaling and a single-nucleotide polymorphism has been associated with autoimmune diseases, including T1D<sup>29</sup>. This single-nucleotide polymorphism in LYP was shown to cause a gain of function resulting in more efficient dephosphorylation of Lck and TCR $\zeta$  as well as reduced calcium mobilization and IL-2 gene transactivation<sup>30</sup>. Thus, the polymorphism causes a more active phosphatase which can suppress TCR signaling more efficiently than 'normal' LYP.

CTLA-4 plays a role in maintaining regulatory T cell (Treg) function as well as down-regulating effector T cell responses<sup>31, 32</sup>. In humans, T1D is associated with a *Ctla-4* variant that results in decreased expression of the soluble form of CLTA-4<sup>31</sup>. In NOD mice, T1D is associated with low levels of a splice variant of *Ctla-4* that lacks the domain which binds with the co-stimulatory markers, CD80/86<sup>32, 33</sup>.

The VNTR element of the insulin gene promoter has been shown in humans to result in decreased thymic expression of the insulin gene<sup>34, 35</sup>. This VNTR allele is not present in NOD mice, but studies in the NOD demonstrate that alterations in thymic

insulin expression contribute to disease<sup>36, 37</sup>. In rodents there are two different insulin genes, *ins1* and *ins2*, which are located on two different chromosomes. The *ins2* gene is thought to be preferentially expressed over *ins1* in the thymus<sup>38</sup>. By selectively decreasing expression of *ins2* in the thymus it has been shown that *ins2* deficient NOD mice exhibit earlier onset of disease compared to wild-type NOD mice<sup>36, 37</sup>.

In humans hypomorphic polymorphisms of *IL2* and *IL2ra* are linked to autoimmune susceptibility<sup>39-42</sup>. In NOD mice, lower levels of IL-2 are associated with disease progression whereby treatment with IL-2 can inhibit the onset of clinical diabetes<sup>43, 44</sup>. In addition, a loss of one copy of the *IL2* allele can accelerate onset of T1D<sup>41</sup>. Though these susceptibility loci are not completely conserved between humans and mice, the similarities between them make the NOD mouse a good model for understanding development of the disease.

### **NOD Mouse Model**

In human T1D, there is an asymptomatic preclinical phase where an autoimmune process is initiated, but the autoimmune process leading to destruction of the pancreatic  $\beta$ -cells is difficult to study due to the inaccessibility of the pancreas. Thus, in order to decipher the initiating and progressive autoimmune events leading to the destruction of  $\beta$ -cells, animal models that develop spontaneous autoimmune disease resulting in destruction of the  $\beta$ -cells, have been utilized.

The NOD mouse strain was developed through inbreeding of the ICR (Swiss) mice in Japan<sup>45</sup>. Dr. S Makino at the Shionogi Research Laboratories in Aburahi, Japan was developing a strain by selecting for dominant cataract with microphthalmia<sup>45-47</sup>. After six generations (F6), a subset of mice began exhibiting high fasting glucose levels and was thus inbred selecting for this trait. At the same time, progeny that

exhibited normal fasting glucose levels were inbred as a euglycemic control strain. Interestingly, the first mouse to spontaneously develop hyperglycemia and exhibit insulinitis actually came from the strain being inbred as the euglycemic control. This female mouse was the founder of the NOD strain <sup>45-47</sup>.

### **NOD Mouse Disease Progression**

The majority of what we know regarding local cellular events that contribute to autoimmune destruction of islets within the pancreas has been learned from the NOD mouse. The progression of  $\beta$ -cell autoimmunity in the NOD mouse is broken down into well defined “checkpoints” <sup>48</sup>. The first checkpoint occurs around 3 weeks of age and is characterized by infiltration of the islets by immune cells <sup>48-51</sup>. Invading immune cells consist of both innate and adaptive immune cells where the majority are CD4+ and CD8+ T cells, B cells, dendritic cells (DC), NK cells, and macrophages <sup>46, 47</sup>. During this first checkpoint infiltration continues until about 10-12 weeks of age, though  $\beta$ -cells remain intact and mice remain euglycemic even in cases with severe insulinitis <sup>48</sup>. The second checkpoint is characterized by a switch in the pathogenic potential of the infiltrating immune cells where massive  $\beta$ -cell destruction occurs resulting in eventual development of hyperglycemia known as overt diabetes <sup>48</sup>.

Both CD4+ and CD8+ T cells can directly mediate islet cell destruction, though some studies suggest that a CD8+ response occurs early in disease resulting in islet death and priming of the CD4+ response <sup>52</sup>. Despite understanding the two checkpoints of NOD disease progression, the initiation of the autoimmune process and the switch in pathogenic potential between Checkpoint 1 and 2 are still not understood.

Some insight into this pathogenic switch has been gained by using BDC2.5 mice, a mouse line with a transgenic TCR that recognizes  $\beta$ -cell antigens. In this mouse

model, the mice exhibit insulinitis yet only 10-20% of the BDC2.5 mice develop overt diabetes<sup>53</sup>. This may be due to the presence of a high number of Tregs contributing to prevention of  $\beta$ -cell death, suggesting a role for peripheral tolerance regulating movement from Checkpoint 1 to 2<sup>54</sup>.

While the NOD model shares many similarities with human disease and many of the discoveries involving pathogenesis have come to light using the NOD model<sup>55</sup>, translating therapeutic approaches from NOD mice to humans has been difficult. These difficulties are most likely due to the fact that there are some differences in the etiology of disease between NOD and humans. For example there are differences in target antigens, the composition of inflammatory infiltrates, as well as an increased expression of MHC class I in humans<sup>56</sup>. Despite these differences, NOD mice still prove to be a good model for describing the role of the adaptive immune response in the pathology of the disease as well as identifying therapeutic targets.

### **T1D Pathology**

Though diabetogenic T cells (CD4+ and CD8+ T cells) are key players in the destruction of  $\beta$ -cells in T1D for both humans and NOD mice, there are many other cell types of both the innate and adaptive immune system that are involved with development and progression of disease.

### **Antigen Presenting Cells**

Antigen presenting cells (APC), including macrophages, dendritic cells (DCs), and B cells, play an important role in bridging the gap between innate and adaptive immunity. APC induce T cell responses by TCR activation, through recognition of peptides presented on MHC molecules, and co-stimulatory signals<sup>57</sup>. In the absence of co-stimulatory signals T cells become anergic and/or undergo apoptosis<sup>58</sup>. Because

APC are able to prime and activate T cells as well as regulate T cell responses, they play a critical role in development of autoimmunity<sup>59, 60</sup>.

### **Macrophages**

Macrophages can be detected in the islets of NOD mice early in disease. Their depletion can prevent the development of disease<sup>61</sup>. In vivo and in vitro studies have demonstrated that they play a pathogenic role in  $\beta$ -cell death through production of TNF $\alpha$  and IL-1 $\beta$ <sup>62, 63</sup>. Interestingly, macrophages in NOD mice are less efficient at engulfing apoptotic cells, which results in accumulation of dying cells or debris that promote inflammatory responses<sup>64</sup>. Some have suggested that these products released by dying cells are the initiators of disease development<sup>65</sup>.

### **Dendritic Cells**

While DCs are integral in T cell activation, they are also critical in peripheral tolerance because they can also induce T cell deletion, T cell anergy, and expansion of Tregs, all of which are critical for the prevention of autoimmunity including T1D<sup>57</sup>. Evidence for a role for DC in peripheral tolerance is highlighted in studies where DC depletion results in autoimmune disease<sup>66</sup>.

In the NOD mouse DC take up islet self-antigens after  $\beta$ -cell death, process, and present these antigens to islet-specific T cells located within the pancreatic lymph nodes (PLNs)<sup>67</sup>. Here DC from NOD mice have an increased ability to activate T cells through higher expression of IL-12 as well as higher expression of co-stimulatory molecules compared to C57Bl/6 mice<sup>68, 69</sup>. In addition there is an increased frequency of type 1 IFN-producing DC in the pancreatic lymph node during T1D initiation, which could contribute to autoimmune T cell activation<sup>70</sup>. These studies suggest that in T1D there is an enhance ability of DC to activate T cells.

On the other hand, the tolerogenic potential of DC in T1D is diminished. For instance, indoleamine 2,3-dioxygenase (IDO) expression by DC in young NOD mice is decreased<sup>71</sup>. IDO expression by DC controls effector T cell expansion by catabolism of tryptophan, which is necessary for T cell proliferation and expansion. Thus less IDO production allows for increased T cell survival<sup>72</sup>. Indeed, over-expression of IDO in NOD can extend islet graft survival<sup>73</sup>.

Other studies have investigated FMS-like tyrosine kinase (Flt3)-ligand, which promotes tolerogenic DC that enhance Treg frequency in the PLN<sup>74,75</sup>. Interestingly, early administration of Flt3-ligand in the NOD is protective, while late stage administration actually expands DC populations exacerbating T1D development<sup>76</sup>. These data suggest that tolerance may only be able to be achieved at early stages of the disease.

## **B Cells**

B cells serve not only as APCs but also as a source for autoantibodies. Autoantibodies have not been shown to have a direct effector function in the destruction of  $\beta$ -cells<sup>77,78</sup>. In fact, a study using B cells that cannot secrete antibodies demonstrated that B cells play an antibody-independent role in the development of disease<sup>79</sup>. On the other hand, B cell deficiency achieved through gene targeting or through antibody mediated depletion prevents development of the disease in NOD mice<sup>80</sup>. Similar effects are seen in humans, whereby B cell depleting regimens improve  $\beta$ -cell function in newly diagnosed patients<sup>81</sup>. Though they do not present antigens as efficiently as DC, it has been suggested that the antigen specific B cells are acting as APC<sup>82</sup>. B cells actually internalize antigen 10,000-fold more efficiently than non-specific cells<sup>83,84</sup>. In

this way, B cells can internalize a much greater range of peptides from the protein or proteins associated with it and diversify the CD4<sup>+</sup> T cell response <sup>85</sup>.

### **Autoantibodies**

Detection of autoantibodies is considered a predictor of T1D where the presence of autoantibodies at younger ages and at higher titers correlates to disease development <sup>78, 86-88</sup>. Islet cell antibodies (ICA) were first observed in the islets of human pancreatic tissue samples <sup>89</sup>. Since then, studies have determined the targets of these autoantibodies to be insulin (insulin autoantibody, IAA), glutamic acid decarboxylase 65 (GAD65), and insulinoma-associated protein 2 (IA-2) [reviewed in <sup>90</sup>]. NOD mice also exhibit autoantibodies towards insulin <sup>77</sup>, but only low levels of autoantibodies against GAD65 and IA-2 <sup>91</sup>. Though autoantibodies are considered a risk factor, they are not considered to be a factor contributing to islet destruction because  $\beta$ -cell specific autoantibodies can be detected in first degree relatives though they may not progress to overt disease <sup>90</sup>. Similarly, in NOD mice, autoantibody transfer from new onset donors cannot transfer disease <sup>82</sup>.

### **T cells**

Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are key players in the destruction of  $\beta$ -cells. This is highlighted by the fact that adoptive transfer of purified populations of CD4<sup>+</sup> or CD8<sup>+</sup> T cells from NOD donors as well as T cells clones generated from infiltrated islets cause disease <sup>92-96</sup>. CD8<sup>+</sup> T cells can directly kill  $\beta$ -cells expressing MHC I through perforin/granzyme secretion <sup>97</sup>. CD4<sup>+</sup> T cells that recognize MHC II presented peptides cause  $\beta$ -cell destruction through production of IFN $\gamma$  and by activation of innate cells such as dendritic cells and macrophages <sup>97</sup>.

Autoreactive CD4+ and CD8+ T cells recognizing  $\beta$ -cell antigens within peripheral blood can be found in both NOD mice and humans with T1D. Here CD4+ and CD8+ T cells recognizing GAD65 and insulin have been identified<sup>90</sup>. Unlike in humans, NOD mice also have CD8+ T cells that recognize IGRP which comprises about 40% of the CD8+ T cells in the islets of NOD mice<sup>98, 99</sup>. Other autoreactive T cell identified include those that recognize heat shock protein 60<sup>98, 100-102</sup>.

### **Immune Regulation**

Ineffective central and peripheral immune regulation contributes to the development of T1D. In NOD mice, there are several studies suggesting ineffective thymic selection and peripheral regulation.

### **Thymic Selection**

In the thymus, T cells undergo positive selection where double positive (DP), CD4+ CD8+, cells interact with cortical thymic epithelial cells (CTE) that express MHC I and MHC II. T cells that have TCR that can recognize MHC I or MHC II receive signals to survive, while T cells with TCR that cannot recognize peptide-MHC complexes die<sup>103</sup>.<sup>104</sup> This is known as positive selection and is a key step in the induction of natural CD4+CD25+ regulatory cells<sup>103, 104</sup>. Following positive selection T cells undergo negative selection where T cells that strongly recognize self-antigen undergo apoptosis<sup>104</sup>.

