I, Kyle J Bednar, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Immunology.

It is entitled:
Therapeutic Approaches to the Treatment of Type 1 Diabetes

Student's name: Kyle J Bednar

This work and its defense approved by:

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Committee member: Kasper Hoebe, Ph.D.

Committee member: Jonathan Katz, Ph.D.
Dissertation Abstract

Type 1 diabetes (T1D) is currently an incurable autoimmune disease, characterized by the destruction of insulin producing islet β cells in the pancreas. The incidence of T1D is rising; between 2001 and 2009 T1D incidence rose by 23% in Americans under the age of 20 and is rising at a rate of 3% annually worldwide. It is believed that both genetic factors and environmental triggers are involved in disease pathogenesis. Animal models have shown that T1D is a result of intricate interactions between innate and adaptive immune responses. These responses produce soluble or contact dependent factors that are toxic to islet β cells. This results in β cell death and uncontrolled blood glucose levels. Pathogenic T-cells are undeniably involved in mediating destruction of islet β cells. Controlling the activation of these cells may be crucial to finding a treatment to this currently incurable disease. There are many ways to control T-cell activation: Control the ability of the innate immune system to prime the aberrant adaptive immune responses, alter the ratio of regulatory T-cells (Tregs) to effector T-cells, and control the adaptive immune response directly through an immunosuppressant.

One explanation for the rising incidence of T1D and autoimmunity in general is the “hygiene hypothesis”, which suggests that insufficient microbial stimulation of the innate immune system skews the adaptive immune responses towards autoimmunity. We hypothesized that a tolerogenic stimulation of the innate immune system could “re-tune” the irregular adaptive immune responses in T1D. Our results demonstrate that stimulation of TLR4 in non-obese diabetic mice with a TLR4 agonistic monoclonal antibody (TLR4-Ab) can prevent and reverse new onset T1D. This reversal was due to preservation of insulin, hypertrophied islets, and decreased insulitis. Antigen presenting...
cells (APC) tolerized using a TLR4-Ab resulted in decreased pro-inflammatory cytokines and co-stimulatory molecule expression, and increased IL-10 production, ultimately resulting in decreased capacity to stimulate antigen specific and non-specific T-cell responses. Finally, treatment of APCs alone was able to prevent disease transfer by pre-diabetic cells, and resulted in increased Tregs. Tolerizing innate immune cells thus caused changes in the adaptive immune responses and reversed T1D.

In another project, our lab has previously shown that NOD mice have a quantitative deficiency in the CD137^+Treg subset. CD137^+Tregs are the major cellular source of soluble CD137 (sCD137), and NOD mice have low serum levels of sCD137. Our hypothesis was that correcting low levels of sCD137 will prevent and/or reverse T1D; therefore we engineered recombinant soluble CD137. Recombinant sCD137 prevented T1D in NOD mice. Insulitis was significantly decreased, while insulin was preserved despite residual insulitis. To understand the protective immune mechanisms of sCD137, we studied highly purified CD4^+ T cells and demonstrated that they are actively suppressed by sCD137 in a CD137L dependent manner. These results support the hypothesis that sCD137 acts in a feedback loop to suppress immune responses. This dissertation will lead to better therapeutics in the future for T1D. Further, both of these therapeutics offer mechanistic insight into disease mechanism which can advance the therapeutic field of T1D.
Acronyms and Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>ALS</td>
<td>Anti-lymphocyte serum</td>
</tr>
<tr>
<td>ATG</td>
<td>Anti-mouse thymocyte globulin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BG</td>
<td>Blood glucose</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD25</td>
<td>Interleukin-2Rα chain</td>
</tr>
<tr>
<td>CCL</td>
<td>CC-Chemokine ligand</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional Dendritic Cell</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for disease control</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete-Freund’s adjuvant</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-lymphocytes associated protein 4</td>
</tr>
<tr>
<td>Ctrl-Ab</td>
<td>TLR4 non-agonistic monoclonal antibody</td>
</tr>
<tr>
<td>CxCL</td>
<td>CXC-Chemokine ligand</td>
</tr>
<tr>
<td>DAISY</td>
<td>Diabetes and Autoimmunity Study in the Young</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DKA</td>
<td>Diabetic ketoacidosis</td>
</tr>
<tr>
<td>DTR</td>
<td>Diptheria toxin receptor</td>
</tr>
<tr>
<td>Flt3</td>
<td>FMS-like tyrosine nase 3</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>FPG</td>
<td>Fasting plasma glucose</td>
</tr>
<tr>
<td>HHS</td>
<td>Hyperosmolar hyperglycemic state</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T-cell costimulator</td>
</tr>
<tr>
<td>ICOSL</td>
<td>Inducible T-cell costimulator ligand</td>
</tr>
<tr>
<td>Idd</td>
<td>Insulin dependent diabetes</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFIH1</td>
<td>Interferon-induced helicase C domain 1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL2RA</td>
<td>Interleukin 2 receptor alpha</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant natural killer T-cell</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>INS</td>
<td>Insulin</td>
</tr>
<tr>
<td>IPEX</td>
<td>immunodysregulation polyendocrinopathy enteropathy X-linked syndrome</td>
</tr>
<tr>
<td>IRF-1</td>
<td>Interferon regulatory factor-1</td>
</tr>
<tr>
<td>iTregs</td>
<td>Inducible Tregs</td>
</tr>
<tr>
<td>JDRF</td>
<td>Juvenile diabetes research foundation</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS binding protein</td>
</tr>
<tr>
<td>liCTLA4</td>
<td>ligand-independent CTLA4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
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</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LYP</td>
<td>Lymphoid protein tyrosine phosphatase</td>
</tr>
<tr>
<td>Mal</td>
<td>MyD88-adapter-like</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of active B-cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Natural killer group 2, member D</td>
</tr>
<tr>
<td>NLRs</td>
<td>Nucleotide-binding oligomerization domain receptors</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>nTregs</td>
<td>Natural Tregs</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pattern associated molecular patterns</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>PD1</td>
<td>Programmed death receptor 1</td>
</tr>
<tr>
<td>PDL-1</td>
<td>Programmed cell death ligand 1</td>
</tr>
<tr>
<td>PLN</td>
<td>Pancreatic Lymph Node</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polyinosine polycytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphotase non-receptor type 22</td>
</tr>
<tr>
<td>RLRs</td>
<td>RIG-I-like receptors</td>
</tr>
<tr>
<td>RAE1</td>
<td>Retinoic acid early transcript 1</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile α and HEAT-Armadillo motifs-containing protein</td>
</tr>
<tr>
<td>sCD137</td>
<td>soluble CD137</td>
</tr>
<tr>
<td>sCTLA4</td>
<td>soluble CTLA4</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single-nucleotide polymorphisms</td>
</tr>
<tr>
<td>STAT-1</td>
<td>Signal transducer and activator of transcription-1</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-like Interleukin 1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like receptor</td>
</tr>
<tr>
<td>TLR4-Ab</td>
<td>TLR4 agonistic monoclonal antibody</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>Tregs</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor inducing IFN</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic-stromal lymphopoietin</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UnTx</td>
<td>Untreated</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
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</table>
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I. Summary of Research

II. TLR-Ab Reverses New Onset T1D

III. sCD137 Prevents T1D and Reduces CD4⁺ Proliferation

IV. Summary Statement

V. References
Chapter 1: General Introduction
Epidemiology of Type 1 Diabetes

Type 1 diabetes (T1D) is one of the most commonly diagnosed endocrine and metabolic conditions among children[1]. T1D disease incidence worldwide is estimated to be approximately 17.4 million people; however this is likely an underestimate due to poor disease diagnosis in under developed countries (http://www.cdc.gov/diabetes/risk/age/youth.html). Diagnosis may be delayed or missed because of subtle symptoms. In many cases acute T1D can be difficult to stabilize without hospital care, and in various parts of the world lack of hospital care presents a serious problem[1]. This may partly explain why it is predicted that 361/100,000 patients with T1D die each year[2]. The incidence of T1D is also rising rapidly worldwide, especially among the youngest children (ages 3-12) with a 3% increase per year [3-8]. According to the Juvenile Diabetes Research Foundation (JDRF) there are currently 3 million people living with T1D in the U.S (http://jdrf.org/about-jdrf/fact-sheets/type-1-diabetes-facts/). T1D incidence is also rapidly rising in the United States; with a 23% increase in T1D between 2001 and 2009 in the population aged 20 or under (http://www.cdc.gov/diabetes/risk/age/youth.html). In the United States, this increase in T1D has resulted in $14.9 billion dollars in health care costs each year due to direct medical costs and lost wages [9]. T1D is not only a financial burden, but in 2010 diabetes was noted as a cause of death on 234,051 death certificates (http://www.diabetes.org/diabetes-basics/statistics/). Finally, even in developed countries with high standard medical practices children with clinical T1D have a 2 times higher mortality rate than non-diabetic healthy children[10]. Therefore better therapeutic approaches are needed for the reversal of T1D.
Type 1 Diabetes Overview and Clinical Diagnosis

T1D is a chronic autoimmune disease caused by complex interactions between innate and adaptive immune responses resulting in the selective destruction of islet β cells in the pancreas. The primary function of islet β cells is to store and release insulin in response to nutrients, hormones, and neuronal stimuli and thus islet β cells play a major role in the maintenance of glucose homeostasis[11]. Aberrant glucose homeostasis is a serious physiological problem that can be fatal. Two common T1D clinical syndromes of T1D caused by high levels of blood glucose are diabetic ketoacidosis (DKA) and hyperosmolar hyperglycemic state (HHS)[12]. DKA results from inadequate insulin production; in response fatty acids are used for fuel, resulting in acidic ketone bodies. Ketone bodies are responsible for most of the symptoms and complications associated with mortality[13]. HHS is a complication of high blood glucose causing severe dehydration and increased blood osmolarity, which can lead to coma or death[14]. These two fatal disease complications usually arise in individuals with undiagnosed T1D, but can also arise due to poor control of established disease. One major reason these fatal complications still occur is that symptoms of T1D do not arise until exceedingly advanced disease in which most β cells have been destroyed; in other words the disease is clinically silent until it reaches an advanced pathological stage. American Diabetes Association (ADA) guidelines for T1D are shown in Figure 1 and include A1C levels, fasting plasma blood glucose (FPG), oral glucose tolerance tests (OGTT), and auto-antibodies. A1C quantifies the amount of hemoglobin-bound glucose. Since red blood cells survive ~ 3 months, the A1C level reflects the average blood glucose level over the past 3 month time span. FPG measures blood glucose
after fasting for 8 hours, thus eliminating any false effect of high blood glucose from a meal. OGTT is usually a two hour or six hour test, depending on the protocol, in which blood glucose is measured before and after drinking a measured dose of glucose. Blood is drawn at several time points in order to identify the patient’s insulin response over time.

![Image: Figure 1. Diabetes Determination](http://www.diabetes.org/diabetes-basics/diagnosis/)  

Auto-antibodies are commonly used to distinguish between type 1 and type 2 diabetes; the presence of multiple auto-antibodies indicates T1D. Type 2 diabetes is described as non-insulin dependent diabetes, where the body ineffectively uses insulin and this can result in exhaustion of insulin producing islet β cells. Type 2 diabetes is often a result of excess body weight and physical inactivity (WHO). A common list of the auto-antibodies found in T1D is listed below (Table 1). Over 95% of T1D patients will have at least one auto-antibody and having multiple auto-antibodies is a predictor of disease. Other symptoms commonly present in T1D, include unexplained weight loss, polyuria, and extreme thirst. Further, with the increases in high through-put genetic screening, screening for genetic markers of T1D has become a more common practice in patients at high-risk for disease development. Finally, metabolomics are being researched to detect differences before disease onset. These predictive markers may help in the treatment of T1D.
<table>
<thead>
<tr>
<th>Test</th>
<th>Abbr</th>
<th>Description</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islet Cell Cytoplasmic Auto-antibodies</td>
<td>ICA</td>
<td>Measures a group of islet cell auto-antibodies targeted against a variety of islet cell proteins</td>
<td>One of the most common islet cell auto-antibodies and is detected at onset of disease in 70-80% of patients with T1D</td>
</tr>
<tr>
<td>Glutamic Acid Decarboxylase Auto-antibodies</td>
<td>GADA</td>
<td>Test for auto-antibodies directed against beta cell protein antigen, but is not specific for beta cells</td>
<td>One of the most common islet cell auto-antibodies and is detected at onset of disease in 70-80% of patients with T1D</td>
</tr>
<tr>
<td>Insulinoma Associated 2-Auto-antibodies</td>
<td>IA-2A</td>
<td>Test for auto-antibodies directed against beta cell protein antigen, but is not specific for beta cells</td>
<td>Detected in about 60% of type 1 diabetics</td>
</tr>
<tr>
<td>Insulin Auto-Antibodies</td>
<td>IAA</td>
<td>Autoantibody targeted to insulin; insulin is the only antigen thought to be highly specific for beta cells.</td>
<td>Detected in about 50% of type 1 diabetic children; not commonly detected in adults</td>
</tr>
</tbody>
</table>

Table 1. Auto-antibodies measured in T1D incidence. Adapted from [http://labtestsonline.org/understanding/analytes/diabetes-auto/tab/test/](http://labtestsonline.org/understanding/analytes/diabetes-auto/tab/test/)

The Non-Obese Diabetic Mouse as a Model for Human Type 1 Diabetes

Non-obese diabetic (NOD) mice spontaneously develop T1D and this strain has served as the primary animal model for T1D for almost 40 years. The NOD model has been used to understand genetic components, disease mechanisms, preventative treatments, biomarkers, auto-antigens, treatment targets and reversal of disease. Further, the NOD mouse has been extensively studied using standard genetic techniques including genetic manipulation, transgenic development, and gene knockout approaches. As with many animal models there are some differences between NOD mice and human patients diagnosed with T1D (Table 2), however it remains an important component of T1D research[15, 16]. One major difference is there is no sex bias in human disease, but in NOD mice only female mice have a high incidence of T1D. This has not been fully explained, but is believed to be due to different levels of hormones such as testosterone and estrogen. Some studies have used human cadavers from patients with T1D, but information found from these studies has been limited due to the scarce amount of samples available. Immune cells found in human insulitis include CD8\(^+\) cytotoxic T-cells, dendritic cells (DCs), macrophages, B cells, and CD4\(^+\) T helper
cells[17]. Similar immune infiltrates are found in NOD mice (details below). However, another major difference between NOD mice and humans with T1D is the amount of insulitis and possibly cell types involved in the destruction of β cells. NOD mice are characterized by an initial stage of non-destructive insulitis, consisting of macrophages and dendritic cells. This stage is followed by T-cells and B-cell infiltration into the pancreas[18]. Infiltration then leads to a T-cell mediated destruction of islet β cells by 4-6 months of age and the extensive amount of inflammation eventually develops into a tertiary lymphoid structure, which is more extensive than human immune infiltrates[19]. NOD mice may therefore have a more aggressive disease pathogenesis compared to the more subtle chronic immune process in humans.