It has been proposed that the I-A<sup>g7</sup> MHC class II in NOD mice and the diabetogenic HLA-DR and –DQ alleles in humans contribute to inefficient negative selection, thereby allowing more autoreactive T cells to make escape into the periphery<sup>105</sup>. I-A<sup>g7</sup> has been shown to poorly bind self-peptides which doesn't allow for a strong interaction with DP cells necessary for negative selection<sup>106, 107</sup>. In addition, in

NOD mice, it has been shown that the CTE are inefficient at inducing CD4+CD25+ Tregs<sup>108</sup>. Together these data demonstrate alterations in central immune regulation in T1D.

### **Peripheral Regulation**

In addition to ineffective thymic selection, there are deficits in peripheral tolerance, which is primarily mediated by Tregs, in T1D. The major function of Tregs is to suppress activation and proliferation of T lymphocytes<sup>109</sup> in order to eliminate excessive activation which could lead to prolonged host damage or autoimmune diseases. Tregs are typically characterized by expression of CD25+, the  $\alpha$ -chain of the high affinity IL-2 receptor<sup>110</sup>, and the transcription factor forkhead box P3 (FoxP3)<sup>111</sup>. Tregs have different mechanisms of suppression through direct interaction with effector T cells or through interactions with APC.

Tregs are able to suppress T cell responses through the inhibitory effects of IL-10 and TGF- $\beta$ . Surface bound TGF- $\beta$  on Tregs induces cell-cell immunosuppression<sup>112</sup>, while soluble IL-10 induces inhibition of signaling through the co-stimulatory molecule CD28<sup>113</sup>. Importantly Tregs are able to inhibit the induction of IL-2 mRNA in effector T cells; an important proliferative signal for T cells<sup>114-116</sup>. Tregs can also mediate cytotoxicity of effector T cells. In humans, activated Tregs express granzyme A and thus can kill CD4+ and CD8+ T cells in a perforin-dependent, Fas-FasL independent manner<sup>117, 118</sup>. In mice, activation of Tregs results in upregulation of granzyme B whereby granzyme-B deficient Tregs had reduced suppressive activity<sup>119</sup>.

A number of studies have shown that Tregs downregulate expression of co-stimulatory molecules on both human and mouse DC<sup>120, 121</sup>. Interactions of CTLA-4, located on the Tregs, with CD80 and CD86, located on the APC<sup>122</sup>, prevents increased

expression of CD80 and CD86 effectively limiting their abilities to stimulate naive T cells through CD28<sup>123</sup>. In addition, CTLA-4 on Tregs interacting with CD80 and CD86 on APC has also been shown to upregulate expression of IDO, which can inhibit T cell proliferation<sup>124</sup>.

The importance of Tregs has been demonstrated using “depletion of regulatory T cells” or DEREK mice which leads to severe autoimmune disease and death upon Treg depletion. DEREK mice express a diphtheria toxin (DT) receptor–enhanced green fluorescent protein fusion protein under the control of the *foxp3* gene locus and depletion of Tregs can be achieved upon injection of diphtheria toxin<sup>125</sup>.

Specific to T1D, as mentioned previously, BDC2.5 mice exhibit insulinitis yet only 10-20% of the BDC2.5 mice develop diabetes, most likely due to regulation by Tregs<sup>53, 54</sup>. In humans, mutations in forkhead box p3 (FOXP3), a Treg transcription factor, have been observed in several autoimmune diseases including T1D<sup>126</sup>. Together these models suggest that Tregs play an integral role in the loss of peripheral tolerance, the immune decision for  $\beta$ -cell destruction, and eventual development of clinical disease.

### **Environmental Contributions**

The incidence rate of T1D has been increasing over the past century faster than can be accounted for by genetics, suggesting that there are non-genetic factors contributing to initiation and or progression of disease<sup>127</sup>. There are a number of observations suggesting an environmental contribution. 1) A low concordance rate and differing rates of onset of T1D between monozygotic twins (65% when followed out to age 60)<sup>17-19, 128</sup> and 2) differences in incidence between the transmigratory population and their homeland counterparts despite genetic similarity<sup>12, 129, 130</sup>. For instance, one study demonstrated an increase in the incidence of T1D within Asian families who

moved to the UK. The study was conducted over 12 years evaluating subjects aged 0-16 years within the resident area. They found that the incidence in the Asian children started to approach the incidence rate found within the UK rather than that of their genetic counter parts <sup>130, 131</sup>.

Some environmental factors implicated in the development of the disease include: dietary factors (such as cereal proteins, cow milk proteins, and vitamin D) <sup>132</sup>, enteroviruses <sup>133</sup>, or changes in gut microbiota <sup>134</sup>. Additionally, some have suggested the “hygiene hypothesis” where exposure to infections early in life helps to ‘train’ the immune system and thus individuals who are not exposed develop autoimmunity <sup>135</sup>. Evidence supporting the hygiene hypothesis is that NOD mice develop diabetes only in a specific pathogen free environment, while housing them in a “dirty” environment dramatically decreases incidence <sup>92, 136, 137</sup>. Each of these environmental factors would closely interact with and effect the mucosal immune system. Thus, many researchers have started to investigate the role of the mucosal immune system in T1D.

### **Mucosal Immunity**

The intestinal mucosal system covers a very large surface area (~300 m<sup>2</sup> in humans) which is exposed to the external environment. Thus, the mucosal immune system faces the unique challenge of combating pathogens, while still maintaining homeostasis in the presence of bacteria and luminal content. In addition to being the first line of defense for the mucosal immune system, commensal bacteria are necessary for development and maturation of the lymphoid tissues within the mucosal immune system <sup>138-143</sup>. Specifically, the commensal microbiota can provide protection by competition with pathogenic organisms, as well as help develop host natural immunity and tolerance <sup>138-143</sup>. After the protection granted from the microbiota, the next line of

defense involves the intestinal epithelial cells that provide a barrier from the external environment.

### **Intestinal Epithelial Cells**

The Intestinal epithelial cell (IEC) not only absorb nutrients from food, but also functions as a barrier to the external luminal content and as acts an innate immune cell<sup>144, 145</sup>. The IEC function to uptake nutrients from the luminal content via two pathways: transcellular and paracellular permeability<sup>146</sup>. Transcellular permeability occurs through specific pumps, channels, and transporters<sup>146</sup>. Paracellular permeability occurs around or between cells and is regulated by tight junctions (TJ)<sup>146-148</sup>. Expression of different TJ molecules allows for selective restrictiveness of the molecules allowed to pass<sup>146-148</sup>.

The functions of tight junctions are twofold. First, TJ can function to restrict paracellular permeability and second, TJ allow IEC to form a barrier. TJ are the most apical complexes on IEC responsible for sealing off access from the lumen. Tight junctions are comprised of transmembrane proteins that interact extracellularly with adjacent cells and intracellularly with adaptor proteins that interact with the cytoskeleton<sup>149-152</sup>.

IEC form a single layer which is covered by both mucus and anti-microbial products produced by specialized cell types within the gastrointestinal track. Specifically, goblet cells secrete mucus which acts as a protective covering limiting movement within the gastrointestinal track and access to IEC. Paneth cells secrete anti-microbial peptides, such as  $\alpha$ -defensins, while IEC secrete the anti-microbial peptides,  $\beta$ -defensins<sup>153, 154</sup>.

The IEC are constantly being replenished, where damaged, infected, or apoptotic IEC move to the tip of villi to be sloughed off and new IEC arise in the crypt and migrate

upwards<sup>153, 154</sup>. Interspersed within the IEC are intraepithelial lymphocytes (IEL) that maintain normal homeostasis. Importantly, while IELs can regulate IEC, IECs also influence IEL T cell development<sup>155-157</sup>.

### **Intestinal Macrophages**

Typically cells within the mucosal immune system regulate homeostasis, but if bacteria do invade the epithelium and the underlying lamina propria (LP) there are various immune mechanisms to prevent systemic infection. For instance, LP residing intestinal macrophages interact closely with the epithelium and quickly take up and eliminate bacteria through the production of antibacterial peptides and reactive oxygen species<sup>158, 159</sup>.

Although these intestinal macrophages are able to efficiently clear bacteria, they do not elicit a strong pro-inflammatory immune response<sup>160, 161</sup>. Activation of toll like receptors (TLR), receptors that recognize pathogen associated molecular patterns (PAMPs), on intestinal macrophages instead induce production of growth factors, such as insulin-like growth factor 1 (IGF-1), which induce proliferation of the epithelium in order to repair breaks in the barrier<sup>162, 163</sup>. In addition to regulating epithelial cell homeostasis, intestinal macrophages have been implicated in generation of FoxP3+ regulatory T cells (Tregs) through expression of IL-10<sup>164</sup>. Thus, if a breakdown in barrier integrity occurs and there is in an aberrantly inflammatory environment, intestinal macrophages may be unable to aid in the development of Tregs resulting in reduced Treg frequency.

### **Intestinal Dendritic Cells**

In general DC sample antigens from the environment and present them to T cells. Upon activation, DC express high levels of MHC, co-stimulatory molecules, and

cytokines necessary for the activation, proliferation, and polarization of T and B cells. In this way, they regulate and direct the adaptive immune response. Intestinal DCs reside in the mesenteric lymph nodes (MLN), peyer's patches (PP), small intestine LP and colon LP<sup>165, 166</sup>. Intestinal DC have specialized properties and function from conventional DC. For example, activated DC from the PP express higher levels of IL-10 and are able to induce higher levels of IL-4 and IL-10 in naïve CD4+ T cells than DC from the spleen<sup>167</sup>.

A subpopulation of intestinal DC expresses CX3CR1 and resides within the LP where they extend dendrites into the lumen and sample luminal content<sup>168</sup>. While CX3CR1+ DC have been described as the most efficient in sampling luminal content, they also polarize naïve CD4 T cells towards a Th17 phenotype<sup>169</sup>. For instance, *in vitro*, CX3CR1+ CD70+ LP DCs induce CD4 T cells to produce IL-17 in the presence of ATP<sup>170</sup>. In addition, in CX3CR1-/- animals, transfer of colitis is attenuated due to decreases in IFN $\gamma$  and IL17A secreting CD4 T cells in the LP<sup>171</sup>. The role of IL-17 producing T cells in mucosal immunity will be expanded on below.

Another intestinal DC subpopulation which express CD103+ are more efficient in the priming CD4 and CD8 T cell responses, than CX3CR1+ DC, primarily due to their ability to migrate to the MLN<sup>172</sup>. But unlike the Th17 phenotype induced by CXCR1+CD70+ DCs, CD103+ DC induce naïve T cells to express FoxP3<sup>173-176</sup>. CD103+ DCs induce FoxP3 expression via the expression of retinoic acid (RA), a metabolite of vitamin A, transforming growth factor beta (TGF- $\beta$ ), and indoleamine-2,3-dioxygenase (IDO)<sup>175, 177, 178</sup>. Retinoic acid, as well as TGF- $\beta$  is made by both CD103+ DCs and stromal cells within the MLN<sup>175</sup> where a substantial proportion of proliferating

T cells are expressing FoxP3<sup>175, 176</sup>. In addition, CD103+ DC induce the expression of intestinal homing markers CCR9 and  $\alpha 4\beta 7$  on T cells. Expression of these intestinal homing markers recruits and retains T cells within the mucosal environment.

### **Regulatory T cells and Mucosal Tolerance**

In the intestine, Tregs play a critical role in maintaining homeostasis and achieving oral tolerance. Antigen specific Treg clones are found in peyer's patches (PP) in orally tolerized mice<sup>179</sup>. Similarly, it has been demonstrated that CD25<sup>+</sup>CD4<sup>+</sup> Tregs are generated in the mesenteric lymph nodes (MLN) in orally tolerized ovalbumin-T cell receptor transgenic mice<sup>180</sup>.

Trafficking of T cells to the GALT plays an important role in mucosal homeostasis, oral tolerance, and prevention of autoimmunity. Expression of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on the endothelium allows for T cells expressing the mucosal homing integrin  $\alpha 4\beta 7$  to traffic to mucosal sites. Homing to the gut via MAdCAM-1 and  $\beta 7$  is necessary for oral tolerance since mice lacking either of these molecules are unable to be orally tolerized<sup>181</sup>. It has been demonstrated that CD4<sup>+</sup> T cells need to migrate to the gut to be exposed to IL10 in order to become fully tolerogenic<sup>181</sup>. Additionally, IL10 expressing myeloid LP cells are able to sustain FoxP3 expression in T cells<sup>182</sup>, suggesting that exposure to the LP environment can stabilize a regulatory T cell phenotype. Other supporting evidence suggest that Tregs migrate to and undergo expansion within the LP<sup>183</sup>. Another mucosal integrin,  $\alpha E\beta 7$ , allows T cells to interact with the epithelium via E-cadherin.  $\alpha E^+CD25^+$  Tregs are more suppressive than  $\alpha E^-CD25^+$ , again suggesting that the mucosal environment supports peripheral immune tolerance.<sup>184</sup>

## Th17 Cells

Th17 cells are a subset of CD4<sup>+</sup> TCRβ<sup>+</sup> T cells that express the transcription factor RORγt, which promotes the transcription of IL17a. Th17 cells do not express the transcription factor T-bet, and thus do not express IFNγ therefore they are considered to be a subset of T cells distinct from the Th1 subset [reviewed in <sup>185</sup>]. Naïve T cells in both mice and humans can be induced to express RORγt, through expression of TGFβ, IL-6, and IL-1β, which then leads to the expression of IL17A and IL17F <sup>186-188</sup>. IL-6 can induce expression of IL-21 which can synergize with the above cytokines to induce expression of the IL-23 receptor (IL-23R) <sup>189, 190</sup>. IL-23 interaction with its receptor on CD4<sup>+</sup> RORγt<sup>+</sup> IL-17A<sup>+</sup> cells expands and fully matures the T cells into Th17 cells <sup>191, 192</sup>. In both mice and humans under steady state conditions, there are a low number of Th17 cells with a majority of them accumulating within the intestine <sup>185, 193, 194</sup>. Here Th17 cells function to maintain the epithelial barrier and to help defend from extracellular bacterial invasion.

Th17 effector cytokines are critical for defense against infections and include IL-17A, IL-17F, and IL-22 <sup>195</sup>. Receptors for IL-17A, IL-17F, and IL-22 are expressed on the epithelium throughout the intestine, therefore Th17 cells provide communication between the mucosal immune system and the intestinal tissue <sup>185, 195</sup>. IL-17A and IL-17F promote production of β-defensin by the epithelium and recruits neutrophils to sites of inflammation <sup>196-198</sup>. IL-17A and IL-17F also induces expression of CCL20 by the epithelium. CCL20 can bind to the receptor CCR6 which is highly expressed on Th17 cells, in order to retain or recruit Th17 cells in the gastrointestinal track <sup>193</sup>. IL-22 also induces expression of antimicrobial peptides from epithelial cells <sup>195, 199</sup>, while promoting epithelial survival, proliferation, and tissue repair within the intestine <sup>200-202</sup>.

Although Th17 cells play a significant role in mucosal homeostasis, several autoimmune models such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD) which were incorrectly considered to be Th1-mediated diseases are now considered Th17-mediated diseases<sup>203-205</sup>. It is now known that IL-23, not IL-12, though they both share the p40 subunit, is required for these diseases and has given rise to a new Th17 mediated pathogenesis paradigm<sup>206</sup>. Unlike Th1 cells, which do not upregulate the IL-23R, Th17 cells express both the IL-23R and the IL-12R and can respond to IL-23 or IL-12<sup>207</sup>. Using an IL-17F reporter mouse model, it was determined that Th17 cells could change their cytokine expression depending on the milieu of cytokines with which they were stimulated<sup>207</sup>. The stimulation with various cytokines resulted in cells that had high expression of IL-17A/F, low expression of IL-17A/F with high levels of IFN $\gamma$ , or cells that expressed both IL-17A/F and IFN $\gamma$ <sup>207</sup>.

Several studies suggest that Th17 cells are able to progress into IFN $\gamma$ -producing cells in vivo. First, transfer of Th17 cells from an IL-17F reporter mouse into immunodeficient hosts induced colitis and shifted the Th17 cells to express reduced levels of IL-17A, lose expression of IL-17F, and upregulate expression of IFN $\gamma$ <sup>207</sup>. Second in an antigen specific model for ocular inflammation, a substantial fraction of Th17 cells adopted a more Th1 phenotype through expression of both IFN $\gamma$  and IL17<sup>208</sup>. Finally, two groups reported that transfer of Th17 transgenic BDC2.5 T cells induced insulinitis and diabetes after adopting a Th1 phenotype<sup>209, 210</sup>. Both groups noted that islet destruction was IFN $\gamma$  dependent, suggesting that Th17 cells producing IFN $\gamma$  are pathogenic in this model of autoimmunity<sup>209, 210</sup>.

Several mechanisms within the mucosa have evolved to control the effector function of Th17 cells including prevention of Th17 differentiation. High concentrations of TGF $\beta$  and/or retinoic acid (RA) promote expression of Foxp3, which inhibits expression of ROR $\gamma$ t, therefore inhibiting Th17 differentiation<sup>175, 190, 211</sup>. Similarly, IL-2 can synergize with TGF $\beta$  to induce Tregs instead of Th17 cells<sup>212</sup>. Specifically, IL-2 induces pSTAT5, which competes with the STAT3 binding site on the IL-17A locus and inhibits IL17a transcription<sup>213</sup>. Prevention of Th17 differentiation is one way of regulating Th17 responses, but once a strong Th17 response occurs additional regulatory mechanisms are called into play. For instance, Foxp3<sup>+</sup> Tregs have been shown to suppress Th17 cells *ex vivo*<sup>214, 215</sup>. In addition, Th17 cells have demonstrated great plasticity in their phenotype and effector function. For instance, Th17 cells typically reside within the ileum, where during strong Th17 responses they are recruited to the duodenum on a CCL20 chemokine gradient<sup>193</sup>. Here effector Th17 cells are eliminated into the lumen due to tissue destruction and diarrhea. The remaining Th17 cells are reprogrammed to a more regulatory phenotype and express high levels of IL-10 and have suppressive capacity through TGF- $\beta$  and CTLA-4<sup>193</sup>. The mechanism through which these cells were reprogrammed is still unclear.

Similar to Th17 cells, Tregs also exhibit plasticity where a dualistic function of TGF $\beta$  is evident since both Tregs and Th17 cells require TGF $\beta$  for differentiation<sup>186-188</sup>. TGF $\beta$  inhibits Th1 and Th2 differentiation<sup>216</sup>, thus in an environment with TGF $\beta$  Th17 or Treg cells are favored depending on whether IL-6 is present or absent. Tregs can be induced to express IL-17 and downregulate Foxp3 by stimulation with IL-6, suggesting that Tregs can become Th17-like<sup>217</sup>. Thus far the reciprocal conversion, from Th17 to

Treg, has not been described, suggesting that the conversion from Treg into Th17-like may be irreversible<sup>218</sup>. This would have important implications for autoimmunity and the autoimmune process, because if the environment favors conversion of Tregs into Th17-like cells it may result in a permanent loss of regulation. Coupled with the IFN $\gamma$  promoting environment, a more inflammatory Th17 phenotype would also prevail.

## **Mucosal Immunity and T1D**

### **Diet and T1D**

Dietary factors seem to have an influence on development of T1D. For example, there is increasing evidence of an association between celiac disease (CD) and T1D. Celiac disease is an intestinal autoimmune disease triggered by exposure to gluten which is found in cereal proteins. After exposure, CD patients exhibit inflammation that leads to destruction of the epithelium which results in loss of villous structure and impairs absorption of nutrients<sup>219</sup>. Both T1D and CD share high susceptibility with the HLA DQ2/DQ8 genotype and they share almost half of the susceptibility alleles associate with T1D<sup>220</sup>.

It has been shown in rodent models for T1D that a cereal based diet promotes development of the disease<sup>221, 222</sup>. In NOD mice, it has been demonstrated that a life-long gluten free diet decreased incidence<sup>223</sup> and that a delayed exposure to wheat and barley proteins also decreases incidence<sup>224</sup>. These studies suggest a relationship between exposure to dietary gluten and the development of T1D.

### **Evidence of Mucosal Inflammation in T1D**

Increasing evidence suggests that the mucosal immune system is involved with development of T1D. It has been observed that pediatric patients with T1D have an increased expression of major histocompatibility complex (MHC) class II as well as

ICAM-1 on the epithelium suggesting activation<sup>225, 226</sup>. Additionally, a higher frequency of IFN $\gamma$  and TNF $\alpha$  positive cells as well as IL-1 $\alpha$  and IL-4 positive cells were found to be located in the lamina propria in patients compared to diabetes free participants<sup>226</sup>. These findings suggest that the gastrointestinal mucosal immune system is activated in T1D patients.

In the NOD mouse, it has been observed that disease causing T cells are located within the MLN as early as 3 weeks of age<sup>227</sup>, where MLN lymphocytes display a higher diabetogenicity compared to the PLN lymphocytes upon adoptive transfer. Additionally, islet infiltrating T cells express  $\alpha 4\beta 7$  which is a gut associated lymph tissue (GALT) specific integrin. T cells during the pre-diabetic phase typically express this integrin whereby  $\alpha 4\beta 7$  blocking antibodies can prevent diabetes<sup>228, 229</sup>. Interestingly, around time of weaning endothelium within the NOD mouse pancreas begins to express MAdCAM-1 which interacts with  $\alpha 4\beta 7$ <sup>228</sup>. This suggests that the change in diet may be contributing to the T cells homing to the pancreas. Some human studies support these findings, where GAD reactive T cells from T1D patients express also express  $\alpha 4\beta 7$ <sup>230</sup> and lymphocytes from pancreas of T1D individuals were able to adhere to both the mucosal and pancreatic endothelium<sup>231</sup>. Together these data suggests that autoimmune activation can occur within the GALT and contribute to migration of auto-reactivity to the target organ<sup>232</sup>.

### **Gastrointestinal Alterations in T1D**

Gastrointestinal (GI) alterations have been observed in both rodent models of T1D and in T1D patients. Biobreeding diabetes prone rats exhibit several GI alterations including increased intestinal permeability, greater crypt depth, abnormal epithelial cell

proliferation, and inflammatory lymphocyte infiltration prior to development of insulinitis and diabetes<sup>233-236</sup>.

Human studies support that there are GI alterations in T1D. A study evaluating patients at varying stages of disease demonstrated that all patients exhibited an increase in permeability and that a compromise in barrier function occurred prior to clinical onset of T1D<sup>237</sup>. Another study examined non-celiac T1D patients where these patients exhibited increased permeability and structural changes in microvilli and tight junctions compared to diabetes-free controls<sup>238</sup>. Finally, it has been demonstrated that pediatric T1D patients with the genotype HLA-DQ2 also exhibit increased gastrointestinal permeability<sup>239</sup>. Together, these studies suggest that a compromised intestinal function or even an intestinal defect may play a role in the disease.

Somewhat recently it was demonstrated that zonulin, a host protein involved with the disassembly of tight junctions may be contributing to the alterations in intestinal permeability observed in T1D<sup>240</sup>. Zonulin can cause disruption of the tight junctions through interaction with proteinase-activated receptor 2 (PAR2) which can lead to activation of the EGF receptor (EGFR) or through direct interaction with EGFR. The activation of EGF receptor leads to increased permeability<sup>240</sup>. Zonulin has been found to be upregulated in a number of autoimmune diseases including CD and T1D<sup>241-244</sup>.

As mentioned previously, studies have shown an increase in intestinal permeability occurs prior to clinical onset of disease. Thus, using the BBDP rat model, it was demonstrated that zonulin dependent increases in intestinal permeability occurred 2-3 weeks prior to onset<sup>245</sup>. Additionally, the study demonstrated that oral administration of the zonulin inhibitor, AT1001, decreased intestinal permeability,

blocked auto-antibody formation, and decreased incidence of disease<sup>245</sup>. These studies showed that in the BBDP rat model for T1D zonulin dependent loss of barrier function contributed to the initiation of the disease.

In humans, preliminary studies suggest that zonulin is upregulated prior to disease onset<sup>246</sup>. Interestingly, this same study reported that about 25% of unaffected family members of patients with T1D exhibited increased zonulin levels and an increase in intestinal permeability without progressing into disease, suggesting that loss of barrier function is not sufficient for disease progression<sup>246</sup>.

### **Hypothesis and Summary**

As describe above, there is increasing evidence supporting a role for the mucosal immune system in the development of T1D. We particularly found evidence of increased intestinal permeability prior to disease onset in both humans and in rodents intriguing. When paired with the evidence of mucosal inflammation in humans and in rodents, the question then became: which occurs first? This question drove us to our hypothesis: that a breakdown in intestinal barrier function results in an inflammatory environment that promotes non-tolerizing conditions.

## CHAPTER 2 MATERIALS AND METHODS

### **Murine Models**

NOD/LtJ mice were bred and housed under specific pathogen-free conditions. Mice were maintained in an American Association of Laboratory Animal Care–accredited facility, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee. NOD/LtJ and C57Bl/6 mice were sacrificed at 4, 8, 12, and 16 weeks of age. At time of sacrifice, intestinal segments, MLN, and PLN were harvested.