**Figure 2.** The degree of pancreatic infiltration in T1D patients is limited compared with the insulitis in NOD mice around diabetes onset. Infiltration in or around the islets of Langerhans in the pancreas comprises CD8 T cells, CD4 T cells, but also B cells, macrophages and very few dendritic cells. In this respect, i.e., the type of cell infiltrating the islets, pancreatic section from human and NOD mice correspond. But the degree to which the pancreas and islets are inflamed, i.e., the number of infiltrating cells that can be found, is much more limited in humans than in NOD mice. A: typical degree of infiltration in recent-onset type 1 diabetic pancreas in humans. Staining of human pancreas sections for insulin (in green) and CD8 T cells (in red) indicates that only low levels of CD8 T cells can be seen in the proximity of the islets. A similar low degree of infiltration is observed for CD4 T cells and B cells in pancreatic sections of T1D patients (not shown). More information can be found in Reference 99. B: typical level of inflammation around diabetes onset in female NOD mice. Staining for insulin (in blue) and CD8 (in brown-red) shows a severe degree of infiltration by CD8 T cells in the islets. The type and degree of infiltration of CD4 T cells at this time point are usually similar. (Van Belle T L et al. Physiol Rev 2011;91:79-118.)

Besides the cell types involved in the islet infiltrates, another similarity between NOD mice and humans is that only insulin-positive β islets are affected by infiltrates[20].
immune molecules and cells that are involved in killing of islet β cells in humans are less studied than that of NOD mice due to the limited number of cadavers and live samples. However, the immune mechanisms involved in human pancreatic β have been shown through *in vitro* cell culture assays. These studies show that similar immune cell cytokines are involved in the islet β cell destruction between NOD mice and human pancreatic cell lines such as IL-1β, INF-γ, and TNF-α[21, 22]. It has also been shown that *in vitro* contact dependent mechanism such as tumor necrosis factor-related apoptosis inducing ligand (TRAIL) pathway can also cause β cell death in human pancreatic cell lines[22]. These *in vitro* studies show that human pancreatic cell death can occur by similar mechanisms as that in NOD mice despite the differences in the number of islet cell infiltrates. Further, a recent study showed that only β cells were affected in T1D patients while α cells remained intact, identical to the NOD mouse model. Human islets are associated with increased major histocompatibility complex (MHC) I and Fas surface expression on islet β cells only, which may account for their selective destruction[23]. α cells secrete glucagon to stimulate glucose production from other organs in times of hypoglycemia[24]. α cells may remain intact because they do not have the proper molecules expressed on their cell surface for destruction[25]. However, although α cells are not destroyed patients with T1D, patients also have dysregulated function of α cells due to hyperglycemic conditions and can be reviewed elsewhere[24]. These human cadaveric studies and *in vitro* culture systems show that human and NOD mice both involve targeted killing of β cells through contact dependent and independent mechanisms. Similarly both species have auto-antibodies specific for pancreatic islet antigens. While auto-antibodies are seen in humans with T1D, not all
humans with auto-antibodies ultimately develop disease. The number of auto-antibodies developed rather than individual auto-antibodies is most predictive of overt T1D[26]. This development of auto-antibodies in humans seems to be more predictive of disease incidence compared to mice; in some cases β cell auto-antibodies are observed in particular mouse strains that do not subsequently develop T1D[27, 28]. Further auto-antibodies as previously mentioned is a distinguishing feature of T1D compared to Type 2 diabetes, although it has been shown that ~11% of patients with Type 2 diabetes can develop auto-antibodies it is not typical[29].

<table>
<thead>
<tr>
<th>Age of Onset</th>
<th>Humans</th>
<th>Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Children / Young Adults</td>
<td>3-6 months</td>
</tr>
<tr>
<td>Insulitis</td>
<td>CD8+ T-cells, Macrophages, B-cells, CD4+ T-cells, and few natural killer cells</td>
<td>CD8+ T-cells, Macrophages, B-cells, CD4+ T-cells, and dendritic cells</td>
</tr>
<tr>
<td>Ketoacidosis</td>
<td>Controlled with insulin</td>
<td>Mild until late stages</td>
</tr>
<tr>
<td>Potential Auto-Antigens</td>
<td>INS, GAD65, HSP60, IAPP, ZnT8, CPE, G6PC2, PDX-1, IA2, HSP90AB1, IA2β, REG3A, ICA1, MRPS31, PRPH, and SOX13</td>
<td>INS, GAD65, IGRP, PDX-1, ICA69, IA2, DMPK, and chromogranin A</td>
</tr>
<tr>
<td>MHC Linked</td>
<td>HLA-II DR/DQ and class I HLA-A/B</td>
<td>MHC class II Abβ/Eaα, class I Kd/Db</td>
</tr>
<tr>
<td>Reported Non-MHC Linkages</td>
<td>&gt;50</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>T Lymphoaccumulative</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Role of T Regulatory Cells</td>
<td>No change in numbers, functional difference likely</td>
<td>No decrease in numbers, potential decrease in suppressive function</td>
</tr>
<tr>
<td>B Cell Required</td>
<td>Likely required</td>
<td>Likely required</td>
</tr>
<tr>
<td>Hemolytic Complement</td>
<td>Dyregulation linked with complications</td>
<td>C5a deficiency</td>
</tr>
</tbody>
</table>

Table 2. Differences and similarities between human and NOD mouse model of T1D. Adapted from Thayer, T.C., S.B. Wilson, and C.E. Mathews, *Use of nonobese diabetic mice to understand human type 1 diabetes*. Endocrinol Metab Clin North Am, 2010. 39(3): p. 541-61. Abbreviations: CPE, carboxypeptidase E; DMPK, dystrophia myotonica–protein kinase; G6PC2 or IGRP, glucose-6-phosphatase 2; GAD2 or GAD65, glutamic acid decarboxylase 65; HSP 60, heat shock protein 60; HSP90AB1, heat shock protein 90-beta; IAPP, islet amyloid polypeptide; ICA1 or ICA69, islet cell autoantigen 1; INS, insulin; NK, natural killer; PDX-1, pancreatic and duodenal homeobox 1; PRPH, peripherin; PTPRN or IA2, protein tyrosine phosphatase receptor type N; PTPRN2 or IA2b, receptor-type tyrosine-protein phosphatase N2; REG3A, regenerating islet-derived protein 3 alpha; Slc30a8 or ZnT8, solute-like carrier 30a8; SOX13, SRY-related HMG-box 13.

Although auto-antibodies likely do not contribute to T1D pathogenesis in the NOD mice, human patients with T1D develop complement-fixing auto-antibodies that are cytolytic to β cells[30]. However, the role of auto-antibodies is still unclear as patients with
immunoglobulin deficiencies develop disease as well. It is possible β cells death is due to a bystander effect of cell activation (see B cell section). Due to the inability to gain patient islet samples during disease progression, there is little evidence in humans to support a specific mechanism of islet β cells destruction. However, the NOD mouse model provides essential insights into early pathogenesis and remains the basis for much of our understanding of the role immune cells play in progression of disease pathogenesis. It is known, through CD4+ and CD8+ adoptive transfer studies in NOD mice, that T-cells are a major contributor to β cell death; neither subset alone is usually able to cause disease development[31]. Furthermore, both human T1D patients and NOD mice develop autoreactive CD4+ and CD8+ T-cells and altered regulatory T-cell (Tregs) function. T-cells involvement in human disease is supported by the reoccurrence of T1D in pancreatic graft recipients from HLA-matched donors – suggesting a role for CD8+ T-cells[32]. Anti-CD3 antibody, which targets and/or tolerizes effector T-cells / increase Tregs, decreased β cell destruction in new onset T1D, again suggesting a role for T-cells[33]. However, anti-CD3 mediated suppression was not long lasting and most patients remained on some sort of exogenous insulin regiment. Based on the few studies done in human disease, it is also believed that the humoral response does not play a specific role in disease pathogenesis, but that B-cells in the pancreatic infiltrates could be acting as antigen presenting cells to enhance autoreactive T-cells which are driving the force behind islet destruction[34, 35]. Therefore, although there are some differences between NOD mice, which are C5a deficient and human T1D patients, there is no evidence that the difference in complement binding plays a major role in human disease pathogenesis. Auto-antigens
are fundamental in initiating an autoimmune response in T1D patients and mice. There are striking similarities in the auto-antigens that are observed in both species. T-cell responses and levels of circulating antibodies to more than 15 different islet antigens have been measured in T1D patients and in high-risk individuals, whereas 8 islet auto-antigens exist the NOD mice[15]. Many of the autoantigens are shared between mice and men[15]. Thus, T1D prone mice and humans have lost tolerance to many of the same antigens and these can be therapeutic targets to cause induction of tolerance towards these autoantigens. To date, no individual auto-antigen has been effective when used in a tolerogenic approach to reverse new onset disease. In summary, the overall similarities between the NOD mice and human T1D patients make NOD an excellent model for T1D research.

**Genetic Components of T1D**

T1D is a chronic and multifactorial disease with a strong genetic component due to the high association with inheritability of T1D in families. Historically, there were only 6 loci that were established to be associated with T1D, before the development of genome wide association studies (GWAS)[29]. One locus with the highest relative risk is the human leukocyte antigen (HLA) region on chromosome 6p21 found in the early 1970s[36-38]. Approximately half the genetic risk for T1D is conferred by the HLA class II genes (HLA-DRB1, -DQA1, and DQB1). These high-risk haplotypes are identified in only 2.4% of newborns but in more than 20% of children affected by T1D. The presence of these haplotypes is associated with a 55% increased risk of developing overt diabetes by age 12 in siblings of previously diagnosis T1D patients[39]. The next locus strongly associated to the disease was the insulin (INS) gene on chromosome
Detailed mapping of this region showed that susceptibility resides in the variable number of tandem repeat polymorphisms in the promoter region\[41\]. From 1996 – 2006, four more loci were identified: cytotoxic T-lymphocyte associated protein 4 (CTLA4) on chromosome 2q33, protein tyrosine phosphatase non-receptor type 22 (PTPN22) encoded on chromosome 1p13, interleukin 2 receptor alpha (IL2RA) found on chromosome 10p15, and interferon-induced with helicase C domain 1 (IFIH1) gene on chromosome 2q24.3\[42-45\]. The latter four genes are not unique to T1D but are also associated with many other autoimmune diseases. CTLA4 is vital for proper negative regulation of immune responses, as highlighted by the severe lymphoproliferative disorder seen in knock-out mice\[46\]. Single nucleotide polymorphisms (SNPs) in the promoter region of CTLA-4 found in patients with T1D may affect the surface expression of CTLA-4\[47\]. PTPN22 encodes the lymphoid protein tyrosine phosphatase (LYP) which is an important negative regulator of T-cell receptor signaling\[43, 48\]. The exact mechanism of how PTPN22 drives disease development is still under much debate. IL2RA is an essential molecule expressed on activated T-cells and natural Tregs; IL2RA encodes the high affinity receptor for IL-2 which stimulates T-cells survival and proliferation upon engagement. Genetic defects in IL2RA may impair Treg function although normal, increased and decreased frequencies of Tregs have been reported in T1D patients\[49, 50\]. IFIH1 encodes a IFN-induced helicase that contributes to recognition of dsRNA and serves as a cytoplasmic sensor for viral infections\[51\]. How viral infections play a role in T1D is still under debate (discussed below), it is possible an overactive IFIH1 creates an enhanced pro-inflammatory environment. The increase in inflammatory mediators activates innate
immune cells, which in turn activates T-cells, resulting in hastened T1D development[52].

Figure 3. Type 1 diabetes loci discovered up until 2013. Adapted from Bakay et. al. 2013. Susceptibility loci discovered before 2007 were generated using non-GWAS methods. Each genetic locus is shown in the year it was discovered. On the right hand side are the susceptibility loci that have been discovered since the advent of GWAS along with the year of discovery. Many of these loci need to be further studied to confirm their role in T1D disease pathogenesis. These loci have established correlation to disease susceptibility either through NOD mouse studies or patient with T1D onset compared to healthy controls.

After these 6 loci were discovered, the advent of GWAS dramatically increased the discovery of T1D associated loci. GWAS studies have now shown upwards of 60 genes that are susceptibility loci/genes for T1D, with a majority of them being related to immune cells [53]. Recently, it has been discovered that many of these genes are also expressed in human islets, further complicating genetic studies[53]. This complicates genetic studies because the function of these genes associated with T1D may also play a role in islets and possibly β cells response to certain inflammatory cytokines. These genes can therefore be important in islet cell responses to immune attack and may
contribute to disease pathogenesis. Therefore, these genes may no longer only affect the immune response, but also islets suggesting a more complex dual role for many of these genes. The elucidation of the role of these loci is fundamental to understanding molecular pathways involved in disease development, which may offer avenues for drugs to be designed to target specific pathways and limit off target affects[54]. Furthermore, identifying these specific candidate genes in patients at risk for T1D could lead to improved disease predictability[55]. Improved early disease recognition would have a massive benefit on individual healthcare, by preventing disease in high-risk individuals rather than trying to reverse active disease. A lesson from the NOD mouse model has shown that it is extremely easy to prevent T1D; however reversal of disease, which is the true clinical question, is much more difficult. Predictive markers, such as HLA and auto-antibodies, have been discovered for T1D in humans and mice; however these markers are not 100% predictive of disease onset and therefore until a 100% predictive marker is found preventative techniques will not work.