### **Gastrointestinal Permeability**

Immediately following euthanasia, the whole small intestine was removed and placed into 150mM NaCl. Sections for the proximal jejunum (located ~5 cm from the stomach, proximal portion of the proximal jejunum), the distal ileum (located just above the caecum, distal portion of the distal ileum), and the distal colon (distal portion of the distal colon) were isolated. Remnants of connective tissue were removed with dissection tools and the intestinal segment was opened along the mesenteric border. Flat sheets of tissue were mounted in modified Ussing chambers having an exposed tissue area of 0.3 cm<sup>2</sup>. Serosal to mucosal flux of [14C] mannitol, specific activity 50-60 mCi/mmol, and [14C] dextran, specific activity 0.5-2 mCi/g, (American Radiolabeled Chemicals, Inc, Saint Louis, MO) were measured across tissues bathed on both sides by 4 ml of buffered saline (pH 7.4) at 37°C circulated by bubbling with 95% O<sub>2</sub> - 5%CO<sub>2</sub>. The standard saline contained the following solutes (mmol/L): 114.8 NaCl, 1.4 KCl, 1.6 K<sub>2</sub>HPO<sub>4</sub>, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 25.0 NaHCO<sub>3</sub>, 10.0 Glucose, 1.0 MgSO<sub>4</sub> 7H<sub>2</sub>O, 1.0 CaCl<sub>2</sub> 2H<sub>2</sub>O. The standard buffer also contained 10 uM indomethacin to inhibit prostanoid production.

A concentration of 1mM of [14C] mannitol and 0.1uM of [14C] dextran in standard buffer was used for experiments. Radiolabeled mannitol or dextran was added to the serosal side and movement measured at 15 minute intervals for 45 minutes by sampling 1 mL of the buffer on the mucosal side. The activity of the samples were then measured on a scintillation counter (Beckman LS6500, Beckman-Coulter Inc). Experiments were performed under short-circuit conditions using an automatic voltage clamp (VCCMC6; Physiologic Instruments, San Diego, CA). The electrical parameters of the tissue were recorded at 15 minute intervals throughout the experiment. Tissue conductance ( $G_t$ ; mS/cm<sup>2</sup>) was calculated as the ratio of the open circuit potential ( $V$ ; mV) to the short circuit current ( $I_{sc}$ ;  $\mu$ A/cm<sup>2</sup>). Fluxes ( $J_{sm}$ ; nmol/cm<sup>2</sup>/hr) of tissues were calculated using the FLUXers Fantasy program developed by Robert Freel, PhD, Department of Pathology, College of Medicine, University of Florida. .

### **Flow Cytometry**

Spleen (SPLN), mesenteric lymph node (MLN), and pancreatic lymph node (PLN) were ground between glass slides to achieve single cell solutions in PBS. Spleen solutions were placed into red blood cell lysis buffer (8.3g NH<sub>4</sub>Cl, 1.0gKHCO<sub>3</sub>, 1.8mL of 5% EDTA, in 1L dH<sub>2</sub>O) for 10 minutes at room temperature, pelleted (1200rpm, 10 min) and resuspended in FACS buffer (1% FBS/PBS/2mM EDTA). Cells were then plated in 96 well plates with 5ug/ml of plate-bound CD3 (eBio 145-2C11) and soluble CD28 (eBio 37.51) for 3 days. Afterwhich cells were incubated for 4 hours with 10ng/ml of PMA, 1ug/ml of Ionomycin and 10ug/ml of Brefeldin A (Sigma).

Polyclonally expanded SPLN, MLN, and PLN cells were stained for extracellular markers in FACS buffer for 30 minutes on ice, pelleted (1200 rpm, 10 min) and washed 3x with FACS buffer. Cells were then fixed and permeabilized with Cytofix/Cytoperm

(Becton Dickinson, Franklin Lakes, NJ ;51-2090KZ) solution for 30 minutes on ice. The extracellular stained cells were washed 3x with Perm/Wash (Becton Dickinson; 51-2091KZ) and stained with intracellular cytokine abs for 1 hour in BD Perm/Wash solution on ice. Samples were pelleted (1200 rpm, 10 min), washed 3x with Cytotfix/Cytoperm and resuspended in FACS buffer containing 4% PFA. Data were acquired using a BD Accuri (Becton Dickinson) and analyzed using BD Accuri C6 software (Becton Dickinson). Antibodies were purchased from BD Bioscience and eBioscience and used at 1:200 dilution for analysis of CD4<sup>+</sup> T cells: FITC anti-CD4 (eBio RM4-5), APC anti-IFN $\gamma$  (eBio XMG1.2), PerCPCy5.5 anti-IL-17A (BD Bio TC11-18H10), and PE anti-IL-4 (eBio 11B11).

### **ELISA**

A capture ELISA was used to measure levels of TNF- $\alpha$  in homogenates of duodenum. Sections of duodenum (~1.2 cm, normalized by weight 0.085g) were obtained and homogenized using a Mini-Beadbeater TM (Biospec Products) and 1.0mm Zirconia/Silica beads (Biospec Products 11079110z). Samples were then centrifuged for 10 minutes at 10000rpm and supernatants harvested. TNF $\alpha$  levels were determined using a BD OptEIA ELISA kit (BD OptEIA TNF- $\alpha$  ELISA kit 559534). 96-well plates were coated with a 1:250 dilution of capture anti-TNF $\alpha$  in coating buffer (8.40g NaHCO<sub>3</sub>, 3.56g Na<sub>2</sub>CO<sub>3</sub>, pH 9.5) and incubated overnight at 4C. Plates were blocked in 10%FBS/PBS for 1 hour. Plates were washed 3x with wash buffer (PBS 0.05% Tween-20). 50ul of supernatant was added per well and incubated at room temperature for 2 hours. Plates were washed 5x with wash buffer and incubated with 1:250 dilution of biotinylated anti-TNF $\alpha$  at room temperature for 1 hour. Plates were washed 5x with wash buffer and streptavidin-horse radish peroxidase (HRP) was added at a 1:250

dilution and incubated for 30 minutes. After washing 7x with wash buffer, plates were developed for 30 minutes in the dark at room temperature using 100ul of TMB substrate solution. Data was acquire on a spectrophotometer (Biotek Epoch) at (450nm) and (570nm) where the final absorbance was calculated as  $\Delta(450\text{nm}-570\text{nm})$ . Cytokine concentrations in culture supernatants were determined using a standard curve.

### **Statistics**

The following data are presented as mean  $\pm$  SEM. Comparisons between NOD and B6 mice were made by unpaired Students t-test. Comparisons between NOD MLN and PLN ages were made by unpaired Students t-test, since ANOVA analysis could not be conducted due to low sample number. A Spearman's correlation and linear regression was made between  $J_{\text{sm}}^{\text{dextran}}$  and  $G_{\text{T}}$ . The results were accepted as significant at  $P < 0.05$ . Statistical analysis was carried out using GraphPad Prism 6 and figures were drawn in GraphPad Prism 6.

## CHAPTER 3 RESULTS

In order to address our hypothesis, we temporally evaluated intestinal barrier function and inflammation. The effect of changes in barrier function and inflammation on MLN and PLN T cell phenotypes was also evaluated. Time points for evaluation were chosen based off of the well-characterized disease checkpoints of disease progression within the NOD mouse <sup>48</sup>. Thus, we evaluated mice at 4, 8, 12, and 16 weeks of age. As a control for our NOD mice, we used the genetically distinct C57Bl/6 mice which do not exhibit autoimmunity (Figure 3-1).

To evaluate intestinal barrier function, we measured transcellular movement of charged ions through and paracellular permeability of the small and large intestine by conducting flux experiments utilizing Ussing chambers. Specifically, the movement of [14C] mannitol across specific sections of intestine measured paracellular permeability, while the electrical parameters were used to determine the movement of charged ions across the tissues. Data generated here would allow us to determine if, when and, where there are alterations in gastrointestinal permeability during T1D disease progression.

To evaluate general intestinal inflammation, we evaluated levels of inflammatory cytokines in homogenized intestine by ELISA. T1D is primarily a T cell mediated disease due to loss of central and peripheral tolerance. In addition, there is strong evidence of autoreactive T cells residing in the MLN at early stages of disease <sup>227</sup>. Thus we also evaluated T cell populations within the MLN and PLN over the course of the disease.

Our final goal was to determine if a rise in the gastrointestinal inflammatory environment was a cause or consequence of loss of barrier function and its effect on peripheral tolerance as measured by T cell phenotypes in the gastrointestinal track and target organ.

### **Intestinal Permeability**

Previous studies have demonstrated alterations in GI function associated with T1D in humans and mice<sup>233-239</sup>, whereby there was increased permeability as measured by lactulose:mannitol leakage. Though these experiments do answer the question of whether there are alterations in permeability, they do not temporally evaluate permeability or evaluate specific sections of intestine for differences in permeability. Thus, whether this is a consequence or cause of immunological phenomenon observed in T1D is still unclear. Therefore, in order to temporally evaluate specific intestinal sections for differences in permeability, we utilized *in vitro* analysis of gastrointestinal charged ion movement and paracellular permeability using Ussing chambers.

Sections of proximal jejunum and distal ileum, both located within the small intestine, as well as distal colon, located within the large intestine were harvested. Tissue sections were mounted in Ussing chambers and measurements for flux of [14C]mannitol ( $J_{sm}^{\text{mannitol}}$ ) across the short-circuited tissue sections were obtained. Electrical parameters were also obtained. Specifically, short circuit current ( $I_{sc}$ ) and open circuit potential ( $V$ ), were measured at 15 minute intervals for 45 minutes. Conductance ( $G_T$ ) was determined as the ratio of  $V$  to  $I_{sc}$  and relates to the permeability of the tissue. [14C] mannitol can be used as a marker for paracellular permeability

while electrical parameters can be used as an indicator of movement of charged ions if no differences in paracellular permeability are present.

### **Movement of Charged Ion Differs between NOD and B6 Mice**

Initially, in conjunction with a collaboration with Dr. Marguerite Hatch, Department of Pathology, College of Medicine at the University of Florida, the short circuit current ( $I_{sc}$ ) and open circuit potential ( $V$ ), were measured and the conductance ( $G_t$ ) calculated in the distal ileum (DI), and proximal jejunum (PJ) (Figure 3-2). It was determined that that  $G_T$  of the proximal jejunum and the distal ileum from B6 mice was higher than that observed in tissues from NOD mice throughout the course of disease (Figure 3-2 C, F). The higher  $G_T$  was most likely due to the higher  $I_{sc}$  also exhibited by tissues from B6 mice (Figure 3-2 B, E). For paracellular permeability, it was determined that there were no differences in the  $J_{sm}^{mannitol}$  of tissues from NOD and B6 mice until 16 weeks of age (Figure 3-2 A, D). Thus, without differences in paracellular permeability, the differences in  $I_{sc}$  and  $G_t$ , indicate smaller movements of charged ions within the small intestine of NOD mice compared to those of B6 mice.

### **Preliminary Flux Trials**

After a period of shadowing Dr. Hatch, I needed to improve my skills with the techniques used for flux measurements. Dissection and mounting of the intestinal tissue are technically demanding aspect of the experiment where incorrectly handled intestine can significantly alter permeability of the tissues. Thus, a series of flux trials was performed in order to evaluate the reproducibility of the assays following my training (Figure 3-3). Initially, we compared  $G_T$  and  $J_{sm}^{mannitol}$  from B6 mice with measurements from previous experiments performed by Dr. Hatch (Table 1). Although my initial experiments had high levels of variation (Trial 1; Figure 3-3), by the third trial of

experiments, the variation was significantly lower and measurements were within similar ranges of those obtained by Dr. Hatch (Table 1). Specifically, within the distal colon, the  $G_T$  lowered each trial and by the third trial measurements had come into acceptable range ( $\sim 15\text{-}20\text{mS}/\text{cm}^2$ ) indicating that handling of the tissue had improved with each trial whereby the integrity was not compromised (Figure 3-3 A). Similar results were seen for the  $G_T$  and  $J_{sm}^{\text{mannitol}}$  of the distal ileum and proximal jejunum (Figure 3-3 B, C, E, F). Specifically, the measurements of mannitol flux across the distal ileum and proximal jejunum was initially very high suggesting that handling of the tissue was not optimal (Figure 3-3 E, F), which significantly improved over the subsequent trials.

### **70kDa Dextran as a Paracellular Permeability Marker**

After evaluating the movement of mannitol as a marker for paracellular permeability and seeing no differences between NOD and B6 mice we decided to evaluate paracellular permeability using a larger molecule. Mannitol is a small molecule ( $\sim 4\text{\AA}$  in diameter) and it is possible that it is not large enough to distinguish small changes in paracellular permeability. Indeed a study investigating the role of tight junctions in size exclusion of macromolecules observed greater fold differences in paracellular permeability when using larger molecules<sup>247</sup>. Thus, we decided to use [14C] 70kDa dextran which is significantly larger ( $\sim 36\text{\AA}$  diameter) than mannitol and therefore should be more sensitive to small changes in permeability.

I initially evaluated [14C] 70kDa dextran flux ( $J_{sm}^{\text{dextran}}$ ) as described for [14C] mannitol (Figure 3-4). Experiments were conducted on the proximal jejunum and distal ileum, as well as the distal colon from B6 mice. I observed similar  $G_T$  measurements as previously shown (Figure 3-3), but I now observed much lower movement of 70kDa

dextran when compared to [14C] mannitol (Figure 3-4). Therefore, I wanted to determine whether 70kDa dextran could be used as a paracellular permeability marker.

To artificially increase paracellular permeability, we conducted experiments in the absence of  $\text{Ca}^{2+}$  (Figure 3-4). Using a  $\text{Ca}^{2+}$  free buffer results in loss of tight junction integrity therefore causing a decrease in electrical resistance or an increase in conductance ( $G_T$ )<sup>248</sup>. Thus, if we decrease tight junction integrity and see an increase in  $G_T$ , then we would expect to see greater paracellular movement of dextran. Indeed when comparing  $J_{sm}^{\text{dextran}}$  to  $G_T$  for experiments done in standard buffer with the experiments done in  $\text{Ca}^{2+}$  free buffer, we determined that dextran movement ( $J_{sm}^{\text{dextran}}$ ) increases as the  $G_T$  increases (Figure 3-4). This positive correlation suggests that dextran could be used as a paracellular marker in future experiments. Due to its larger size it may provide more sensitive marker of paracellular permeability.

### **Elevated TNF $\alpha$ in Duodenal Tissues of NOD Mice when Compared to B6**

Evidence of intestinal inflammation has been observed in rodent models for T1D and in human patients<sup>225-232</sup>. In addition, inflammatory cytokines, such as TNF $\alpha$ , cause increased permeability by modification of tight junctions and even cell death, while regulatory cytokines, such as IL-10, have been shown to support barrier function [reviewed in<sup>249</sup>]. Thus, in order evaluate mucosal inflammation at key disease checkpoints<sup>48</sup>, we initially determined TNF $\alpha$  levels in the duodenal lysates of NOD and B6 mice by ELISA (Figure 3-5). At both early and late stages of disease, we observed elevated levels of TNF $\alpha$  in the duodenum of NOD mice compared to B6 mice (Figure 3-5). First, this suggests that there is inflammation in the intestine of NOD mice even at early stages in disease. Second, this suggests that there could be effects on tight

junctions in NOD mice, which were not detected in the evaluation of paracellular permeability as measured by movement of [14C]mannitol (Figure 3-2).

### **T cell Population Differences between NOD and B6 Mice**

As outlined in the background, the mucosal environment can regulate and polarize naïve T cell responses. Thus, we evaluated T cell phenotypes within the draining lymph nodes of the gastrointestinal track (MLN). In addition, we wanted to determine the evolution of T cell inflammation/phenotype in the MLN and the target organ over the course of disease. Thus we also evaluated T cell phenotypes within the draining lymph nodes of the pancreas (PLN). In order to evaluate T cell phenotypes, we first started by evaluating populations of CD4<sup>+</sup> T cells. We initially characterized the phenotype of CD4<sup>+</sup> T cells from the MLN and PLN via flow cytometry. Specifically the percentage of IFN $\gamma$ <sup>+</sup> (Th1), IL-17A<sup>+</sup> (Th17 – IL17 only) and IFN $\gamma$ <sup>+</sup> IL-17A<sup>+</sup> (Th17 – IL17 IFN $\gamma$ ) were determined (Figure 3-6 C). Th1 T cells have been shown to be key mediators in the pathogenesis in T1D<sup>92-96</sup>. On the other hand depending on the phenotype of Th17 cells they play an important role in mucosal homeostasis or play a role in induction and maintenance of autoimmunity<sup>250</sup>.

Overall a higher frequency of CD4<sup>+</sup> T cells which were polarized to express cytokines were observed in both the MLN and PLN of NOD mice when compared to B6 mice (Figure 3-7). Specifically, in the MLN, a higher frequency was observed at 4, 8, and 12 weeks of age (Figure 3-7 A), while in the PLN, increased frequencies were observed at all checkpoints of disease in NOD mice (Figure 3-7 B). Interestingly, the frequency of CD4<sup>+</sup> T cells within the MLN of NOD mice which were polarized to express cytokines (at least those we evaluated) appears to decline over the course of the disease (Figure 3-7 A). Conversely, the frequency of CD4<sup>+</sup> T cells within the PLN

appears to increase over the course of the disease (Figure 3-7 B). These data suggest that there is increased inflammation within the MLN at early checkpoints prior to the increased inflammation observed at later checkpoints in the PLN.

### **Th17 and Th1 Differences between NOD and B6 Mice**

As has previously been published, Th1 cells are elevated in the PLN which increase in frequency over the course of the disease in NOD mice and have been demonstrated to significantly contribute to  $\beta$ -cell destruction<sup>251, 252</sup>. Indeed NOD mice exhibited higher frequencies of IFN $\gamma$  only CD4<sup>+</sup> T cells in the MLN at both 4 and 8 weeks of age when compared to B6 (Figure 3-8 C). Although, after 8 weeks of age this population sharply declined in the MLN of NOD mice, but was still higher than the frequencies observed in B6 mice at 12 weeks of age and was normalized by 16 weeks of age (Figure 3-8 C). In the PLN, NOD mice had higher frequencies of IFN $\gamma$  only expressing CD4<sup>+</sup> T cells at every checkpoint in the disease when compared to B6 mice (Figure 3-8 D). As expected, this population continued to rise within the PLN of NOD mice over the course of the disease (Figure 3-8 D). It is plausible that elevated Th1 cells within the MLN could be contributing to intestinal inflammation within the NOD mice at early checkpoints or could be a result of intestinal inflammation causing improper Th1 polarization.

As mentioned previously, Th17 cells are predominantly found within the intestine and play an integral part in regulation of the mucosal immune system. Th17 cells that express only IL17 and are more regulatory in nature<sup>250</sup>, while Th17 cells that co-express IFN $\gamma$  and IL-17 have been implicated in a number of different autoimmune diseases including T1D<sup>206, 253</sup>. In the MLN, NOD mice exhibited a higher frequency of IL17<sup>+</sup> only CD4<sup>+</sup> T cells at each disease checkpoint when compared to B6 mice (Figure

3-7 A). Again within the MLN this population decreased over the course of the disease progression (Figure 3-8 A). On the other hand, similar frequencies of IL17<sup>+</sup> only CD4<sup>+</sup> T cells were observed in the PLN of B6 and NOD mice at 4 and 8 weeks of age (Figure 3-8 B). Interestingly there was a rise in frequency of IL17 only expressing cells in the PLN of NOD mice at 12 weeks of age compared to B6 mice which remained elevated to at least 16 weeks of age (Figure 3-8 B). As IL17 has been shown to have synergistic effects with IL-1 $\beta$  and IFN $\gamma$  by increasing their ability to induce cytokine induced death<sup>254</sup>, it is possible that the elevated levels of IL17 only expressing cells at later checkpoints in the PLN could be contributing to  $\beta$ -cell destruction.

The frequencies of IL17<sup>+</sup> IFN $\gamma$ <sup>+</sup> expressing CD4 T cells in the MLN of NOD mice were higher at earlier checkpoints at disease when compared to B6 mice (Figure 3-8 E). In addition, their frequencies declined over the course of disease in the NOD mouse (Figure 3-8 E). In the PLN, similar frequencies of this population were observed at 4 weeks of age between NOD and B6 mice (Figure 3-8 F). By 8 weeks of age NOD mice exhibited a higher frequency of IL17<sup>+</sup> IFN $\gamma$ <sup>+</sup> when compared to B6 mice which continued to rise over the course of the disease (Figure 3-8 F). Again, in NOD mice, it seems there is elevated inflammation in the MLN at early checkpoints, and within the PLN at later checkpoints as measured by frequencies of IL17<sup>+</sup> IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells.

### **NOD MLN and PLN T cell Populations over the Course of the Disease**

We next evaluated the temporal evolution of these T cell populations within the MLN and PLN of NOD mice. Interestingly for each population of T cells, IL17<sup>+</sup> only, IFN $\gamma$ <sup>+</sup> only, and IL17<sup>+</sup> IFN $\gamma$ <sup>+</sup>, a decrease in frequency in the MLN over time and an increase in frequency in the PLN over time was observed (Figure 3-9). There are a few explanations that are possible and can be further pursued in future experiments. First, it

is possible that these cells are migrating from the MLN to the PLN as the disease progresses. Second, due to the known plasticity of these cells it is possible that T cells residing at either location are changing phenotypes over the course of the disease.

### **Decreased Treg Frequency in the MLN and PLN of NOD Mice**

Previous studies have shown a decrease in the frequencies of Tregs in NOD mice<sup>255</sup> which results in alterations in peripheral tolerance. In addition, the induction of Tregs can be regulated by the mucosal immune system<sup>179, 180</sup>. Thus, we also evaluated the expression of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs within the MLN and PLN of NOD and B6 mice over the course of disease (Figure 3-6 D). As previously published, NOD mice exhibited lower frequencies of Tregs at each stage of the disease in the MLN and PLN when compared to B6 mice (Figure 3-10). Interestingly, the frequency of Tregs within the MLN decreased as B6 mice aged, though relatively stable frequencies were observed in the PLN (Figure 3-10). While there were lower frequencies of Tregs within the MLN of NOD mice compared to those observed in the PLN, but there was no change in either lymph node over the course of disease progression (Figure 3-10). The observed lower frequencies of Tregs, could be insufficient to suppress inflammation, and thus explain why NOD mice exhibit higher frequencies of T cells expressing inflammatory cytokines when compared to B6 mice.<sup>25</sup>

Table 3-1. Gastrointestinal Barrier Function Analysis.

<sup>a</sup>	<b>G<sub>T</sub> (ms/cm<sup>2</sup>)</b>	<b>J<sub>sm</sub><sup>mannitol</sup> (nmol/cm<sup>2</sup>/hr)</b>
<b>Distal Ileum</b>	31.39 ± 2.34 n=7	31.6 ± 5.4 n=8
<b>Proximal Jejunum</b>	20.94 ± 1.84 n=15	24.13 ± 2.71 n=14
<b>Distal Colon</b>	19.44 ± 1.58 n=6	31.78 ± 3.95 n=5

<sup>a</sup>data generated by Dr. Marguerite Hatch, Department of Pathology, University of Florida  
G<sub>T</sub> = the ratio of the open circuit potential (V; mV) to the short circuit current (I<sub>sc</sub>; μA/cm<sup>2</sup>)  
J<sub>sm</sub><sup>mannitol</sup> = serosal to mucosal movement of mannitol

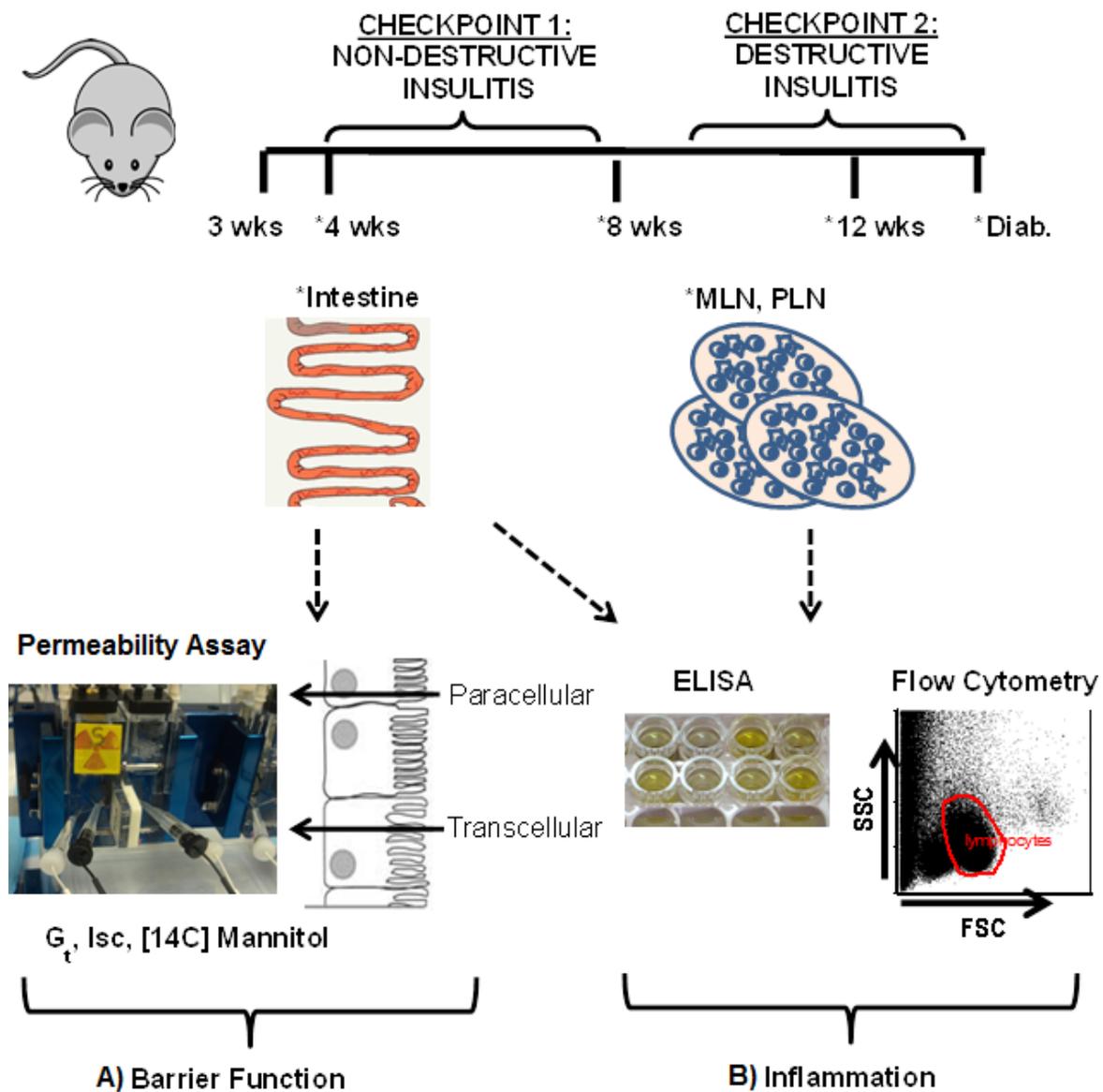


Figure 3-1. Temporal Evaluation of Intestinal Barrier Function and Inflammation  
 Intestinal sections along with mesenteric and pancreatic lymph nodes were harvested at indicated disease checkpoints. A) Using chambers were used to conduct paracellular permeability and transcellular movement of ions to evaluate barrier function ex vivo. B) Gastrointestinal inflammation was assessed via soluble mediator expression and T cell phenotypes nodes at indicated disease checkpoints. Soluble mediators were evaluated in homogenized tissues via ELISA. T cell phenotypes were evaluated via flow cytometry from mesenteric and pancreatic lymphocytes.