**Parallels Between Human Genetics and the NOD Mouse Model**

The NOD mouse model has allowed for narrowing of genetic linkage to relatively small regions which confer protection or increase disease incidence, known as *Insulin dependent diabetes (idd)* regions. Each of these regions contains 1 or more candidate genes. Similar to human disease, the MHC susceptibility region on chromosome 17 was first identified in mice and was given the designation *idd1*. *Idd1* contains a number of genes known to contribute to disease pathogenesis including both MHC class I and class II[56]. The MHC haplotype in NOD mice is unique as it is composed of $H2^{g7}=K^d$, $Aa^d$, $Ab^{g7}$, $E^{null}$, $D^b$ and contains a unique I-Aβ locus, histidine at residue 56, serine at
residue 57; homologous to diabetogenic HLA-DQ\(\beta\) non-aspartic acid\(^{57}\) containing alleles\(^{[57]}\). The role of MHC genes in disease development was proven by replacing histidine and serine residues with proline and aspartic acid residues at position 56 and 57 respectively within the I-A\(\beta\) chain and preventing T1D development in the NOD mice\(^{[58-60]}\). Further this unique haplotype allows for a differential and unstable binding of auto-antigens which may result in inefficient negative selection and escape of auto-reactive T-cells from the thymus\(^{[61, 62]}\). The Idd3 region encodes the cytokines IL-2 and IL-21 which are strong candidates for T1D susceptibility in humans and mice\(^{[63-65]}\). IL-2 expression levels in NOD mice are abnormally low and this could result in lower numbers or inability of suppressor cells to function properly resulting in poor control of disease initiation or progression. Multiple low dose IL-2 treatments reduced severity of insulitis and inhibited T1D development. Similarly, further reduction of IL-2 through deletion of the IL-2 gene accelerated T1D development\(^{[66-68]}\). This is not unique to the NOD mouse model; as mentioned above patients with T1D have variations in IL2RA, and this genetic difference could lower IL-2 signaling, causing diminished Treg responses \(^{[69]}\). Research on IL-21 is more limited than IL-2, but IL-21 is highly expressed in NOD mice, and NOD mice deficient in the IL-21 receptor are protected from disease initiation\(^{[70]}\). IL-21 may cause disease by inducing specific inflammatory cytokines, mainly IL-17, but also including IFN-\(\gamma\), monocyte chemoattractant protein (MCP)-1, MCP-2 and interferon-inducible protein 10. This induction of inflammatory responses may exacerbate disease through recruiting leukocyte infiltration and in turn the destruction of islets\(^{[65]}\). Also, IL-21 is important for efficient initial activation of auto-reactive CD8\(^{+}\) T-cells\(^{[71]}\). Idd5.1 is another locus that
contains multiple genes of interest through their connection with human diabetes, including CTLA4 and inducible T-cell costimulator (ICOS). CTLA4 is expressed by a variety of immune cells including antigen presenting cells (APCs) and is involved in inhibiting T-cell activation. It was found that although CTLA4 is linked to both human and mouse disease; however the mechanism differs between the species. In NOD mice there are four different isoforms. One isoform is the ligand-independent CTLA4 (liCTLA4) which contains a SNP in exon 2 that causes liCTLA4 to be expressed at reduced levels. The reduction in liCTLA4 consequently decreases the threshold for T-cell activation and increases disease susceptibility[72, 73]. Similarly, Humans expressed lower levels of a CTLA4 isoform, soluble CTLA4 (sCTLA4). This reduction in sCTLA4 is believed to contribute to human disease susceptibility and makes an attractive target for immunotherapy[74]. Again, while CTLA4 dominates the literature on the Idd5 region, some studies suggest that ICOS contributes to disease pathogenesis as well. NOD mice have increased levels of ICOS which can contribute to enhanced T-cell activation and ICOS-/- NOD mice are protected from T1D[75, 76]. The mouse ortholog of PTPN22, Ptpn8, may influence disease in NOD mice, but this association has yet to be confirmed[77]. Ptpn8 encodes non-receptor class 4 subfamily of protein-tyrosine phosphatase family. In particular the protein encoded is a lymphoid-specific intracellular phosphatase that associates with the molecular adapter protein CBL. CBL may be involved in regulating the T-cell receptor signaling pathway[78, 79]. Therefore, mutations in this gene may allow for enhanced activation of T-cells including auto-reactive T-cells which could contribute to T1D disease pathogenesis[80, 81]. The Idd9 locus has been further split into 3 separate regions: Idd9.1, Idd9.2, and Idd9.3. The
genes in the *Idd9.1* region are currently unknown, but are associated with increased B-cell activation, low numbers of induced iNKT cells, along with reduced Treg development and activity in these congeneric mice[82-84]. Candidate genes for *Idd9.2* include CD30 and tumour necrosis factor receptor 2 (TNFR2), but these need to be confirmed. The candidate gene within the *Idd9.3* congeneric mouse is CD137, which will be discussed in more depth later[85]. These are just a few of the most commonly studied *Idd* loci, there are greater than 40 susceptibility loci identified in the NOD mouse, and more are likely to be found. The best resource for up-to-date information on T1D disease susceptibility loci in mice and humans and the orthologous regions between them is the T1DBase (http://t1dbase.org/page/Welcome/display)[86]. Although all the regions identified in these studies do not contain orthologous regions, many loci have led to immune pathogenic insight relevant to human disease. This pathogenic insight can then lead to targeting of common pathways for reversal of disease, or allow for novel biomarkers to identify patients that have an increased risk for T1D.

**Environmental Influences on T1D pathogenesis: infections**

While it is clear that genetics are important in T1D pathogenesis, there is increasing evidence that environmental factors play a significant role. This environmental effect is best demonstrated by the increased disease incidence in developed countries[87, 88]. In developed countries there is decreased exposure to pathogens because of the increased hygienic living conditions. Further, the dramatic surge of T1D prevalence cannot be explained exclusively by genetics as a populations genetics do not change radically enough between generations to account for this steep increase in T1D [89, 90]. These environmental effects may explain the ‘hygiene hypothesis’ which predicts that
“increased hygienic living conditions, the use of antibiotics and sterile food preparation will result in decreased exposure of the developing immune system to positive microbes, thus favoring an increased susceptibility to immune-mediated disorders”[91].

The immunological mechanism of how this occurs is still ill defined, however it is postulated that it could be due to a variety of factors such as stimulation of immunoregulatory CD4+ T-cells, modulation cytokines through TLRs, and antigen competition[91]. Another key piece of evidence for the influence of the environment in T1D is the varying disease penetrance and differences in age of disease onset between monozygotic twins[92]. Environmental factors in disease onset are not exclusive to T1D and have been shown for many autoimmune diseases[93, 94]. Infections in NOD mice have shown varying outcomes in T1D development; specifically different types of infections, viruses, bacteria, fungus, and helminthes, play differential roles and mechanisms in preventing or exacerbating T1D. A number of viruses have been positively associated with T1D onset and several mechanisms have been invoked to

Figure 4. from http://jdrf.org/research/prevent/. T1D incidence has doubled every 20 years. Data for Finland are from the Finnish National Public Health Institute; data for Sweden are from the Swedish Childhood Diabetes Registry; data for Germany are a compilation of two reports; data for Colorado are from the Colorado IDDM Registry, the Barbara Davis Center for Childhood Diabetes, and SEARCH for Diabetes in Youth.
explain these correlations, but there are no definitive infectious agents proven to cause disease[90]. Enteroviruses, a single stranded RNA virus family, have the most robust viral associations with T1D. There have been multiple studies linking these viruses to the onset of T1D, and they are considered a risk factor for pre-diabetic children[95, 96]. This viral family may be involved in both the initiation phase as well as disease progression. One group found that the rate of primary islet autoimmunity (detection of auto-antibodies) was significantly increased following detection of enterovirus RNA in the serum of human patients (blood samples from the DAISY)[97]. Another group showed that enterovirus RNA was associated with increased risk of developing primary islet autoimmunity and preceded islet auto-antibodies[98]. This data is in direct contrast with the NOD model, in which insulitis serves as a prerequisite for full onset diabetes involving enteroviruses[99]. Therefore, due to the limitations of human studies it is possible that susceptible individuals have underlying insulitis for years before a viral challenge “pushes” them to full onset diabetes / hyperglycemia. An extensive review of viruses and T1D pathogenesis is beyond the scope of this dissertation, but has been reviewed elsewhere[100]. There is also evidence that certain viruses (i.e. lymphocytic choriomeningitis virus, coxsackievirus) are able to prevent T1D by upregulation of programmed cell death ligand 1 (PDL-1) and induction of increased Tregs which control diabetogenic T effector cells[101-103]. To complicate matters further, various viral antigens such as CpG DNA and polyinosinic-polycytidylic acid (Poly(I:C)) are able to prevent T1D in NOD mice through decreasing T-cell proliferative responses and induction of Tregs, respectively[68, 104]. Therefore, it remains unclear whether viruses and their products cause initiation and destruction of T1D, or if they play a protective
role, or both. It is likely a matter of timing and stage of disease progression that determine these parameters. Bacteria are also a major area of research in preventing or accelerating T1D. In recent years it has been demonstrated that the gut microbiome plays a major role in initiating and maintaining immune responses to pathogens and autoimmunity. NOD mice housed under specific germ free conditions have a higher incidence of T1D compared to NOD mice housed under non-germ free conditions[105]. This is in agreement with the ‘hygiene hypothesis’ discussed above, and suggests that specific bacterial strains or families can promote or inhibit T1D. Probiotic treatment of NOD mice can elevate IL-10 levels and reduce the incidence of T1D[106]. Due to these promising gut microbiota results, a current clinical trial using probiotics is underway in high-risk children for T1D and is showing minor positive results[107]. However, it is important to note that the gut microbiota can also promote T1D. A positive correlation between increased incidence of disease and the numbers of gram-positive aerophilic and anaerobic bacteria was observed in NOD mice[108]. The differences in gut microbiota and causative linkages need to be explored in depth further. A paper by Wen et. al.[109] shed light on a possible mechanism in which the microbial balance may affect autoimmunity. NOD mice lacking MyD88, an essential component to TLR signaling, were protected from T1D. Furthermore, CD4+ T-cells expressing the highly diabetogenic TCR receptor BDC2.5 (NOD.BDC2.5 transgenic mice) failed to expand in the pancreatic lymph nodes (PLN) when transferred into MyD88/- mice. This suggests that abnormal recognition of certain gut microbiota may be indispensible for T1D in the NOD mouse model. However, in partial conflict with this data is the finding that bacterial infections and components have been found to prevent T1D, mainly through
the activation of TLRs. Bacterial infections such as *Salmonella* or *mycobacterium avium* were able to prevent T1D through inhibition of T-helper cells type 1 (Th1) and IFN-γ mediated responses, while causing phenotypic changes in DCs, upregulation of PDL-1 and indoleamine 2,3 dioxygenase (IDO) production or through the induction of “suppressor” cells[110-112]. Further, full fledged infections are not needed for prevention; numerous bacterial proteins/ligands have been shown to prevent T1D by induction of regulatory responses, either through natural “suppressor cells”, NK cells, NKT cells or Tregs. Induction of regulatory cells leads to production of suppressive cytokines and up regulation of cell surface ligands involved in preventing disease onset and progression including, but not limited to IL-10, transforming growth factor β (TGF-β) and PDL-1[113-119]. Therefore, infections and their linkage to T1D are not entirely clear. It may depend on the timing, dose and specific location of the infection or insult that proves to enhance or prevent T1D. Further, studies need to be done to account for these factors.

**Other Environmental Influences on T1D**

Other environmental factors that have been associated with T1D incidence in humans include: geographical location and diet. It has been demonstrated that there is an inverse relationship between disease incidence and proximity to the equator[93]. However, this relation cannot be attributed to a specific cause, although it is believed to be due to temperature and UV radiation mediated induction of Vitamin D[120-123]. The role of dietary components in T1D incidence has arisen in connection with the advent of various “fad” diets (i.e. Atkins) and increased health concerns about the “western” diet[109]. These dietary components are all believed to have influence on
mucosal immunity and gut microbiota (described above)[93]. One of the main ideas is that dietary antigens can influence susceptibility or protection from T1D through the release of pro-inflammatory or anti-inflammatory cytokines which skews the balance of effector cells and regulatory cells[108, 124].

**Disease Time Line and Pathogenesis**

In the NOD mouse model, disease pathogenesis can be classified into three phases: initiation, expansion, and destruction. In all three phases of T1D, multiple immune cells and cross talk between immune cells are needed for disease progression. Many “timelines” for disease progression and β cell death have been published. One early hypothesis was the linear islet β cell decline model[125], which suggested that in genetically susceptible individuals, when they encounter an environmental trigger, T1D is initiated and pathogenesis begins. Once triggered T1D progresses linearly and β cell-mass declines steadily over time. Another model is called ‘benign’ T1D in which a person never develops clinical T1D, but may have inflammation and some islet β cell destruction. This model is based on the finding that many patients at high genetic risk for T1D never develop full onset T1D. It is thus possible that predisposing genetic mutations alone may not in fact cause clinical disease[126]. However, while the ‘benign’ model suggests there are multiple regulations and the linear model suggests once the disease process is initiated there is an inability to stop disease progression; both models do not take into account the complex interactions of the immune system or the variability of time during the pre-diabetic phase. Therefore, a new model incorporating these variables is termed “relapsing-remitting”[126, 127], and suggests that there is disequilibrium between autoreactive T-cells and Tregs which develops over
time. This disequilibrium eventually leads to a decrease in β cell-mass, suggesting that during the progression of disease pathogenesis, the amount of functional insulin producing β cells fluctuates over time. Susceptible individuals may have ongoing subclinical insulitis for years until there is an environmental trigger and/or when epitope spread occurs allowing for enhanced destruction of β cells which causes hyperglycemia. Epitope (determinant) spreading is the development of immune responses to multiple additional endogenous epitopes after a primary response that releases multiple self antigens[128]. This massive destruction leaves few islet β cells left to control glucose homeostasis. However, islets can temporarily counteract the loss of β cell-mass by proliferating, which may explain the initial transient phase of reduced insulin requirement after diagnosis, known as the “honeymoon phase”. The ability for β cells to proliferate is not only found in patients with T1D, but can occur upon acute reversal of disease in NOD mice[129]. One interesting aspect of the relapsing-remitting model is that the therapeutic window for treatment of the disease is diminutive. While the time from diabetes diagnosis to full onset does fluctuate between individuals, immune intervention must be initiated before all of the islets have been destroyed. If there are residual islets and they are able to proliferate in response to stress, the preservation of these islets after treatment may allow for reduced exogenous insulin requirements, or even a slim chance for normal glycemia to be obtained. Watkins et. al generated five major categories of markers that are either currently used or forthcoming: genetic, autoantibody, risk score quantification, cellular immunity, and beta-cell function. The current standard used to assess T1D onset or predisposition focuses on autoimmune pathology and disease-associated autoantibodies. Research studies in general go
beyond autoantibody screening and assess genetic predisposition, and quantitate risk of developing disease based on additional factors. However, there are few currently used techniques that assess the root of T1D: beta-cell destruction. Thus, novel techniques are being developed with the potential to gauge degrees of beta-cell stress and failure via protein, RNA, and DNA analyses. A full review of these techniques can be found elsewhere[130].

**Initiation:** The initiation phase of T1D begins with genetics and a person must be genetically predisposed to develop T1D. There are multiple theories of how the disease process actually begins, such as ‘natural’ apoptosis of islet β cells due to changes in metabolism brought on by weaning; or triggering by a pathogenic insult, such as a virus[131]. All theories for the initiation phase agree that β cell antigens are released from the pancreatic islets and genetic predispositions drive diabetogenic autoimmunity and disease progression. APCs in the pancreas, specifically conventional or classical dendritic cells (cDCs), endo/phagocytose the newly released β-cell antigens, and function to induce immune activation or promote tolerance[132]. The reason APCs of the NOD mice do not promote tolerance will be discussed in more detail later; briefly, NOD mice have genetic defects in APCs that promote a proinflammatory or activated state. Once β cells have been endo/phagocytosed, cDCs travel to the draining pancreatic lymph node (PLN) to prime pathogenic islet β cell antigen specific CD4⁺ and CD8⁺T-cells (diabetogenic T-cells)[133].
Figure 5. Timeline for T1D. This figure represents the β-cell mass or function (represented by the yellow line) and describes the immunological events that take place during these times, along with the anatomical location in which the immunological events are taking place. Adapted from Van Belle T L et al. Physiol Rev 2011;91:79-118. Once the yellow line of beta-cell function falls into the blood glucose 200mg/dL, the individual is clinically diagnosed with type 1 diabetes. Before this time it is extremely hard to predict T1D in the clinic. A pre-requisite of T1D is genetic susceptibility and an environmental trigger. The changes in weaning or environmental insult cause beta cell death and release of β-cell antigens. These antigens are engulfed by resident antigen-presenting cells (APC) and transferred to the pancreatic lymph node (PLN). The environmental trigger or genetic defect causes a pro-inflammatory environment which favors effector T-cell responses over Treg function. β-cell antigens presented in this pro-inflammatory environment initiate the conversion of B cells into plasma cells. These plasma cells then secrete insulin auto-antibodies. Auto-reactive CD4 and CD8 T cells are stimulated to proliferate and migrate into the pancreas. During this phase, Treg cells cause inactivation on disease development. The destruction phase which involves perforin, IFN-γ, and tumor necrosis factor (TNF)-α, causes a massive decrease in insulin producing islet β-cells. The killing also causes the release of new β-cell antigens that are engulfed by APCs and get presented in the PLN. This causes epitope spread of CD4 and CD8 T cells and B cells. After epitope spread, more β-cell death occurs and usually results in severe depletion of β-cell mass. However, it has been shown that stress on the islets can cause some beta-cell proliferation, so that the β-cell mass temporarily resurrects. Tregs can overpower and dampen the effector response, but in T1D disease pathogenesis this does not occur. The fluctuation between destructive responses and the alleviation by immune regulation coupled with beta-cell proliferation possibly creates what is known as “relapse-remitting” (yellow line). Eventually, the destructive responses over power everything and T1D is diagnosed when only 10–30% of insulin producing β-cells remain. The remission after clinically diagnosed diabetes is termed the “honeymoon phase” a temporary state of relative self-sufficient insulin production.

Adapted from Van Belle T L et al. Physiol Rev 2011;91:79-118.
**Expansion:** In the draining PLN cDCs and macrophages promote the expansion of diabetogenic T-cells through antigen presentation with increased co-stimulation and inflammatory cytokine production (eg IL-12)[134]. During this expansion phase, B-cells also may play a role as an antigen presenter. While the role of B-cells is still under much debate, it is undeniable that B-cells produce auto-antibodies against major β cell antigens such as insulin[35]. Since, the immunopathological events are clinically silent until the point of auto-antibody production in humans, inhibiting the initiating event is extremely difficult. However in NOD mice, which have a relatively homogenous age of onset, this stage of activation of diabetogenic T-cells can be inhibited through a variety of mechanisms. One of these mechanisms is inhibiting co-stimulatory molecules on cDCs surface such as CD80 or CD86[135]. Another mechanism is engagement of inhibitory receptors such as PDL-1[136]. Finally, there is evidence that that certain cell types can prevent disease through the recruitment of tolerogenic DCs, plasmacytoid DC, and Tregs. These suppressor cells can act on diabetogenic T-cells to down regulate their pathogenic effector functions through multiple mechanisms including IL-10, IDO, TGF-β, and ICOSL[133]. In the natural course of disease, however, the diabetogenic T-cells are not inhibited and they therefore migrate to the pancreas and destroy islet β cells.

**Destruction:** Once diabetogenic T-cells enter the pancreas destruction of islet β cells can occur through a variety of pathways, both contact independent and dependent.

**Contact independent** pathways consist of cytokines and chemokines, which in some instances cause islet β cell damage and apoptosis. Cytokines such as IL-1β, IL-12, IFN-
γ, and TNF-α secreted by immune cells in the pancreas, from both innate and adaptive immune cells (macrophages, DCs, CD4+ and CD8+ T-cells, and natural killer (NK) cells), may cause direct damage of islet β cells[133]. These cytokines in most instances have a detrimental effect on islet β cells; however in certain scenarios cytokines may induce self defense mechanisms. One such example is IFN-γ; IFN-γ can induce IDO production from islet β cells, allowing them to control some of the diabetogenic T-cells and thus decrease damage[137]. Chemokines on the other hand such as CXC-chemokine ligand 10 (CXCL10), CC-chemokine ligand 2 (CCL2) and CCL20 can recruit macrophages and other inflammatory cells into the insulitis lesions[138]. These inflammatory cells then cause a positive feedback loop in destruction of islet β cells.

Islet β cells express different TLR receptors including, but not limited to TLR2, TLR4, TLR3, and TLR9. TLR2 recognizes peptidoglycan which is found on the surface of most bacteria and TLR4 recognizes another crucial bacterial component lipopolysaccharide (LPS). Stimulation of TLR2 and TLR4 have been shown to cause apoptosis of islet β cells, but there is some controversy whether this damage is a result of a bystander effect, in which cells of the immune system are activated by these ligands and secretion of inflammatory cytokines causes the damage to islet β cells; or if ligation to TLR2 and TLR4 on islet β cells directly causes their death[139, 140]. Our results suggest that TLR4 does not cause damage to islet β cells indirectly or directly (data not shown), however further studies need to be performed to confirm this result. TLR3 and TLR9 are both intracellular TLRs, TLR3 binds to viral double stranded RNA or a synthetic analog (Poly(I:C)). Poly(I:C) is used in a variety of experiments to show protection from T1D as well as damage to islet β cells. TLR9 binds to DNA containing
hypomethylated CpG motifs expressed by viruses and bacteria, but it is unclear whether it protects from or exacerbates disease. The role of the TLR family in T1D has been reviewed [141].

**Contact dependent** mechanisms of islet β cell apoptosis includes a variety of immune cells such as NK cells, CD4+ and CD8+ T-cells. NK cells can bind to islet β cells through the retinoic acid early transcript 1 (RAE1) ligand in conjunction with natural killer group 2, member D (NKG2D), and leads to direct islet β cell damage and apoptosis. NK cells can also interact with islet β cells via their NKp46 receptor, which engages an unknown ligand on the surface of islet β cells and causes NK cell degranulation[142]. This degranulation releases factors such as granzymes and perforin which causes apoptosis. CD4+ and CD8+ T-cells are activated by the presentation of islet auto-antigens by MHC class II and class I, respectively, on APCs. Further, islet β cells themselves can express auto-antigens on the surface through MHC class I which stimulates diabetogenic CD8+ T-cells through their T-cell receptor (TCR). Once activated, CD8+ T-cells perform cytotoxic effector functions such as IFN-γ production, granzyme and perforin release causing death of β cells. CD4+ diabetogenic T-cells on the other hand interact with DCs which present islet β cell antigens via MHC II, this DC:T interaction can cause expression of FasL. The islet β cells can express Fas on their surface and the interaction between Fas and FasL can mediate apoptosis through caspase-8 activation[143]. However, there is contradicting data suggesting that Fas deficiency in NOD mice actually accelerates disease and is therefore dispensable for T1D pathogenesis[144]. There are also contact dependent mechanisms to control the killing of islet β cells by diabetogenic T-cells. The most well studied inhibitor of contact
mediated apoptosis of islet β cells is PD-L1 which is expressed on the surface of islet β cells. PD-L1/PD-L2 expression only on antigen-presenting cells is insufficient to prevent the early onset diabetes that develops in PD-L1/PD-L2(-/-) non-obese diabetic mice. PD-L1 expression in islets protects against immunopathology after transplantation of syngeneic islets into diabetic recipients. Therefore, PD-L1 on islet β cells inhibits pathogenic self-reactive CD4+ T cell-mediated tissue destruction and effector cytokine production[145, 146].

**Innate Immune Cells in T1D**

This discussion of disease pathogenesis clearly illustrates that both the innate and adaptive immune responses are necessary for the progression of T1D. To avoid possibly damaging chronic inflammatory responses to the host, the immune system requires precise regulation when combating pathogens while maintaining homeostasis and tolerance to self. The innate immune system consists of macrophages, DCs, NK, iNK T-cells, γδ T-cells, and neutrophils which recognize potentially dangerous molecules via germ line encoded receptors and elicit rapid immune responses that function as the first line of defense against foreign pathogens[147]. These cells can then lead to downstream responses through the stimulation of antigen-specific cells of the adaptive immune system. In order to respond quickly to pathogens and foreign antigens the innate immune system recognizes conserved molecular patterns almost always restricted to pathogens by using a variety of pattern-recognition receptors (PRR). Examples of PRRs include TLRs, complement receptors, mannose receptors, nucleotide-binding oligomerization domain receptors (NLRs), RIG-I-Like receptors (RLRs), and Fc receptors. TLR4 will be discussed more in depth later as it is a major
focus of our research. Besides the role of the innate immune system in the development of protective immune responses to pathogens, the innate immune system is important for the development of adaptive autoimmune responses and is also pivotal in recognition of self-antigens. APCs, such as DCs and macrophages, efficiently capture and process self-antigens from infected or dead cells and present them to autoreactive T-cells by means of cross-presentation. If the presentation of self-antigens occurs in the absence of signals that promote APC maturation, these APCs remain immature and normally induce T-cell tolerance by deletion or anergy[148]. However, in the case of an inflammatory environment, such as found in T1D, cross-presentation (cross-presentation is the uptake and presentation of exogenous antigens on MHC class I rather than on MHC class II) leads to the ability to break self-tolerance by promoting APC maturation. Clearly defects in the innate immune system play a major role in the pathogenesis of T1D [149].

**Macrophages:** Macrophages play a critical role in recognition and elimination of pathogens through the use of PRRs such as TLRs to detect pathogen-associated molecular patterns (PAMPs)[150]. The recognition of pathogens through innate receptors leads to activation and effector functions including antigen processing / presentation, scavenging for dead cells or foreign substances, and release of inflammatory mediators[150, 151]. However, macrophages can play a detrimental role to the host, due to the non-specific nature of the response and can cause extensive damage if uncontrolled. This has been demonstrated in different diseases including T1D[152], rheumatoid arthritis[153], degenerative diseases[154], sepsis[155], and others[156]. In T1D macrophages along with DCs are the first to infiltrate the pancreatic
islets well before the infiltration of NK cells or diabetogenic T- and B-cells in NOD mice and BB rats[18, 157-159]. Not only are macrophages the first cells that infiltrate into the pancreas, they are necessary to disease pathogenesis. It has been reported that DCs and macrophages are essential for the retention of lymphocytes in peri-insulitis lesions prior to full onset of progressive insulitis[160]. Alterations in macrophages can change the course of disease and this has been demonstrated through a variety of mechanisms including blocking specific adhesion molecules[161, 162], depletion of macrophages[163, 164], or genetically modifying the NOD system to change the underlying immune defects. NOD mice have underlying innate immune defects that may drive disease. Macrophages from NOD mice inherently make more IL-12 then the non-obese resistant (NOR) strains[134]. Furthermore, macrophages from NOD mice exhibit developmental and functional defects that would be expected to promote the development of auto-reactive T-cells[62, 165]. Bone marrow cells from NOD mice develop reduced numbers of mature macrophages in vitro and this is correlated with the inability of NOD macrophages to stimulate a syngeneic mixed lymphocyte reaction. NOD macrophages also have a reduced capacity to stimulate the expansion and/or function of Tregs[166]. Human T1D patients and NOD mice both have enhanced macrophages prostaglandin synthase 2, which could limit the ability to produce Tregs via its negative effects on IL-2 production[167]. The difference in IL-2 signaling effects the maintenance of T-cell tolerance by means of differential T-cell activation: high levels of IL-2 signaling activate Tregs, and lower levels lead to the stimulation of effector T cells[168]. Mice that have a disruption in IL-2 signaling develop autoimmunity due to the lack of Tregs[169-171]. In conjunction with the lower IL-2, macrophages from NOD
mice secrete elevated levels of proinflammatory cytokines including IL-12(p70), TNF-α and IL-1β, attributable in part to hyperactivation of Nuclear Factor kappa-light-chain-enhancer of activated B-cells (NF-κB)[172]. High levels of IL-12 produced by NOD macrophages promote the differentiation of diabetogenic T-cells in the PLN as well as in the pancreatic lesions. NOD macrophages have defects in the phagocytic clearance of apoptotic cells, which could synergistically promote inflammation mediated by cellular contents released from apoptotic cells or allow for the uptake by DCs to be efficiently presented as auto-antigens in the PLN[173]. Finally, as previously described in the “destruction” section macrophages have the ability to directly kill β cells in the pancreas through soluble mediators. There is however evidence that alternatively activated macrophages may play a beneficial role in T1D. Alternatively activated macrophages do not require priming from pathogens, but can be stimulated by IL-4 and IL-13. They can then produce molecules that work towards resolution of inflammation rather than exacerbation of inflammation[174]. Alternatively activated macrophages can decrease hyperglycemia, insulitis, and inflammation in the pancreas[175], while producing higher levels of immunomodulating cytokines IL-10, TGF-β, and reduced amounts of proinflammatory cytokines. They also express high levels of PDL-1 and PDL-2 which can cause inhibition of activated T-cell proliferation[174]. In summary macrophages play an important role for disease initiation through presenting antigens, producing inflammatory cytokines, inducing diabetogenic T-cell response, and directly killing islet β cells. However, macrophages may be able to prevent disease if they become alternatively activated or regulatory macrophages. Targeted induction of alternatively activated macrophages may warrant more attention for the reversal of T1D.
**Conventional or Myeloid Dendritic Cells:** Dendritic cells play a major role in priming the adaptive immune response. Immature DCs phagocytose antigens in the periphery and migrate to the regional lymph node. In the lymph node DCs present antigen to T- and B-cells either inducing an immune or tolerogenic response. If DCs present antigens to T-cells in a “steady or resting” state this leads to the induction of tolerance, however if DCs present antigens in an “activated or inflammatory” state, this induces T- and B-cell activation. Therefore, DCs play a critical role in T-cell immunity and tolerance. As previously mentioned DCs and macrophages are the earliest islet infiltrating cells in both humans with T1D and NOD mice. In NOD mice, a specific subset of DCs, conventional DC (cDC) or myeloid DCs (mDC) (CD11c⁺CD11b⁺CD8α⁻), are responsible for capturing and transporting β cell antigens from islet cells to the PLN to prime diabetogenic T-cells[131, 176]. Presentation of islet auto-antigens to BDC2.5 T-cells in vivo requires mDCs. Further, after the deletion or removal of mDC using a CD11c promoter driven diphtheria toxin receptor (DTR) in NOD mice, the NOD.CD11c.DTR mice were no longer able to advance to full onset T1D or activate T-cells[177]. Further, work has been done in this area and merocytic dendritic cells (CD11c⁺CD11b⁻/loCD8α⁻PDCA⁻) cross present and prime both CD4⁺ and CD8⁺ T-cells to cell-associated antigens[178]. A comprehensive review of DCs in T1D, and the mDC subset, can be found elsewhere[179]. Interestingly, in human T1D patients, DC subsets exist in normal frequency and phenotypic states compared to healthy controls. These cells were isolated from peripheral blood and therefore may be quite different within the pancreas[180]. Another caveat to this experiment is that most DC work was done after isolation of the cells or after they were transformed *in vitro* which could affect the
functional characterization of these cells. DCs, specifically mDCs, play an important role in disease initiation. mDCs have a relatively mature phenotype in NOD mice and this may be due to maturation signals received by remodeling of pancreas following β cell apoptosis, these mature mDCs then prime diabetogenic T-cells[181]. Furthermore, macrophages in NOD and humans have increased levels of TNF-α. TNF-α plays a critical role in the initiation of T1D by causing DC maturation, which in turn causes sufficient co-stimulation for diabetogenic T-cell activation[182, 183]. Similarly, studies in NOD mice have revealed that mDC exhibit a hyperinflammatory state, and these cells have an increased capacity to produce IL-12. IL-12 is a main driver for the induction of diabetogenic T-cell Th1 responses in T1D disease progression[184, 185]. In conjunction with increased IL-12, mDCs in NOD have higher expression levels of co-stimulatory molecules such as CD80 and CD86[186]. A possible explanation for the contribution of NOD DCs in T1D disease pathogenesis may be that the underlying defects in NOD DCs predispose them to selectively reduce co-stimulatory signals required for the expansion of Tregs, but not of effector T cells. Further studies on these issues will provide significant insights into the mechanism of how mDCs can promote T1D[147]. Another area of interest is in modulation of DCs in order to provide a therapeutic effect for the treatment of T1D. mDC are not only able to expand diabetogenic T-cells, but also have the ability to promote peripheral tolerance by inducing T-cell deletion, T-cell anergy, or expansion of antigen specific Tregs[132]. In the context of T1D, mDCs have the ability to induce the expansion of islet specific auto-antigen Tregs, these cells are then able to mediate disease development[187]. Further, therapeutics directed to modulate the functions of mDCs such as granulocyte colony-
stimulating factor (G-CSF) or FMS-like tyrosine kinase (Flt3)-ligand treatment increase
the number of plasmacytoid DCs (pDCs) and/or tolerogenic mDCs in the spleen and
PLN. These DCs consequently expanded Tregs which can control diabetogenic T-
cells[188-190]. Of note most T1D therapies described in the NOD literature involve
prevention, rather than reversal of disease. Many reagents are only able to prevent
disease but not reverse active disease; this is the case with G-CSF and Flt3 ligand
administration. The treatment is only effective very early in the pre-clinical disease
process, when low numbers of non-epitope spread diabetogenic T-cells are present
[191]. mDCs are not the only DC cell type that has been implicated in T1D for disease
pathogenesis and prevention.