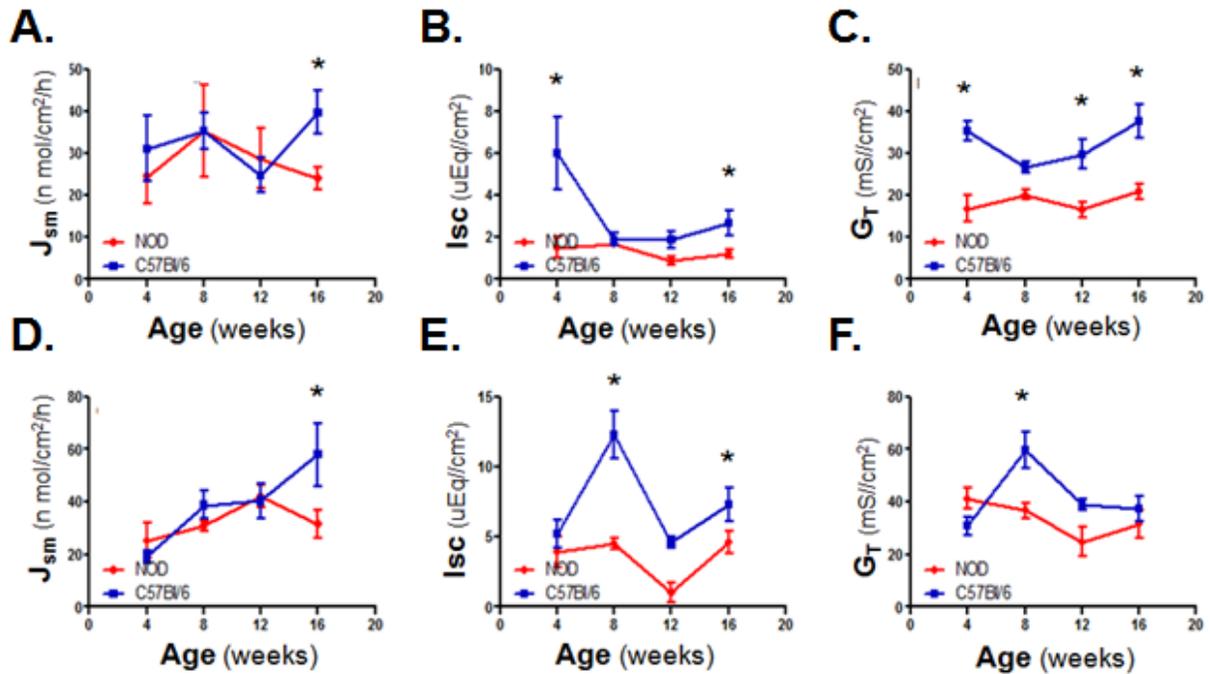


Figure 3-2. Decreased Gastrointestinal Movement of Charged Ions in NOD mice. Intestinal sections of proximal jejunum A,B,C) and distal ileum D,E,F) were harvested and placed into Ussing chambers. [<sup>14</sup>C] Mannitol movement and electrical parameters were measured over a 45 minute time period. A,D) Measurement of [<sup>14</sup>C] mannitol movement from the serosal to the mucosal ( $J_{sm}$ ) side of the tissue. B,E) I<sub>sc</sub> or short circuit current is indicative of ion movement. C,F)  $G_T$  measurements ( $G_T$  is the ratio of voltage to I<sub>sc</sub> (short circuit current)). n=4-15 tissues per strain per age. \*p value = <0.05, Student's T-test

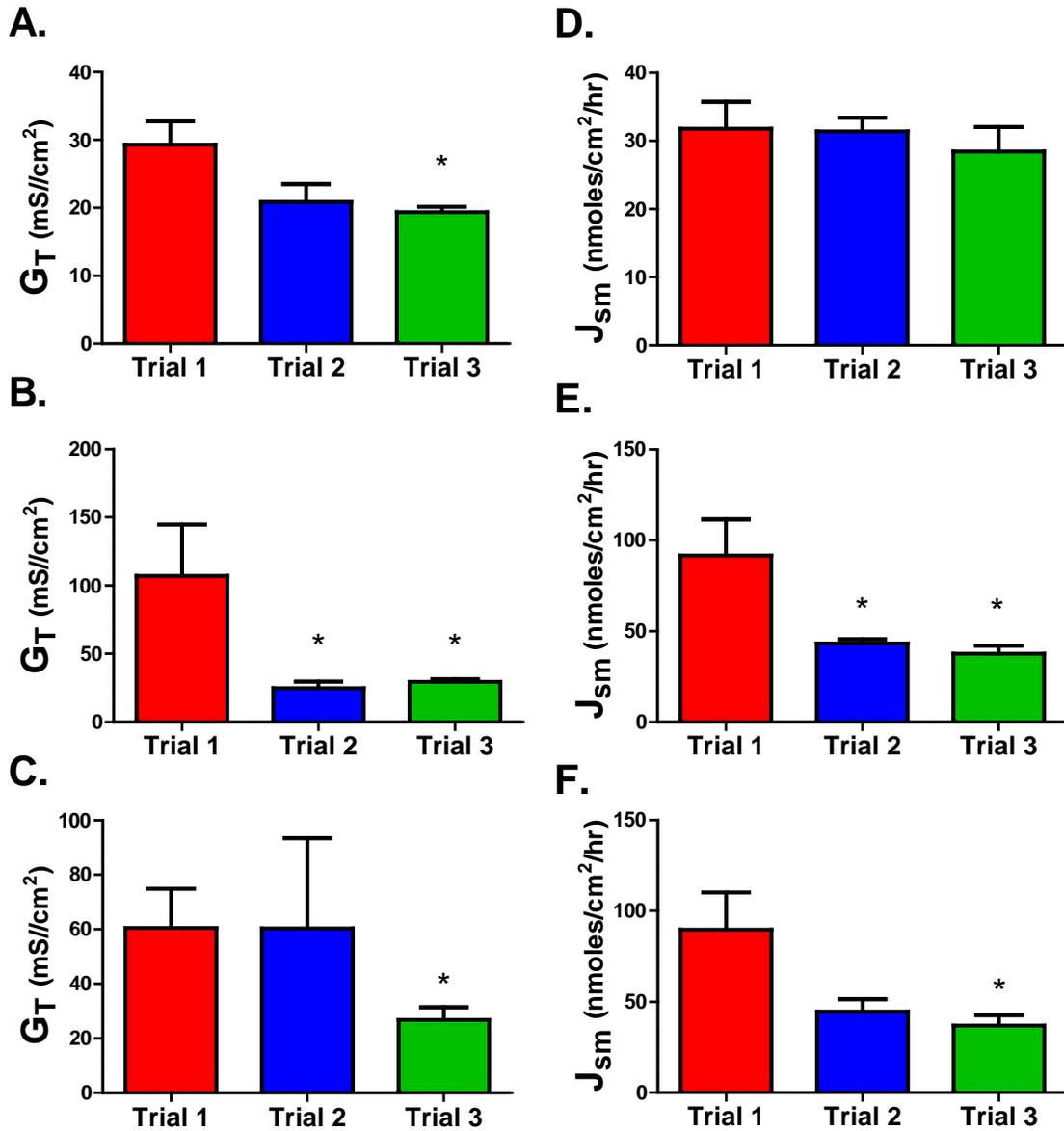


Figure 3-3. Optimization of Gastrointestinal Permeability Assays. Intestinal segments of the A,D) distal colon, B,E) distal ileum, and C,F) proximal jejunum were harvested from C57Bl/6 mice and placed in Ussing chambers. [14C] Mannitol movement and electrical parameters were measured over a 45 minute time period. A,B,C) Conductance ( $G_T$  = ratio of voltage to  $I_{sc}$  (short circuit current)) was measured. D,E,F) Paracellular permeability was measured by tracking serosal to mucosal movement of [14C] mannitol ( $J_{sm}$ ).  $n=5-6$  tissues per trial. \* $p < 0.05$  vs. Trail 1, Student's T-test.

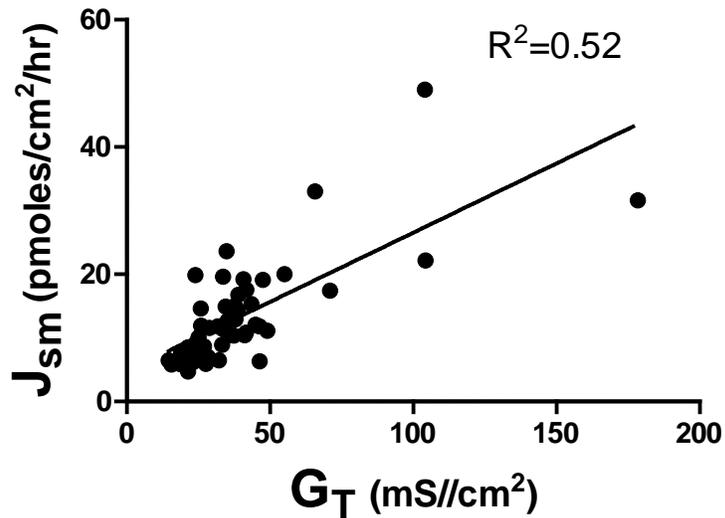


Figure 3-4. Evaluation of 70kDa Dextran as a Paracellular Permeability Marker. Distal colon, proximal jejunum, and distal ileum segments were harvested from C57Bl/6 mice and mounted in Ussing chambers. [<sup>14</sup>C] Dextran movement and electrical parameters were measured over a 45 minute time period and analyzed. Movement of [<sup>14</sup>C] dextran from the serosal to the mucosal side ( $J_{sm}$ ) of the tissue was evaluated.  $G_T$  (ratio of voltage to  $I_{sc}$  (short circuit current)) was calculated. Assays were conducted in both standard and  $Ca^{2+}$  free buffer. Linear regression ( $R^2=0.52$ ) and Spearman's correlation ( $p$  value  $<0.001$ ) was performed between  $J_{sm}$  and  $G_T$  in all tissues.

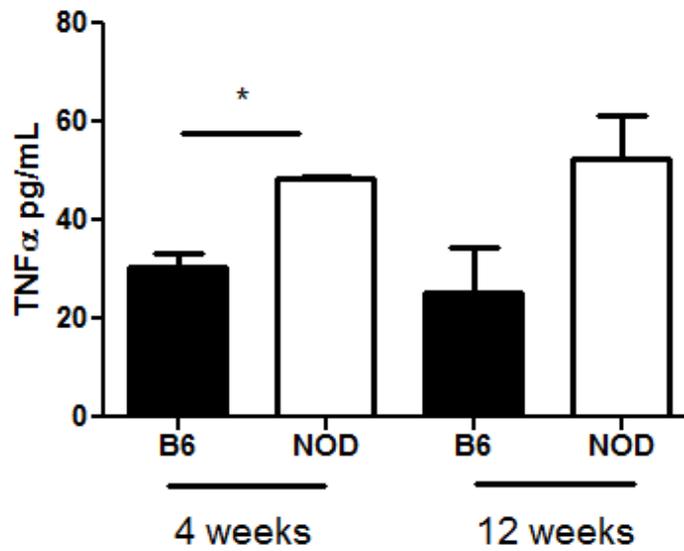


Figure 3-5. NOD mice exhibit Elevated Duodenal TNF $\alpha$  Levels. Duodenum samples (~1.2 cm length, normalized by weight 0.085g) were harvested at indicated disease checkpoints and homogenized via bead beating. TNF $\alpha$  levels were measured by ELISA. \*p value = 0.0011, Student's T-test.