**Plasmacytoid DCs:** Plasmacytoid DCs (pDCs) have been implicated in disease
pathogenesis due to their ability to detect viral RNA and DNA through TLR7 and TLR9,
which induces large amounts of anti-viral cytokines such as Type 1 IFNs that are
destructive to islet β cells[192]. Further, type 1 IFNs are increased in the pancreatic
islets in both human and rodent models with T1D compared to healthy controls [193,
194]. pDCs are increased in the PLN upon initiation of T1D[195]. However, it is
possible that this is a feedback response to induce regulation of diabetogenic T-cell
responses, which will be discussed later. As with many cell types pDCs can also play a
role in disease prevention. pDCs can mediate protection through the expression of
various molecules implicated in tolerance induction such as PDL-1, ICOS, and IDO. A
protective role for pDCs was shown in a T1D transfer model using naïve diabetogenic
CD4^+/CD8^+ T-cells transplanted into NOD.scid recipient mice along with pDCs. pDCs
prevented T1D onset likely by inducing IDO production in the pancreas that inhibited the

diabetogenic T-cell response[177]. IDO regulates effector T-cell expansion by catalyzing oxidative catabolism of tryptophan, and free tryptophan is essential for effector T-cells. Young NOD mice appear to be defective in IDO expression and over expression of IDO leads to extended islet graft survival[196, 197]. In summary, DCs can play both a pathogenic and protective role in T1D; the timing as well as the stage of T1D progression are important parameters that may affect the differential responses seen in DCs subsets.

**Natural Killer Cells:** NK cells are involved in antiviral and anti-tumor responses mainly through direct killing of target cells or indirectly by producing IFN-γ[198]. Thus NK cells could contribute directly or indirectly to islet β cell death. NK cells infiltrate the pancreas of NOD mice and diabetic patients[199-201]. In diabetic patients, their presence in insulitis has been associated with coxsackievirus B infection. NK cells are found in the insulitis areas in coxsackievirus B infection, therefore it is not surprising, NK cells are involved in diabetes initiation in mouse models induced by coxsackievirus infection[202]. NK cells isolated from NOD mice have a more activated phenotype with higher expression of CD25, CD69, PD1 and natural killer cell lectin-like receptor group G (NKG2D)[199]. Aside from surface molecules, NOD NK cells also proliferate more, produce more IFNγ and express higher levels of the degranulation marker CD107a on their cell surface, further reflecting a more activated phenotype[203]. NK cell ligands, recognized by NKG2D and NKp46, are expressed by the pancreatic β cells of NOD mice and humans after diabetes onset [142, 203] and these molecules could play a key role in the destruction of pancreatic β cells. Depletion of NK cells using various antibodies against NK1.1 and asialo-GM1 results in the prevention of NOD T1D[201,
Further, the depletion of Treg cells in the BDC2.5 TCR transgenic NOD mice resulted in an increase in the percentage of activated pancreatic NK cells that produced large amount of INFγ, which promoted effector functions of diabetogenic T-cells[205]. Controversy surrounds the area of NK cells; several studies have reported a protective role of NK cells. Interestingly, impaired NK cell function was described in the blood of diabetic patients and in lymphoid tissues of NOD mice[206-208]. However, the defects seen in patients may result from long term hyperglycemia/disease pathology, rather than cause the disease. In contrast, NOD mice present with defects in NK cells before disease onset. Prevention of diabetes in NOD mice induced by complete Freund adjuvant injection is NK cell dependent[117]. Finally, a protective role for NK cells has been observed in an islet transplant model; NK cells induced allograft islet survival by killing DCs in a perforin dependent manner[209]. The deleterious or beneficial role of NK cells in the development of diabetes again probably depends on the context and stage of insulitis.

iNKT cells: iNKT cells are innate like T-cells that express an invariant TCR restricted to non-polymorphic CD1d molecules. Upon TCR stimulation, these cells promptly produce large amounts of various cytokines and chemokines that will in turn influence both the innate and adaptive immune system. Previous reports on iNKT cells show that they have a beneficial role in autoimmunity and T1D in particular[210, 211]. The beneficial effect of iNKT cells on T1D development and disease progression was demonstrated in a variety of different ways all involving increased iNKT frequency. The first demonstration of this was through the introduction of a Vα14-Jα18 transgene in the NOD mouse model, this subsequently increased the number of iNKT cells and reduced
the incidence of T1D[212]. Secondly, adoptive transfer of iNKT cells, increasing their frequency in NOD mice was sufficient to reduce the incidence of T1D[213]. Finally, to increase the frequency of iNKT cells, activation of iNKT cells using an exogenous ligand α-galactosylceramide prevented diabetes incidence[214-217]. The mechanism of protection from T1D by iNKT cells is unclear, it was originally shown that iNKT cells promote a Th2 response in the NOD mice and that a Th2 response in the setting of T1D is protective from disease development[215, 217, 218]. However, later studies indicated that prevention by iNKTs was a result of inhibiting differentiation of effector cell. This inhibition of effector function reflected anergy of diabetogenic T-cells which in turn failed to cause islet destruction in the pancreas. It is also thought the islet antigen specific CD4+ T-cell become anergic in the PLN and pancreas causing CD4+ T-cell to differentiate into Tregs. It is hypothesized that iNKT cells do not inhibit T-cells directly, but recruit tolerogenic DCs (as opposed to activating DC) into the PLN of NOD mice[219, 220].

**γδ T-cells:** While there has been very little work on γδ T-cells in T1D, there is increasing evidence that they are involved in the development of autoimmunity. The number of γδ T-cells is significantly reduced in NOD mice and in prediabetic high-risk patients with pancreatic β cell impairment [221, 222]. In one study, immunization with insulin prevented the development of T1D by induction of immune tolerance associated with regulatory CD8+ γδ T-cells[223]. There is also significant acceleration of T1D by neonatal thymectomized NOD mice, supposedly largely due to impaired development of γδ T-cells [221]. As the involvement of mucosal immunology is explored in depth the role of γδ T-cells may be expanded in the setting of T1D.
Neutrophils: Neutrophils are controversial in their role for T1D development without any outcomes being fully confirmed[224]. Several studies have explored these controversial roles of non-specific immune responses in T1D, in which neutrophils show defects in chemotaxis, phagocytosis, killing of bacteria and release of superoxide in T1D patients and animal models, but this could be due to the effect of disease and not causal[224-227].

Conclusion: Innate immune cells beyond doubt have a role in prevention or exacerbation of disease. Interestingly, based on studies in the NOD mouse model, innate immune cells often seem to play protective roles in the early phases of disease, before expansion of diabetogenic T cell clones. However, few studies have studied the effects of innate immune cells in reversal of disease. There may be fundamental innate immune defects that establish and maintain persistent autoreactive T-cell responses. These innate immune defects have been highlighted both in rodent models of T1D as well as in T1D patients. It is thus possible that correcting or ‘retuning’ the innate immune response will have beneficial effects in controlling autoreactive adaptive immune responses. Further investigations are needed to decipher why these cells are implicated in the development of T1D in absence of exogenous stimuli. T1D is further associated with immune deficiencies of innate cells rendering them unable to induce tolerance against islet antigens. Moreover, chronic low activation of these innate immune cells in the pancreas through continued β cell death and/or persistent virus infection promotes their pathogenic functions. Increasing the knowledge of regulating mechanisms of T1D by innate cells would open promising therapeutic approaches[228]. This avenue is explored by manipulating the TLR4 pathway in Chapter 2.
TLR4 Signaling

TLR4 is a membrane-bound protein that recognizes an integral component of gram-negative bacteria, lipopolysaccharide (LPS). TLR4 is 1 of 13 murine TLRs that have been identified to date, while 10 TLRs have been identified in humans [229]. TLR4 is shared between the two species. Activation of TLRs can upregulate multiple pro/anti-inflammatory cytokines, chemokines, and co-stimulatory molecules as a result of various intracellular signaling pathways[229]. TLR4 consists of a three components: an N-terminal ligand recognition domain, a single transmembrane domain, and a C-terminal cytoplasmic signaling domain. Stimulation of TLR4 with LPS requires a series of interactions between: LPS binding protein (LBP), CD14, MD-2, and TLR4[216]. LBP is a soluble shuttle protein which can directly bind LPS and facilitates the interaction between LPS and CD14[216]. CD14 is a glycosylphosphatidylinositol-anchored protein, which also exists in a soluble form, but CD14 facilitates the transfer of LPS to the TLR4/MD-2 receptor complex and modulates LPS recognition. MD-2 is a soluble protein that non-covalently associates with TLR4 and is involved in causing downstream signaling cascades[230, 231]. Upon binding of LPS, TLR4 forms a homodimer and recruits downstream adaptor molecules through the interaction with the Toll-like interleukin-1 receptor (TIR) domain. The TIR domains in TLR4 is critical for downstream signaling as a single point mutation in the TIR domain can abolish signal transduction[232]. There are five TIR domain-containing adaptor proteins associated with TLR4 signaling: Myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (also known as Mal, MyD88-adapter-like) (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule
(TRAM), and sterile α and HEAT-Armadillo motifs-containing protein (SARM)[233]. It is important to note that TLR4 signals via two distinct pathways, the MyD88 dependent and MyD88-independent pathways. MyD88 dependent and independent pathways can be summarized in the figure below (Figure 6) and will not be described in detail. Briefly, the MyD88-dependent pathway was shown to be responsible for pro-inflammatory cytokine expression, while the MyD88-independent pathway mediates the induction of Type I interferons and interferon-inducible genes[234]. TLR4 signaling results in production of a large amount of inflammatory mediators. A specific example of this is the activation of NF-κB. NF-κB regulates many different genes including, but not limited to the production of inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, IL-12, IL-17, TNF-α, IFN-γ, inducible nitric oxide synthase (iNOS), and intercellular adhesion molecule 1 (ICAM-1)[235]. Most of these cytokines have been implicated in T1D disease pathogenesis, either through the initiation phase, expansion of effector T-cells or destruction of islet β cells. Other transcription factors (AP-1, CREB, IRF5, IRF3, and IRF7) also play a role in the induction of inflammatory mediators after induction of NF-κB and these cytokines include but are not limited to type 1 interferons, IL-10, IL-23, COX2, and monocytes chemoattractant protein-1 (MCP-1) [235]. Due to the induction of what is mostly considered pro-inflammatory cytokines, TLR4 signaling is usually associated with inflammation when stimulated. However, there is also a unique cellular effect that can be experienced by TLR4 expressing cells when stimulated with low doses of LPS, known as endotoxin tolerance[236]. Endotoxin tolerized cells have a decreased inflammatory response when re-challenged with LPS and exhibit enhanced anti-inflammatory properties[237]. Therefore manipulating TLR4 responses can cause
decreases in inflammatory cytokines and an increase in negative regulators of the immune system such as IL-10. TLR4 stimulation can thus result in tolerogenic APCs. These tolerogenic APCs then can have an impact on disease outcome through suppression of effector T-cell responses and expansion or induction of Tregs.

Figure 6. The TLR4 signaling pathway. LPS shuttled to CD14/MD-2 by LPB. CD14/MD-2 accessory molecules are needed for LPS binding. Upon binding LPS, TLR4 dimerizes, and recruits downstream adaptor molecules such as MyD88/MAL and TRIF/TRAM to induce signaling. The activated MyD88 then activates IRAK1, IRAK2, TRAF6, TAK1, TAB2/3 and the IKK complex, while TRIF/TRAM signals through RIP1 to TRAF6 to cause this downstream response. Both of these pathways converge at NF-κB. The cytoplasmic NF-κB complex is maintained inactive by IκB, which is in turn degraded by ubiquitin (Ub) proteasomes, resulting in the translocation of NF-κB into the nucleus. Besides activating NF-κB, TAK1, TAB2/3 also phosphorylates MAPKs to cause further activation of p38 and JNK, which allows for the translocation of CREB and AP-1 into the nucleus to cause further production of inflammatory mediators. The TRIF/TRAM pathway not only activates NF-κB but also triggers TRAF3, TBK1 and IKKe, to induce IRF3 and IRF7. These produce large interferon responses.
Immune Cell Tolerance

The best *in vivo* example of endotoxin tolerance is in hepatic immunity. The liver is constantly exposed to low doses of LPS from the gut. This exposure leads to APC tolerance rather than activation[238]. Tolerization of cells is extremely important in transplant immunology, however these same properties can be applied to autoimmunity. Immature DC express high endo-and phagocytic capacity, low levels of MHC class II and co-stimulatory molecules (CD40, CD80 and CD86), and low T-cell stimulatory ability[239]. These immature DCs are associated with induction of T-cell anergy and generation of Tregs. Mature DCs, on the other hand, express high levels of MHC class II, CD80 and CD86, all of these co-stimulation molecules have a strong migratory capacity for T-cell proliferation. These mature DCs also upregulates the lymphoid homing receptor CCR7 and secretes the inflammatory Th1 cytokine, IL-12[239].