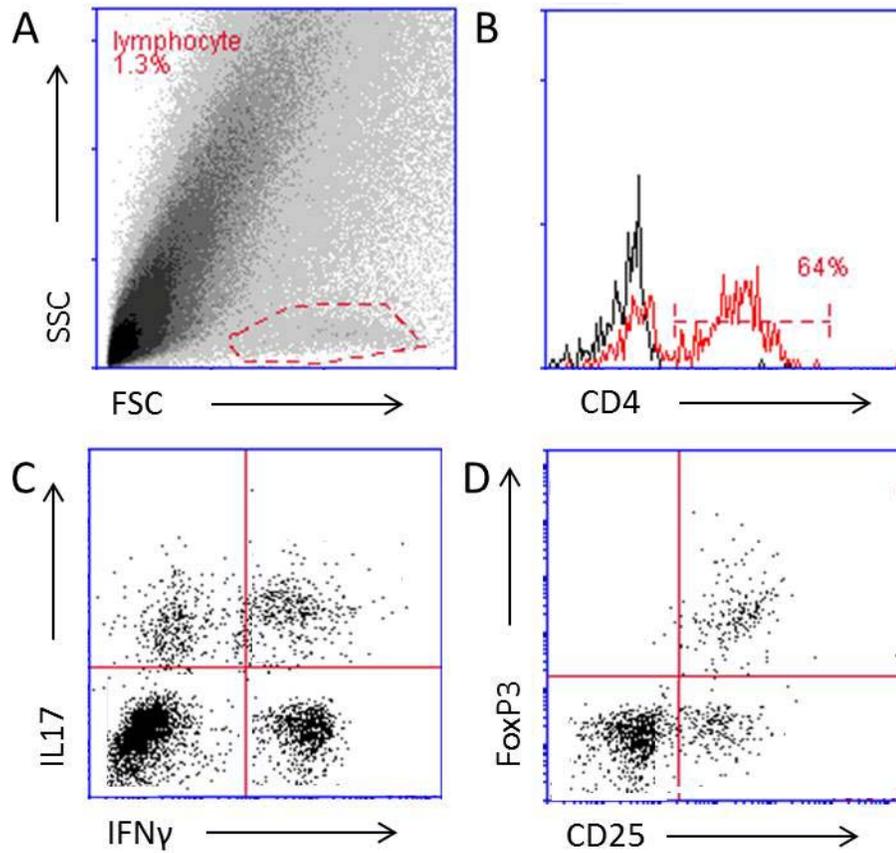


Figure 3-6. Gating schemes for Flow Cytometric Analysis. MLN and PLN lymphocytes were harvested. T cells were expanded using plate bound anti-CD3 and soluble anti-CD28 for 3 days followed by incubation with PMA/Ionomycin and Brefeldin A for 4 hours. A-B) T cells were probed for B) CD4 and C) intracellular cytokines (IL17 and IFN $\gamma$ ) or D) CD4, CD25 and the intracellular marker FoxP3. Panels C and D are gated on the red highlighted regions in panels A and B.

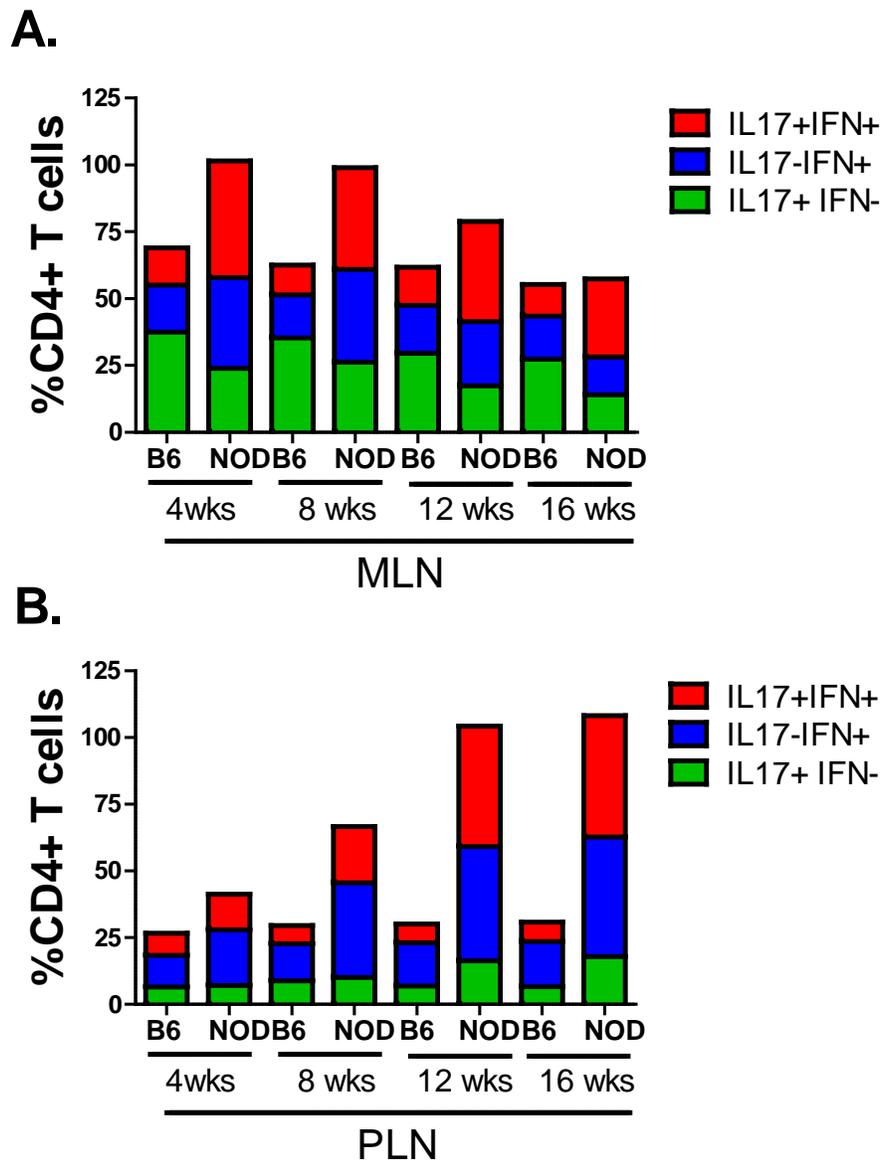


Figure 3-7. Frequencies of Polarized T cells in the MLN and PLN. A) MLN and B) PLN lymphocytes were harvested at indicated disease checkpoints. T cells were expanded using platebound anti-CD3 and soluble anti-CD28 for 3 days followed by incubation with PMA/Ionomycin and Brefeldin A for 4 hours. T cells were probed for CD4 and intracellular cytokines (IL17 and IFN $\gamma$ ). n=2 mice per age per strain.

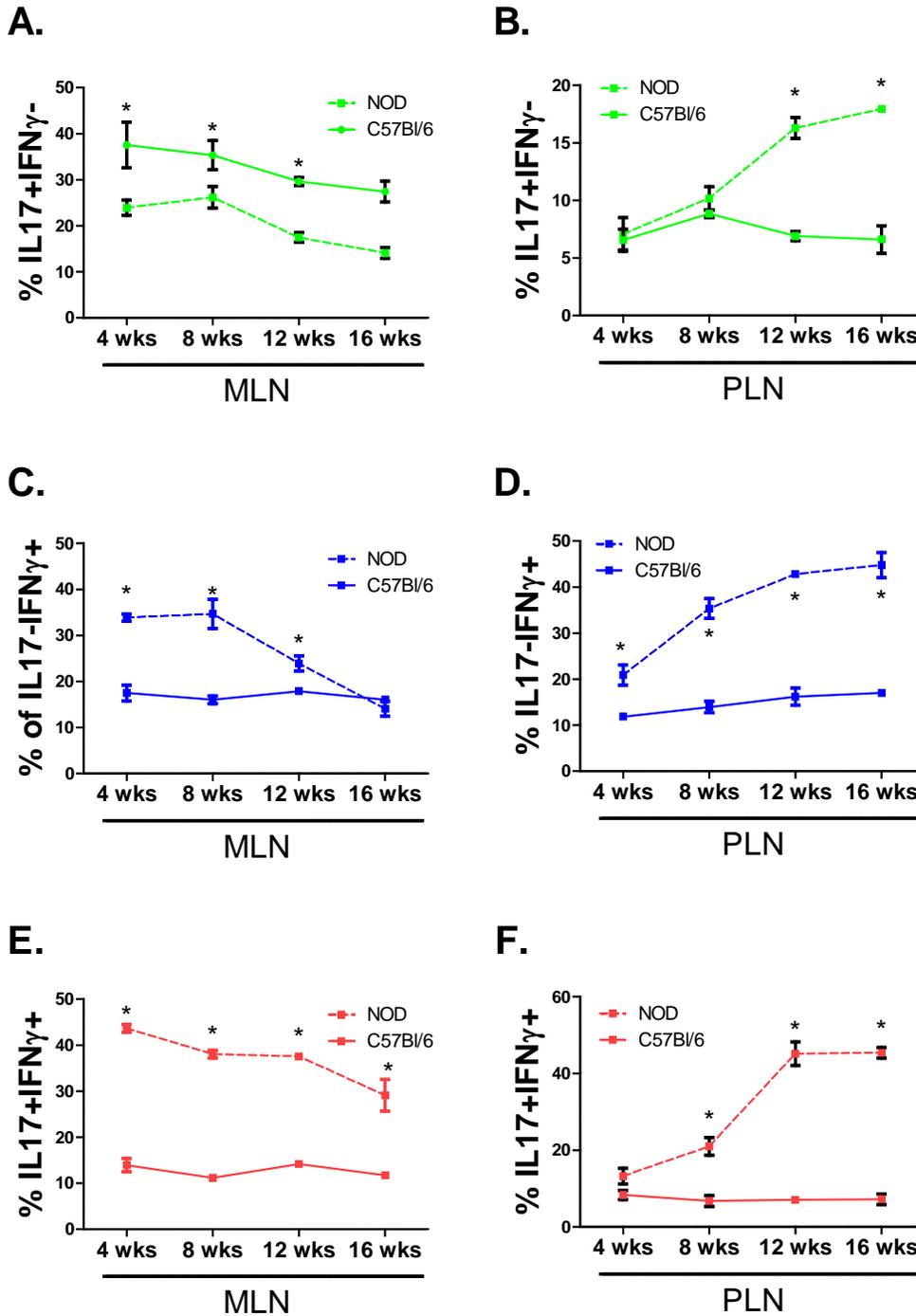


Figure 3-8. Frequencies of IL17 expressing T cell populations. A,C,E) MLN and B,D,F) PLN lymphocytes were harvested at indicated disease checkpoints. T cells were expanded using platebound anti-CD3 and soluble anti-CD28 for 3 days followed by incubation with PMA/Ionomycin and Brefeldin A for 4 hours. T cells were probed for CD4 and intracellular cytokines (IL17 and IFN $\gamma$ ). n=2 mice per strain. \*p= <.05, Student's T test.