Therefore, immature DCs have been established as the prototypical tolerogenic DC. Tolerogenic DCs suppress antigen specific responses in transplant tolerance and therefore reduction in these response could have a significant impact in the field of autoimmunity[240]. APCs are essential for the maintenance of both central and peripheral tolerance. Central tolerance is achieved through negative selection in the thymus and is a highly efficient process mediated by APCs and can cause induction of nTregs (see Tregs section below for details). DCs in the periphery can migrate to the thymus, and can generate Tregs and/or cause negative selection. This shows that some foreign antigens can eventually be expressed in the thymus and become tolerized through central tolerance *in vivo*[241]. In the NOD mouse model, bone marrow derived DC treated with thymic stromal lymphopoietin (TSLP), acquire a tolerogenic phenotype,
induce the conversion of naïve T cells into functional CD4⁺CD25⁺Foxp3⁺ iTregs, and provide protection from T1D[242]. This result shows that by causing induction of tolerogenic DCs and/or inducing functional Tregs in autoimmune diabetes it is possible to prevent hyperglycemia. This could then be translated to the reversal of disease; this hypothesis for disease reversal is seen in Chapter 2 by using an innate immune stimulating antibody to TLR4 causing reversal of T1D through induction of tolerogenic APCs. In peripheral tolerance, tolerance to self antigens can be achieved through a variety of mechanisms in the absence of inflammation, including T-cell depletion, induction of T-cell anergy, iTreg generation, expression of surface molecules (CTLA4, PDL1) and production of cytokines (IL-10, TGF-β, and IDO)[239]. In a mouse model of collagen-induced arthritis, LPS-stimulated DC upregulated IDO expression, induced markers for Tregs (Foxp3, TGF-β1 and CTLA-4) in vivo, and improved arthritis scores when injected after immunization. These mice were given low doses of LPS, which may have induced endotoxin tolerance (although this paper did not test for endotoxin tolerance) and allowed for lower expressions of MHCII, CD40, CD80 and CD86. These “semi-mature” DCs had decreased capacity to secret IL-12p70, IFN-γ, TNF-α, and IL-10. This model thus shows that regulatory mechanisms of DCs through “stimulation” of an innate immune receptor can cause changes in adaptive immunity and reversal of established disease[243]. This further suggests that in other autoimmune disease or in models of transplant tolerance, the induction of tolerance through innate immune stimulation may allow for increased iTregs or nTregs, which in turn could cause reversal of disease or long term graft survival, respectively. There is a dynamic interplay between DCs and T-cells in the induction of tolerance. It has also been shown that
CD4^+CD25^+ Tregs activated with LPS could induce IDO expression in DCs through a CTLA4 independent, but cytokine dependent mechanism [244]. Although Tregs responsiveness to LPS is controversial, it was shown that Treg cultures given LPS increased IL-10. IL-10 is a known regulator of IDO expression and upon LPS stimulation of Tregs and increased IL-10 production this resulted in the upregulation of IDO in DCs[244]. However, LPS from the original stimulation could have contaminated the co-cultures, as no wash was mentioned, and this could have played a role on DCs directly. This low level stimulation with LPS could then have caused a tolerogenic phenotype and increased IDO production. The influence DCs have on Treg induction is well established. It has been previously published that loss of DCs in vivo results in increased risk of autoimmunity through a reduction/loss of Tregs while there was an increase in Th1 and Th17 T-cells[245]. IL-10 contributes to the induction of anergy, the development of suppressive T cells and plays a fundamental role in the reciprocal effects of tolerogenic DCs and iTregs. CD4^+ T-cells stimulated through ICOS resulted in IL-10 production, while blocking of the ICOS/ICOS-L interaction abolished induction of IL-10 and reduced the CD4^+ anergy previously seen with ICOS stimulation. Tolerogenic DCs produce larger amounts of IL-10 and this can cause the induction of Tregs that also produce IL-10, a subset of Tregs referred to as Tr1 cells[246]. pDCs can also increase their surface expression of ICOS-L and upon ligation increase their ability to produce large amounts of IL-10. Increased IL-10 production allows for increased Tr1 cells, similarly to tolerogenic DCs[247]. In a few studies DCs can inhibit memory T-cells[248-250] and this inhibition underscores the potential tolerogenic DCs to regulate autoreactive or alloimmune responses in vivo.
**TLR4 Monoclonal Antibodies**

For the manipulation and in depth study of TLR4 signaling, Bahrun et. al produced monoclonal antibodies against TLR4/MD-2 in TLR4-/- mice[251]. This resulted in 6 monoclonal antibodies, 3 of which we will focus on: UT12, UT15 and UT18. Each of these antibodies bind to the TLR4/MD-2 complex, however UT12 and UT18 (TLR4-Ab) caused downstream signaling as shown by NF-κB activation, cytokine production, and co-stimulatory molecule upregulation, while UT15 (Ctrl-Ab) showed no agonistic effects on the cell types treated. These antibodies have no contaminating LPS and they do no inhibit LPS binding to the TLR4/MD-2 complex [251]. The TLR4-Abs and Ctrl-Ab bind to different portions of the TLR4/MD-2 complex as shown using a competitive binding assay. Therefore signal transduction may depend on the location of binding or the affinity of binding to the TLR4/MD-2 complex. Further, in another study by this group, the TLR4-Ab was able to induce endotoxin tolerance[252]. This tolerance was sustained with decreased LPS mediated IL-6 and TNF-α after tolerization with the TLR4-Ab. This suggests that there is a decrease in immune responses towards TLR4 ligands (LPS) when TLR4-Ab is administered prior to the ligand. Also, TLR4-Ab protected from septic shock for up to 9 days, suggesting a stable effect on immune cells, while the Ctrl-Ab had no effect. Further this response was considered stable because LPS tolerogenic pre-treatment lasted at most 3 days, a relatively transient effect compared to the TLR4-Ab. What is currently unknown in these studies is what happens to the TLR4-Ab as well as TLR4 on the surface of cells after the antibody initially binds to TLR4/MD-2. It is possible that the TLR4-Ab needs to be present throughout tolerance induction. Multiple scenarios may be taking place during this time.
and this needs to be studied in depth. For instance, the TLR4-Ab could bind and releases relatively quickly causing low level stimulation and long term tolerance induction. Another possibility is that TLR4 is down-regulated upon TLR4-Ab stimulation, allowing for long term endotoxin tolerance[253]. Finally, the TLR4-Ab could bind and be endocytosed causing a differential downstream signaling effect which results in long term tolerance or upon being endocytosed causes destruction of TLR4/MD-2 reducing the recycling of TLR4/MD-2 to the surface effectively lowering the amount of TLR4 on the surface, which as previously mentioned induce long term endotoxin tolerance[253]. It may be possible that low doses of LPS given every few days or consecutively can cause tolerance[254, 255], however this is not as clinically efficacious as LPS can cause septic shock[256], but our antibody does not cause stimulation above tolerogenic levels (data not shown). In a different model of tolerization, TLR4-Ab can modify a model of allergic asthma[257]. In this allergic asthma model, endotoxin tolerance after treatment with TLR4-Ab, can effect antigen presentation through regulation of MHCII, CD80, CD86 and CD40. TLR4-Ab also inhibited the expansion of ovalbumin specific Th2 and Th17 cells. This correlated with significantly suppressed allergic airway inflammation when the TLR4-Ab was given before OVA-inhalation. The authors hypothesized that induction of endotoxin tolerance by activating TLR4 signaling pathways could directly cause decreases in inflammatory response and directly suppress T-cells through tolerogenic DCs. An interesting question that was not raised in this paper, is what if the TLR4-Ab was not given prophylactically, but given after disease initiation or onset? This will be tested in Chapter 2.
**TLR4 in T1D**

Editorial - *Immunotherapy*

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**Targeting innate immunity for treatment of Type 1 diabetes**

“The role of innate immunity in Type 1 diabetes may explain the rapidly rising incidence of Type 1 diabetes since the mid-20th century.”

Keywords: immune tolerization • innate immune therapy • reversal of Type 1 diabetes

• TLR4 • Type 1 diabetes

Type 1 diabetes (T1D) is an autoimmune disease perpetuated by intricate inter-actions between innate and adaptive immune responses, which collaborate to cause destruction of insulin producing islet $\beta$ cells. Antigen-specific CD8+ and CD4+

T cells ultimately destroy pancreatic islet $\beta$ cells; however, clinical trials directed at dominant T-cell epitopes in T1D (e.g., glutamic acid decarboxylase or insulin) have failed to reverse disease [1]. This does not necessarily mean that antigen-specific therapies will not work, but highlights the need to uncover new therapeutic approaches and/or combination therapies. Why have T-cell directed therapies failed? There may be fundamental innate immune defects that establish and maintain persistent autoreactive T-cell responses. These innate immune defects have been highlighted both in the mouse model of T1D, nonobese diabetic (NOD) mice, as well as in T1D patients reviewed elsewhere [2]. It is thus possible that correcting or ‘re-tuning’ the innate immune response will have beneficial effects in controlling autoreactive adaptive immune responses.
The role of innate immunity in T1D may explain the rapidly rising incidence of T1D since the mid-20th century [3,4]. The ‘hygiene hypothesis’ explains the rising incidence of T1D, as well as other autoimmune diseases, by positing that the lack of innate immune stimulation early in life primes the adaptive immune system toward autoimmunity. The NOD mouse model supports this hypothesis, since mice housed in colonies with a greater bacterial burden have lower incidence of T1D compared with mice housed in ‘clean’ cages [5]. This suggests that innate immune stimulation may be beneficial in the prevention of T1D. There is indeed a large literature on efficacy of therapeutic agents which stimulate or modulate the innate immune system to prevent T1D in NOD mice. However, although predictive biomarkers for T1D are being investigated, preventative therapeutics are currently inutile until an accurate marker is found. The unsolved clinical problem remains reversal of new onset disease. New onset T1D presents with elevated blood glucose levels, polyuria, failed glucose tolerance test and weight loss; these symptoms all arise after destruction of pancreatic islet β cells have already progressed. These clinical features are present in the NOD mouse model and our lab (and others) has changed our focus from T1D prevention to reversal, which is a much more difficult task. Some adaptive immune modulatory agents have reversed T1D and induced hypertrophy of islet β cells in the NOD mouse model [6], but with limited effect in humans (generally only moderate prolongation of C-peptide serum positivity [7]). Given increasing evidence of the role of innate immunity in T1D, we therefore investigated therapeutic effects of modulators of innate immunity in T1D. One innate immune molecule of particular interest is TLR4 due to its known ability to directly modulate innate immunity with subsequent activity on adaptive immune responses [8]. TLR4 is
one of twelve murine TLRs. TLRs recognize conserved structural components exclusively expressed on microbial pathogens and are an essential part of the innate immune response. TLR4 responds to lipopolysaccharide (LPS), a preserved component of Gram-negative bacterium cell walls. LPS binding to TLR4 causes downstream signaling mediated by MyD88 and Trif adaptor molecules; this leads to production of inflammatory modulating cytokines TNF-α, IL1-β, IL-10 and others [8,9].

“…modulating how the innate immune system affects the adaptive immune response with innate immune-directed therapies is a promising new approach to treating autoimmunity.”

TLR4 has long been considered a ‘proinflammatory’ molecule and as such potentially involved in the initiation of T1D. TLR4 is mainly expressed on islet β cells compared with islet α cells and signaling through TLR4 may selectively damage β cells during T1D progression and thus contribute to disease pathogenesis [10]. Furthermore, in a Streptozotocin-induced diabetes model, TLR4-/- mice presented with lower levels of inflammatory cytokines in the serum as well as reduced levels of signaling molecule activity including MyD88, Trif, NF-κB and others, suggesting that TLR4 contributed to the inflammatory state of T1D [11]. In addition to this mouse data, human data have suggested an overactivation of the innate immune system in the development of T1D. T1D patients have been reported to express increased ligands for TLR4 which could cause excessive signaling and a pro-inflammatory state [12]. Finally, patients at risk for the development of T1D have dysregulated TLR-induced IL-1β and IL-6 responses in comparison to healthy individuals [13].
On the other hand, there are data suggesting TLR4 may in fact be beneficial in prevention of T1D. Complete Freund’s adjuvant (CFA), which contains both TLR2 and TLR4 ligands, given at an early age (5 weeks) prevented T1D and resulted in decreased in vitro splenic lymphocyte proliferation [14]. However, the mechanism of this effect is still unclear. CFA may induce antigen presenting cells to increase Tregs, which in turn can control autoreactive T cells [15]. However, this does not prove that TLR4 is essential for disease prevention as CFA contains a mixture of TLR ligands. Therefore, to rule out the role of TLR2, ultra-pure LPS was administered to NOD mice multiple times (5–8 weeks) and this treatment regimen prevented T1D possibly by a downstream increase of Tregs [16]. Consequently, these results suggest that treatment with TLR4 ligands before disease development enhances establishment of tolerance to β cell autoantigens. Consistent with these data, in one study TLR4-/- on an NOD background accelerated T1D compared with TLR4-sufficient littermates [17]. However, all of these data were only in the context of prevention, but the true clinical question remained unanswered: can TLR4 agonists reverse new onset T1D?

Due to conflicting data in the literature showing that targets directed toward TLR4 have either pro- or anti-inflammatory properties depending on administration, timing and dose, Bahrun et al. prepared and characterized a TLR4 monoclonal antibody with extended signaling via TLR4 (weeks vs 8s for LPS) in order to test effects of TLR4 activation in a variety of diseases [18]. Our lab has recently found that this monoclonal agonistic antibody to TLR4 can reverse new onset T1D (polyuria, elevated blood glucose levels and weight loss) in a high percentage of NOD mice. Control nonagonistic TLR4 antibody showed no effect on disease progression; mice rapidly progressed to
end-stage T1D (blood glucose >500 mg/dl). NOD mice with successfully treated new onset T1D had improved islet pathological scores and increased insulin area compared with controls [Bednar KJ et al. Reversal of new-onset Type 1 diabetes with an agonistic TLR4/MD-2 antibody (2014), Submitted].

The mechanism by which TLR4 stimulation reverses new onset T1D is still unknown. Mechanistic insight may come from the TLR4 literature, which shows that 'endotoxin tolerance' may decrease TLR4-mediated inflammatory signals and enhance regulatory signals. Repeated low doses of LPS can induce tolerance rather than activation of immune cells. These endotoxin tolerized cells have a decreased inflammatory response to re-challenge, and produce less inflammatory cytokines (TNF-α and IFN-β) and more anti-inflammatory cytokines (IL-10) [19,20]. Therefore, reversal of new onset T1D may be mediated by TLR4-induced tolerance of the innate immune cells, which enhances the regulatory component of the adaptive immune response. We showed this by treating NOD.scid mice (which do not have T or B cells) with agonistic TLR4 antibody, and then transferring pre-diabetic NOD CD4+ and CD8+ T cell into the treated NOD.scid mice. Antibody-treated mice showed significant protection from the transfer of T1D compared with untreated controls. Furthermore, treatment with TLR4 antibodies increased numbers and percentages of Tregs [Bednar KJ et al. Reversal of new-onset Type 1 diabetes with an agonistic TLR4/MD-2 antibody (2014), Submitted].

TLR pathways may have enormous potential in the treatment of autoimmune diseases depending on the timing of administration and the type of response elicited from any given dose. Further there are already US FDA approved TLR4 agonists such as monophospholipid A, and this may allow for quick translation to human disease [21]. It
is possible that combination therapies are needed to treat the disease, for instance using an innate immune modulating therapeutic along with an agent targeting adaptive immunity, such as anti-CD3. Other agents directed to innate immune cells may also be effective; for example, α1-antitrypsin therapy has recently shown some clinical effect, acting to down-regulate the amount of TLR-induced IL-1β responses in recently diagnosed T1D patients [22]. Therefore, modulating how the innate immune system affects the adaptive immune response with innate immune-directed therapies is a promising new approach to treating autoimmunity.

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References

Adaptive Immune Cells in T1D.