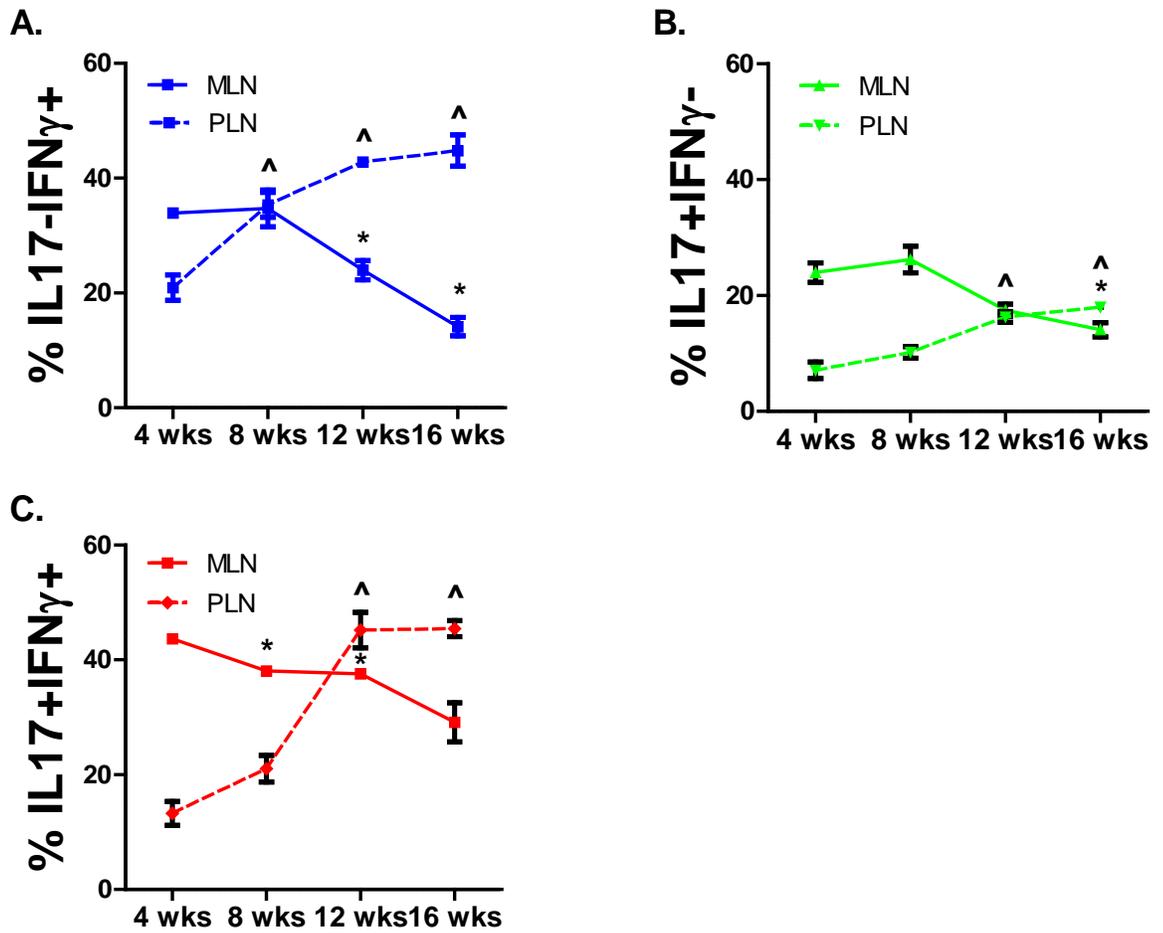


Figure 3-9. Distribution of T cell populations within the MLN and PLN of NOD mice over the course of disease. MLN (solid line) and PLN (dotted line) lymphocytes were harvested at indicated disease checkpoints from NOD mice. T cells were expanded using platebound anti-CD3 and soluble anti-CD28 for 3 days followed by incubation with PMA/Ionomycin and Brefeldin A for 4 hours. T cells were probed for CD4 and intracellular cytokines (IL17 and IFN $\gamma$ ). A) IL17 only expressing T cell frequencies B) Th1 IFN $\gamma$  only expressing T cell frequencies (Th1) and C) IL17 and IFN $\gamma$  expressing T cell frequencies. n=2 mice per strain, \*p= <0.05 vs. MLN 4 weeks, ^p= <0.05 vs. PLN 4 wks. Student's T-test. ANOVA was unable to be completed due to low number of samples.

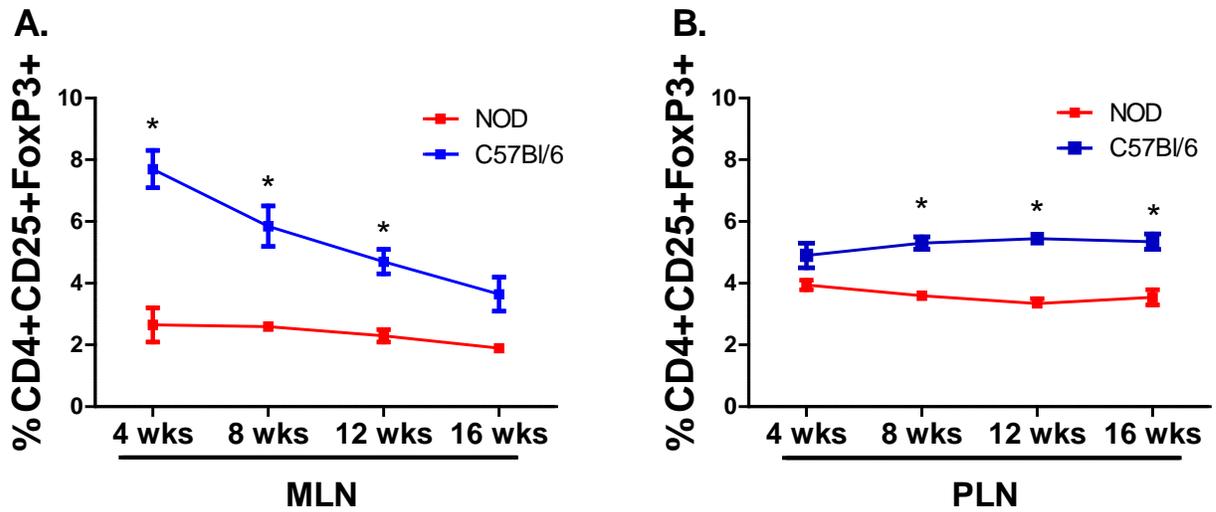


Figure 3-10. Decreased Frequencies of Tregs in the MLN and PLN of NOD mice. A) MLN and B) PLN lymphocytes were harvested at indicated disease checkpoints. T cells were expanded using platebound anti-CD3 and soluble anti-CD28 for 3 days followed by incubation with PMA/Ionomycin and Brefeldin A for 4 hours. T cells were probed for CD4, CD25 and the intracellular marker FoxP3. n=2 mice per strain \*p= <.05 vs. NOD at same time point, Student's T-test. ANOVA was unable to be completed due to low number of samples.

## CHAPTER 4 DISCUSSION

Recent studies mentioned above have implicated a role of mucosal immunity in the development of T1D. Several studies conducted on rodent models for T1D as well as human studies have shown increased intestinal permeability and gastrointestinal inflammation associated with T1D. Thus, we hypothesized that a break in barrier function results in an inflammatory environment which promotes non-tolerizing conditions. To address this hypothesis we temporally evaluated gastrointestinal barrier function and inflammation as well as the effects of these changes on MLN and PLN T cell phenotypes (Figure 3-1). Our study determined that NOD mice exhibit decreased movement of charged ions within the intestine when compared to B6 mice. We also determined that intestinal inflammation occurs early in the progression of disease while pancreatic inflammation occurs later in the progression .

**Barrier function.** We initially measured barrier function through permeability experiments conducted in Ussing chambers using [14C] mannitol movement as a marker for paracellular permeability. NOD mice exhibited similar paracellular movement of [14C] mannitol compared to B6 mice for both the proximal jejunum and distal ileum (Figure 3-2 A, D). This finding was unexpected as prior studies using lactulose:mannitol tests observed increased intestinal permeability in BBDP rats [229, 230] as well as in humans [233-235]. There are a few potential reasons as to why we observed no paracellular differences between NOD and B6 mice. Our initial thoughts were that mannitol was too small to determine paracellular differences and so we investigated using a larger marker, 70kDa dextran (Figure 3-4). A second explanation could be that in NOD mice T1D progression does not involve a breakdown in paracellular

permeability. . This explanation is unlikely as we see elevated intestinal inflammation in NOD mice (Figures 3-5, 3-6) and inflammatory cytokines increase permeability of tight junctions <sup>249</sup>. Despite an absence of differences in paracellular permeability, we did find that NOD mice exhibited differences in the transcellular movement of charged ions.

NOD mice displayed decreased  $G_T$  and  $I_{sc}$  compared to B6 mice, but with measurable differences in mannitol paracellular permeability we concluded that NOD mice had decreased transcellular movement of charged ions (Figure 3-2 B, C, E, F). We wanted to determine whether decreased movement of charged ions is contributing to intestinal inflammation or whether intestinal inflammation is causing decreased movement of charged ions. The decreased movement of charged ions was relatively constant throughout the disease (Figure 3-2 B, C, E, F). Intestinal inflammation on the other hand was present early in disease as measured by  $TNF\alpha$  levels (Figure 3-5) and polarized T cell populations (Figure 3-6), but decreased over time suggesting that there is not a correlation between movement of charged ions and intestinal inflammation. Though we could not determine if there is a correlation between the two, the decreased movement of charged ions proved to be an interesting finding. Firstly as mentioned above, the incidence of T1D has been rapidly increasing over the past century faster than genetics can account for suggesting that environmental factors are likely contributing to disease development. A recent paper determined that increased NaCl promotes development of pathogenic Th17 IL-17A secreting cells that contribute to autoimmune disease <sup>256</sup>. What's interesting about this finding is that processed foods have almost 100 times higher salt content than similar home-made meals <sup>257, 258</sup>. Decreased movement of charged ions may contribute to an inability to deal with

increased NaCl content which could lead to development of pathogenic Th17 cells. Indeed we see increased frequencies of IL-17 IFN $\gamma$  positive T cells (Figure 3-7 E) and decreased frequencies of IL-17 only T cells in the MLN of NOD mice compared to B6 mice (Figure 3-7 A). Though differences in the movement of charged ions start early and stay relatively similar throughout disease, inflammation of the intestine and pancreas exhibit changes over the course of the disease.

**Inflammation.** As mentioned above, we evaluated general inflammation through ELISA analysis of homogenized duodenum for TNF $\alpha$  levels. We found that NOD mice exhibited elevated levels of TNF $\alpha$  at early and mid-stage checkpoints (4 and 12 weeks of age) compared to B6 mice (Figure 3-5). Further checkpoints must be completed to determine if the TNF $\alpha$  levels decrease over time similar to the T cell inflammation we see within the intestine. We saw elevated frequencies of polarized T cells in the MLN compared to B6 mice at early checkpoints which decreased in frequency over time (Figure 3-6 A). When specifically looking at IL17 only, IFN $\gamma$  only, and IL17 and IFN $\gamma$  expressing CD4 T cells we see a similar trend where initially there are higher frequencies and over time they reduce in the MLN of NOD mice (Figure 3-8). Together these suggest that there is intestinal inflammation occurring early in disease. Also to note, NOD mice exhibited lower frequencies of IL17 only Th17 cells compared to B6 mice in the MLN (Figure 3-7 A), while they exhibited higher frequencies of IFN $\gamma$  only and IFN $\gamma$  and IL17 expressing T cells (Figure 3-7 C, E). Once again, a more polarized Th1 T cell population could be indicative of intestinal inflammation or could be a result of intestinal inflammation. In contrast, we saw low frequencies at early checkpoints of polarized T cells and higher frequencies at later checkpoints in the PLN of NOD mice

compared to B6 mice (Figure 3-6). Additionally in the PLN, each specific T cell population increased over time for NOD mice meaning that pancreatic inflammation doesn't occur until later checkpoints in the disease (Figure 3-8). When compared to B6 mice, NOD mice had elevated frequencies of IFN $\gamma$  only and IFN $\gamma$  IL17 Th17 cells in the PLN at all checkpoints (Figure 3-7 D, F), while IL17 only cells did not increase until after 8 weeks of age (Figure 3-7 B). We saw a steady rise in the frequency of IFN $\gamma$  only Th1 cells in the PLN of NOD mice, which have been shown to significantly contribute to  $\beta$ -cell destruction T1D (Figure 3-8 B) <sup>251, 252</sup>. The IL17 only and IL17 IFN $\gamma$  populations sharply rose between 8 and 12 weeks of age and continued to increase out to 16 weeks of age (Figure 3-8 A, C). The sharp rise in these populations suggests that at this stage in the disease there are factors present that influence Th17 differentiation. To summarize, we saw a trend of early inflammation occurring within the intestine, which decreased over the course of the disease, while in the pancreas inflammation increased over the course of the disease. There are a few potential explanations for this trend.

First, it is possible that the T cells residing within the MLN are trafficking to the PLN over the course of the disease. This would explain why the frequencies decline in the MLN and rise in the PLN. When looking at specific populations, for example IL17 and IFN $\gamma$  expressing Th17 cells (Figure 3-8 C), the correlation between the decline and rise is not distinct. This would suggest that this trend is most likely not due to trafficking, though a tracking experiment to see if the T cells migrate from the MLN to PLN would most likely be necessary to rule out this explanation. Second, it is possible that the T cells residing in the MLN or PLN are simply changing phenotypes over the course of the disease. Due to the plasticity of Th17 cells mentioned above and the importance of the

environment with which they are in, this explanation is most likely. The question then becomes how does the initial intestinal inflammation contribute to pancreatic inflammation? What's interesting about our findings is that the initial inflammation within the intestine suggests a non-tolerizing environment. A non-tolerizing environment would result in decreased Tregs (Figure 3-9) as well as decreased tolerogenic DC and therefore polarized T cells (Figure 3-6). We know that luminal antigen can be processed and presented in the PLN, suggesting that intestinal DC likely migrate to the PLN<sup>259</sup>. Through decreased Tregs and non-tolerizing DC it is possible that the intestinal environment contributes to pancreatic inflammation. Thus, for future experiments we will be investigating DC populations and phenotypes in the MLN and PLN over the course of the disease.

To summarize, we could not determine a correlation between a breakdown in barrier function and intestinal inflammation although, we plan to do future flux experiments using a larger paracellular marker. We found that NOD mice exhibited decreased movement of charged ions at all stages of disease development, which potentially could be contributing to intestinal inflammation. We also found that there was increased intestinal inflammation occurring at early stages in disease while pancreatic inflammation occurred at later stages in disease.

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## BIOGRAPHICAL SKETCH

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