The adaptive immune response, specifically Th1 T-cells are essential for the progression and establishment of overt diabetes. However, the balance of effector to regulatory cells is extremely important. The role of B-cells is not entirely clear, but they may play a significant role in disease pathogenesis and their auto-antibody production is used for the diagnosis of T1D. Central and peripheral tolerance or activation are key features to disease pathogenesis of T1D and influences the adaptive immune response. We will summarize the role of adaptive immune cells in the following subsections.

B-cells: B-cells have been identified in the beta-cell infiltrates in human biopsies as well as in NOD mice[258]. Although B-cells are dispensable for the initiation of disease and insulitis, however, NOD mice lacking B-cells had a decrease in overt diabetes development[259-261]. Depletion of B-cells using monoclonal antibody, anti-CD20 in NOD mice or rituximab (Rituxan/Mabthera; Genentech/Roche/Biogen Idec), induced partial protection from disease development and transiently improved β cell function in a subset of patients[34, 262]. These studies, however are confounded by the fact patients with X-linked agammaglobulinaemia can develop T1D[263]. A role for auto-antibodies in promoting T1D development is supported by evidence that preventing transmission of maternal antibodies to NOD offspring was protective against T1D[264, 265]. Auto-antibodies have also been shown, indirectly, to enhance the effector functions of DCs and NK cells, since FcRγ/- NOD mice are protected from disease. This suggested that auto-antibodies may augment β cell destruction through binding antigens and promoting FcRγ-mediated antigen uptake by APCs and/or promoting antibody-dependent cellular cytotoxicity of β cells[266]. However, these studies showed
that individual FcRγ may contribute to disease, but did not take into account that FcRγ can associated with other immune signaling molecules. For instance, could the effect of FcRγ/- be due to the ability of FcRγ to associate with dendritic cell immunoactivating receptor (DCAR) which can cause downstream signaling of ITAMs[267]. Therefore removing this association could decrease DCs ability to cause activation of T-cells. It has also been shown that FcRγ can association dectin-2, a pattern recognition receptor for fungi which can stimulate a Th2 response, known to be protective in T1D, therefore losing this association as well could decrease disease incidence[268]. Finally, a review of these receptors associated with FcRγ can be found elsewhere[269]. Therefore based on these findings, the conclusion that antibodies may contribute to disease pathogenesis indirectly using the FcRγ/- NOD mice may not illustrate the complex role and involvement of other immune receptors in the conclusions made by the authors. The secretion of auto-antibodies is not required for T1D development, since NOD transgenic mice expressing only membrane-bound B-cell receptors develop insulitis and overt T1D. The ability of B-cells to present antigen may also be an important feature for the development of T1D. However, the antigen-presenting role of B-cells must occur after disease initiation because B-cell deficient mice still develop insulitis[204]. It is possible that after initiation of disease, B-cells can present antigens to diabetogenic CD4⁺ and CD8⁺ T-cells in the insulitis lesions, resulting in the rapid expansion of T cells and subsequent destruction of islet β cells[270]. While B-cells can clearly contribute to disease pathogenesis and development in NOD mice and human patients, it is clear alternative pathways exist to progress to end-stage diabetes.
**CD8^+ T-cells:** CD8^+ T-cells recognize pathogen derived peptides presented by MHC class I molecules, once activated these cells proliferate and differentiate into cytotoxic T-cells. In the case of T1D, APCs present auto-antigens to CD8^+ T-cells in the PLN and once activated they migrate to the pancreas[126]. In the pancreas they respond to β cells presenting auto-antigens on the surface which induce cytotoxic functions of CD8^+ T-cells. CD8^+ T-cells are involved in the direct killing of islet β cells through attacking and releasing effector molecules, such as perforin or granzyme B, to β cells carrying auto-antigens on MHC I[126]. Deficiency of MHC class I is sufficient to arrest disease development and prevent β cell destruction[271]. IL-21 is required for efficient initial activation of autoreactive CD8^+ T cells, which in turn allows for killing of β cells[71, 272]. However, the verdict is not in on the role of IL-21 in disease initiation or stability. CD8^+ T cells recognize an insulin-derived epitope (B:15–23) in the islets of NOD mice as early as 3 weeks of age. However, this T cell clonal population declines as epitope spreading begins with emergence of CD8^+ T-cells with multiple specificities[273]. Furthermore, in a majority of T1D patients (9 out of 10 patients tested), there is a specific defect of CD8^+ T cell recognition of HLA-E/Hsp60sp, which was associated with failure of self/non-self discrimination[274]. Thus CD8^+ T-cells play a major role in the destruction of islet β cells and pathogenesis of T1D.

**Th1 T-cells:** Th1 T-cells provide help to both B-cells and CD8^+ T-cells. Th1 cells are responsible for cell-mediated immunity and phagocyte-dependent protective responses, which can also lead to islet β cell destruction and accelerate the course of T1D through cytokine production. Th1 skewing in NOD mice is partially responsible for disease pathogenesis, for instance, as previously described NOD macrophages produce large
amounts of IL-12, which skews the CD4⁺ T-cell helper cells towards a Th1 phenotype. This Th1 phenotype allows for the secretion of IFNγ and IL-2, which has downstream effects on the recruitment, proliferation and activation of B-cells and CD8⁺ T-cells. IFNγ will stimulate resting macrophages to produce toxic effector molecules that are involved in the destruction of islet β cells such as TNF-α, IL-1β, inducible nitric oxide synthase (iNOS) and free radicals[275, 276]. The lack of IFN-γ is known to delay T1D onset and transfer of diabetogenic T-cells from NOD mice into IFN-γ or IFN-γ receptor deficient NOD mice resulted in less efficient disease pathogenesis [277]. This decrease in disease pathogenesis is associated with the inability of T-cells to penetrate the pancreatic islets and influences homing to the pancreas. The role IFN-γ plays in disease development is under debate[278], however it is clear that Th1 cells are pathogenic in T1D. The role of IFN-γ in T1D can be further found in these reviews and is out of the scope of this dissertation [279, 280]. To further establish the role for CD4⁺ T-cells in the induction of T1D, presence of CD4⁺ T-cells in insulitis lesions has been demonstrated, thus suggesting their role in disease development and direct modulation of inflammatory activity[281-283]. Even though Th1 cells show a disease initiating and propagating phenotype, this is complicated by the fact that IFNγ can play a dual role in disease pathogenesis by both causing destruction of β cells via the signal transducer and activator of transcription-1(STAT-1) pathway and in protection of β cells through the interferon regulatory factor-1(IRF-1) pathway, which may stimulate β cell growth[284].

**Th2 T-cells:** Th2 cells mainly produce IL-4/IL-13 and IL-10, which are responsible for enhancing antibody production, eosinophil activation and inhibition of several macrophage functions[285, 286]. Changing the CD4⁺ T-cell helper populations from a
Th1 phenotype to a Th2 phenotype protects from disease development[287]. This has been demonstrated through approaches such as anti-CD28 stimulation, which promote and enhance the islet infiltrating Th2 cells and induced secretion of IL-4 preventing onset of disease[288]. Transgenic expression of IL-4 in dendritic cells of NOD mice skews the immune response towards a Th2 phenotype and subsequently prevents overt diabetes by their migration into the pancreatic islets. This IL-4 transgenic model shows protection from the development of overt diabetes, but still shows residual insulitis in the pancreas[289]. Incidence of T1D is decreased in NOD mice after regulated delivery of IL-4 to pancreatic β cells in vivo using an adenoassociated vector expressing IL-4 under the control of the mouse insulin promoter[289]. Another cytokine that is secreted by Th2 cells is IL-10, an immunoregulatory cytokine, which has multifunctional effects. Several lines of evidence suggested that IL-10 is important in establishing immune tolerance in NOD mice[290]. Th1 and Th2 T-cell subsets have been studied extensively. It is believed that Th1 cytokines lead to disease exacerbation, while Th2 cytokines protect from T1D.

**Th17 T-cells:** The role of Th17 T-cells in T1D is still under debate. These cells are distinguished from other T cell subsets through their production of IL-17. Th17 cells play important roles in other autoimmune disease such as multiple sclerosis and systemic lupus erythematosus[291, 292]. Specifically in T1D, children with new onset T1D have an increased production of IL-17 by peripheral blood T-cells[293]. This correlated with animal studies in which IL-17 expressing cells are found in the pancreas of NOD mice and that inhibition of IL-17 delayed the onset of T1D, but incidence of T1D remained normal[294]. Others have reported that Th17 might induce local inflammation
and thus hasten the destruction of β cells[277]. Therapeutic agents targeting the IL-17 molecule or direct inhibition of IL-17-producing cells regulates autoimmune diabetes, suggesting that IL-17 is involved in T1D[295]. However, there are very few studies to prove a mechanistic role for Th17 T-cells in disease development and progression. One study has shown that transfer of highly purified Th17 cells could cause diabetes in NOD.scid recipients in an equal amount of time as Th1 cells.[296]. However, the caveat to this experiment is that Th1 cells transferred disease, but disease could be prevented by neutralizing Th1 hallmark cytokine IFNγ, while Th17 transferred disease regardless of neutralizing its hallmark cytokine, IL-17. Purified Th17 in this transfer system of diabetes quickly changed to IFNγ producing cells after transfer. This was an effect of IL-12 and the switch was shown both in vivo and in vitro after exposure to IL-12. Therefore, it is still not entirely clear what role Th17 plays in the setting of T1D[297]. It is proposed that Th17 cells may enhance the ability of Th1 cells to cause disease[280].

**T-regulatory Cells (Tregs):** Treg are a subset of CD4⁺ T cells that were initially characterized in the mouse by the constitutive expression of the interleukin-2Rα chain (CD25)[298, 299]. Tregs were shown in further studies to express low levels of the IL-7Rα chain (CD127)[300, 301]. A more definitive marker of Tregs is the transcriptional factor forkhead box P3 (Foxp3), which plays a crucial role in Treg differentiation, function, and biology in both mice and humans[302]. Lack of functional Foxp3 is associated with the development of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), a severe autoimmune disease that ultimately leads to death[303]. Deficits in Treg number and function are frequently described for autoimmune and inflammatory diseases[304, 305]. Finally, the Treg subset of CD4⁺
cells has been further narrowed down to subsets. The major subsets studied include natural Tregs (nTregs) and inducible Tregs (iTregs). nTregs are thought to be derived from the thymus and are positively selected for on MHC class II, resulting in a high avidity selection. iTregs are converted peripheral CD4+ cells that arise from TCR stimulation in the presence of immunosuppressive cytokine, most notably TGF-β[306].

To distinguish these two subsets of Tregs, Helios and Nrp-1 are used; Helios and Nrp-1 expression indicates an nTreg while null expression of these indicates an iTreg or in some cases just Nrp-1 expression[307]. Both of these Treg populations can produce immunosuppressive cytokines and inhibit immune cell activation through a variety of pathways reviewed elsewhere[306, 308, 309]. Two major ways Tregs can control the function of effector T-cells are through contact dependent and independent mechanisms [308, 309]. Briefly, Tregs can mediate contact suppression through specific surface molecules such as CTLA-4. CTLA-4 binds co-stimulatory molecules CD80/CD86 on antigen presenting cells and causes down modulation of co-stimulatory molecules and activating cytokines while increasing anti-inflammatory cytokines such as IDO[310].

The Tregs affect on DCs can then cause inhibition of effector T-cell proliferation and activation; knocking out CTLA-4 results in uncontrolled polyclonal CD4+ T-cell expansion, and ultimately leads to death early on in life [46]. FasL-dependent cytotoxicity is also important for Tregs. FasL can impair the survival of Th1 cells which as previously described are imperative for the development of T1D[282]. Soluble Treg factors include IL-10, TGF-β, IL-35, IDO and soluble CD137 (sCD137), all of these soluble mediators have been indicated in inhibition of Th1/Th2 responses, can inhibit APCs activation or induce tolerogenic APCs, and cause induction and/or maintenance
of iTregs and nTregs[311, 312]. As mentioned Tregs play a major role in controlling immune response and autoimmune diseases, this holds true for T1D. BDC2.5 TCR transgenic mice contain only nTregs[313]. BDC2.5 is a specific T-cell clone that was generated in NOD mice[314] because their specific TCR showed antigen specificity toward islet cells, proliferated and released IL-2 when challenged with islet cells[315, 316]. nTregs in the NOD.BDC2.5 mice confer lower disease incidence due to the large number of antigen specific Tregs, however with the removal of these Tregs through deficiencies in Foxp3, allow for the development of T1D significantly earlier then NOD Foxp3 deficient counterpart[317]. In a similar fashion elimination of Tregs in NOD mice led to enhanced spontaneous development of T1D and reconstitution or transfer of Tregs could prevent the development of disease[318-320]. These data clearly suggest that Tregs play an important role for controlling disease pathogenesis and progression. Therefore, the balance of Tregs to effector T-cells in the PLN, pancreas and periphery modulates the speed and progression of T1D.

**Soluble CD137 and its Role in T1D**

As mentioned above, The NOD mouse model allows for the identification of genetic intervals that are associated with T1D, known as *Idd* loci. *Idd* congenic mice are bred to contain small intervals of B6/B10 resistant genes that affect disease outcome on a predominately NOD background. These congenic mice show decreased susceptibility to diabetes and thus confirm that the resistance genes were “captured” in the congenic interval[321]. Therefore, congenic mapping has led to the identification of candidate genes along the interval introduced into the congenic mouse. One of these intervals is a 1.2Mb interval known as *Idd9.3* and NOD.*Idd9.3* congenic mice have a 40% reduction
in incidence of T1D [85]. *Idd9.3* contains 15 genes in total with the candidate gene being *Tnfrs9* which encodes CD137. T cells expressing the NOD allele have decreased T-cell signaling in response to CD137 mediated stimulation and produce less IL-2 in comparison to their congenic B6/B10 counterparts (NOD.*Idd9.3* congenic mice). There are 3 coding variants different between NOD and B10 *Tnfrs9*, two nonsynonymous SNPs and an alanine insertion in the NOD[322]. These SNPs and substitutions could affect the expression of CD137. CD137 is an inducible member of the TNF receptor superfamily and is mainly expressed on activated T-cells. Its main function is to act as a co-stimulatory molecule and augment T-cell proliferation, function and survival in the absence of CD28 signaling[323-325]. It has been previously shown that anti-CD137 antibodies have differential effects on mouse models of homeostasis and disease. *In vivo* anti-CD137 antibody (3H3) has the ability to expand both CD4$^+$ and CD8$^+$ T-cells and increase their effector functions[326]. However, in murine models of disease such as collagen induced arthritis and lupus, anti-CD137 antibody blocked the development of disease and reversed established disease, respectively[327, 328]. These results demonstrate a possible immune regulatory mechanism of anti-CD137 antibody which has a direct effect on T-cells. This was further demonstrated by our own lab and others that a subset of Tregs constitutively express CD137 on the surface, which will be referred to from here on as CD137$^+$Tregs[329]. CD137 is one of the genes that is under the control of the Foxp3 promoter[330]. CD137 signaling promotes proliferation and survival of nTregs *in vitro* and with sufficient IL-2 signaling enhances Treg function [331]. It is not clear whether Tregs in NOD and humans with T1D differ in numbers or functionality, however it is known that Tregs can suppress disease onset. Our lab has
made some seminal discoveries in the CD137 field in T1D, we have previously demonstrated that treatment of NOD mice with anti-CD137 antibody prevented autoimmune diabetes but did not eliminate insulitis [332]. Pathogenic cells were not eliminated from the anti-CD137 treated non diabetic mice, since T cells could still transfer disease into NOD.scid mice. Anti-CD137 antibody treated splenocytes had significantly increased numbers of Tregs (CD4^+CD25^+) and transfer of these cells completely prevented diabetes in the recipient mice. However, the conventional T-cells (CD4^+CD25^-) in the anti-CD137 antibody treated group offered no significant protection. In contrast to this result, treatment of NOD.scid mice with anti-CD137 antibody after transfer of diabetic CD4^+CD8^+ T-cells did not prevent disease, but hastened its onset. This shows that CD137 expression on autoreactive memory cells can mediate destructive effects. “These results implicate a role for the Idd9.3 candidate gene product, CD137, in type 1 diabetes pathogenesis and suggest that the balance of CD137 stimulation between Tregs and conventional T-cells may be a potential target for therapy in humans”[332]. Anti-CD137 antibody is an activating antibody that caused an increase in CD137^+Tregs. It does appear to block the ability of T-cells to proliferate or interact with its conjugate ligand, however this has not been tested in depth. In a more recent study, our lab has shown that NOD.Idd9.3 congenic mice have an increased accumulation of CD137^+Tregs with age compared to NOD mice[315]. Further, CD137^+Tregs are functionally more suppressive in both contact dependent and independent mechanisms. One interesting aspect of CD137 biology is it can be produced in two isoforms: full length CD137 that is expressed on the cell surface and a soluble from which the transmembrane exon 8 is spliced out[333]. Soluble CD137
(sCD137) is increased in autoimmune diseases and is thought to be part of a negative feedback loop as sCD137 inhibits T-cell proliferation and thus could control over-activation of pathogenic cells in autoimmunity or specifically T1D[334-336]. We have shown that CD137⁺Tregs are a major cellular source of sCD137 and sCD137 can cause suppression through interacting with CD137L in a transwell assay. Further, older NOD.Idd9.3 congenic mice have significantly increased serum sCD137 compared with NOD mice. This increase in functionally superior CD137⁺Tregs and their ability to produce immunoregulatory sCD137 may be one reason for the reduced disease incidence in the NOD.Idd9.3 congenic mice[337]. We followed up on this data under the assumption that the addition of purified sCD137 in NOD mice may modulate pathogenic T-cells and affect disease; this is explored in Chapter 3.

**Paradox in anti-CD137 antibody treatment and sCD137**

The apparent paradox is: how can anti-CD137 antibody treatment prevent disease if it binds sCD137, but sCD137 is responsible for disease protection in both the congenic NOD.Idd9.3 mice and in NOD mice treated with recombinant purified sCD137? In anti-CD137 antibody treatment, the antibody binds to Tregs that constitutively express cell surface CD137. Binding to CD137 increases the number of CD137⁺Tregs which are more suppressive, secrete sCD137, and reduce activation of diabetogenic T-cells. This in turn prevents disease. However, there are a few unanswered questions in this model: Does the anti-CD137 antibody become internalized after binding CD137 on the surface of Tregs? Do sCD137 and anti-CD137 antibody form complexes and signal through activating and/or inhibitory Fc receptors? Finally, does anti-CD137 antibody bind to sCD137 and signal via CD137L? These questions need to be answered in
subsequent experiments. In the sCD137 model presented in Chapter 3, sCD137 binds to CD137L expressing cells, including diabetogenic T-cells, causing down regulation of their activation and preventing T1D. In summary, anti-CD137 antibody binds CD137^+Tregs, increases their number and protects from T1D, while sCD137 binds to CD137L expressing diabetogenic T-cells, decreases their activation and prevents T1D.

**Current Therapies for Type 1 Diabetes**

In short, there are no current therapies that reverse T1D in patients. The current approach to T1D involves managing the disease after all or most of the beta cells have been destroyed and the patients are totally dependent on external insulin administration to achieve acceptable glycometabolic control. Some individuals still may experience episodes of hypoglycemia due to poor monitoring of glucose levels(ADA). It is undeniable that the quality of life for individuals using glucose sensing technology is improved, but the effect this technology has on diabetic complications is not completely clear[338]. Another viable option that has been proposed is islet transplantation, however this has had very limited success because of the shortage of donors and the fact that individual must remain on chronic immune suppression[339]. Even being under immunosuppression, the islet cells eventually fail and do not cause normaglycemia. Therefore, as proposed in this dissertation alternative therapies for T1D are necessary. Although prevention of T1D in NOD mice has been relatively easy to achieve, this has not translated into clinical trials in humans. This is partially due to the fact that no biomarker is able to predict the development of T1D. Biomarkers and the ability to predict disease is an area of intense research and has been reviewed elsewhere[130], but until a marker is found the true clinical question is in reversal of
disease. There have been examples in the NOD mouse model where hyperglycemia (usually defined as a blood glucose reading between 200 and 300mg/dL) was established and the disease was reversed either transiently or with repeated administration of the therapeutic. However, these therapies have failed to successfully translate to human patients due to a variety of factors such as the inability to test specific auto-antigens in the NOD model, the heterogeneity of the human disease (onset and progression is relatively uniform in the NOD model and between colonies), the choice of dosing, and the time of intervention all of which need to be explored further[340]. Many investigators have realized that there is a narrow therapeutic window before islets can no longer be rescued. The major areas of therapy will be briefly discussed below.

**Antigen Specific Therapy:** Antigen specific approaches are of potential interest in all autoimmune diseases because they only target the pathogenic T-cells and spare protective immunity to fight off infections and cancers. This approach usually involves the induction of tolerance to a specific epitope(s) known to be involved in disease progression (in the case of T1D e.g. insulin). However, as described in the chapters above, tolerizing to one antigen may not be able to stop disease progression due to epitope spreading[341]. Further, the auto-antigens in mice and in humans are differentially expressed and thus have slowed progression to preclinical trials for antigen specific therapies. In accordance with this Culina et. al describes multiple trials with antigen specific therapy and all have essentially failed to achieve stable reversion of disease[342]. Instead these types of clinical trials may be more suited for people that are at a high risk for T1D development.
Anti-Inflammatory: The inflammatory environment of T1D (e.g., increased IL-12, IFN-γ, TNF-α) has created opportunities for anti-inflammatory therapies. In NOD mice, anti-inflammatory drugs have had some success in reversal of disease through decreasing insulitis, enhancing β cell regeneration, and improving insulin sensitivity[343]. However, these results are transient and disease recurs. Targeting inflammatory cytokines in human trials has failed, e.g., using anti-TNF-α (Entanercept), Rapamycin, and IL-2, and targeting of IL-1β (Canakinumab and Anakinra)[344]. These anti-inflammatory drugs may also lead to increased risk of infection. Anti-inflammatory drugs in humans have seen very little success and thus may need to be included in a “combination” therapy. Targeting one specific cytokine will likely not cause disease reversal. A full review of these anti-inflammatory therapies in T1D can be found elsewhere[345].

Lymphocyte Targeting: Since T-cells are the main mediators of disease in the NOD animal model, different therapeutic approaches to T cells have been tried: targeting T-helper cells (GK1.5 antibody), depletion of T-cells using anti-lymphocyte serum (ALS) or anti-mouse thymocyte globulin (ATG) and tolerization / depletion using anti-CD3[344]. Targeting of B-cells using anti-CD20 (Rituximab had modest beneficial effects [34]. In human clinical trials there has been some success with targeting lymphocytes. Specifically, anti-CD3 (anti-CD3, hOKT3gamma1 (Ala-Ala), Teplizumab) reduced insulin requirements and increased C-peptide levels in some T1D patients. These individuals also had lower frequency of Th1 cells and IFN-γ producing CD8⁺ cells[346]. Rituximab also improved β cell function, increased C-peptide levels, lowered HbA1c, and reduced insulin requirements[262]. Finally, CTLA4-Ig (Abatacept) had only mild effects on C-peptide levels and did not result in insulin independence[347]. While these targeted
treatments against T-cells and B-cells have shown some efficacy, very few patients became insulin independent. Furthermore, the depletion of T- and B-cells is not antigen specific and has potential side effects of infections and cancer. It has also been proposed that “combination” therapies that target multiple pathways in disease pathogenesis need to be targeted to treat T1D, this type of therapy has shown success in other autoimmune diseases[348].

**Cellular Therapies:** Currently there are three main cell types that are being considered for the reversal of T1D: islet transplants, Tregs and stem cells. Islet transplant are needed to restore insulin production in the individual. It has been well established that not dampening or turning off the immune response to islet cell antigens leads to the destruction of the transplant and due to this complication, this therapy has limited potential[349]. Therefore, utilizing islet transplants with other therapies may prove beneficial in the future. As previously described Tregs have the ability to dampen the immune response, tolerize immune cells, and cause antigen specific suppression. However, due to the plasticity of T-cells there is a concern regarding the stability of Tregs in an inflammatory environment[349]. Stem cells have shown promise as of late in multiple studies and clinical trials. NOD mice treated with BALB/c stem cells showed reversal of disease in 88% of hyperglycemic mice, similarly treatment with non-obese diabetes resistant (NOR) stem cells also had a pronounced effect on hyperglycemia which showed long-term reversal of T1D[350, 351]. This opened up the field for stem cell treatments for the cure of T1D. However, safety concerns do exist with this type of therapy because of the potential oncogenic transformation of stem cells[352]. Clinical trials have been done using autologous hematopoietic stem cell therapy for the
treatment of T1D. Most trials to date have had success in the treatment showing a high percentage of individuals that are no longer dependent on insulin. The success does not come without major concerns, these trials place patients on immunosuppressive drugs (Cyclophosphamide and ATG) during the duration of the study[344]. Furthermore, these studies have not been followed out long enough to address the concern of oncogenic factors. While these trails have shown success in reducing insulin dependency further follow up and trials need to be done.

**Conclusion:** Clinical trials in humans have shown some promise of disease reversal using different types of therapies as well as combination therapies, but to date there still is no cure for T1D. The real issue is: how to curtail pathogenic T-cells without eliminating protective immunity? The ability to manipulate the adaptive immune system using innate immune stimulation or molecules that target adaptive activated cells specifically, rather than total immunosuppression, may be important to control T1D.

**Summary**

Type 1 diabetes is an autoimmune disease that results in the destruction of insulin producing islet β cells in the pancreas. Clinically, patients do not present with disease symptoms until destruction of islet β cells has already begun; making intervention of disease pathogenesis difficult. However, due to the rapidly rising disease incidence, screening children whom are at a high risk for the development of T1D has become more common and earlier diagnosis may be possible. To date there is no specific biomarker that can be detected in pre-diabetic mice or humans, but earlier detection techniques are being developed which may help to clinically intervene by saving the
remaining insulin producing islet β cells. The NOD mouse model is a standard tool in developing novel biomarkers for the detection of disease and is a pivotal model for T1D research. Prevention of disease in NOD mice has failed to show significant translatable potential in humans with T1D. However, disease reversal of T1D in NOD mice has shown some clinical efficacy in humans (eg anti-CD3). In order to design improved therapeutics for the reversal of disease, NOD mice have been essential and are an excellent model for human T1D. Further, T1D is a complex multi-factorial disease that involves both genetic components and environmental triggers.

Genetic studies have revealed specific genes (such as HLA, IL-2RA, INS, CTLA4, PTPN22, and IFIH1) linked to the disease pathogenesis in T1D. Additionally, genetic innate immune defects are seen in both patients with T1D and NOD mice. These underlying innate immune defects cause persistent auto-antigen exposure and exacerbation of disease. Being able to correct these defects and/or “re-tune” the immune system away from autoimmunity is an interest of our lab (Chapter 2). Genetically linked components of the adaptive immune system have also been linked to T1D disease pathogenesis. Some genetic components (eg IL-2RA, CD137) are linked to Treg dysfunction and Tregs are extremely important in disease regulation (Chapter 3). The genetic components of T1D have been well established, and it is becoming increasingly evident that environmental triggers are important for disease onset.

Different environmental triggers have the ability to skew the immune system towards or away from autoimmunity in NOD mice. In support of the need for an environmental trigger, the dramatic increase in T1D prevalence cannot simply be explained by genetics alone because the variation between generations would not account for this
abrupt increase in disease incidence. Exposure to pathogens early on in life relates to the ‘hygiene hypothesis’ which “predicts that increased hygienic living conditions, the use of antibiotics and sterile food preparation will result in the continued segregation of the immune system from positive microbial exposure, thus favoring an increased susceptibility to immune-mediated disorders”[76]. These microbial pathogens could then alter the balance of Tregs to effector T-cells. T1D occurs as the result of dysregulation of auto-reactive T-cells and regulatory cells which develop over time. This disequilibrium eventually leads to a decrease in β cell-mass, and during disease pathogenesis, the amount of functional insulin producing β cells fluctuates over time (Figure 5). This fluctuation may account for the difference in time until clinical disease onset in some patients. T1D is usually classified as having three phases of disease development: initiation, expansion and destruction. While much of the focus resides on T-cells and their ability to cause destruction of islet β cells, innate immune cells contribute heavily to disease onset and maintenance. Innate immune cells, specifically APCs, process apoptotic islet cells to be presented as antigens to T-cell and in the PLN. This presentation allows APCs to expand auto-reactive T-cells which then migrate back into the pancreas where they will have effector functions and destroy islet β cells. In the destruction phase, APCs are necessary to provide stimulus to T-cell allowing them to continually be activated and destroy islet β cells. Underlying defects in the innate immune system allows for over-stimulation of the adaptive immune system. Therefore providing a mechanism of tolerance may reverse the disease by lowering the number of activated cells, causing increased regulation and preservation of islets.
One innate immune signal molecule, TLR4 (Figure 6), has the ability to create a tolerogenic environment. Endotoxin tolerized cells show reduced production of inflammatory cytokines and increased anti-inflammatory cytokines. It was thus our rationale that treating with a TLR4 antibody causing long term induction of tolerance will be beneficial in the treatment of an otherwise over-activated immune system (Chapter 2). The adaptive immune system also plays a major role in disease progression and destruction. NODlidd9.3 mice have decreased disease incidence compared to NOD mice and the candidate gene within this Idd region is CD137. These congenic mice (NODlidd9.3) mice have increased CD137+Tregs, which are functionally superior Tregs and secrete an immunosuppressive molecule known as sCD137. Also, NODlidd9.3 mice have elevated levels of sCD137 which may cause decreased disease incidence. Our lab then went on to show that correcting this decrease in sCD137 was able to prevent disease in NOD mice (Chapter 3). Prevention of disease is thought to be through reducing T-cell proliferation as sCD137 down regulates CD4+ T-cells in an APC independent manner, CD137L dependent manner. The addition of sCD137 can therefore counteract the low levels of this immunomodulatory molecule seen in patients and NOD mice (Chapter 3).

Therapies that are commonly studied today involve antigen specific therapies, anti-inflammatory drugs, lymphocyte depletion or targeting, and cellular therapies (eg Treg, islet, and bone marrow transplantation). While some of these therapies have shown promise in disease reversal, none are currently available. Therefore, improved therapeutics approaches and understanding of disease reversal are needed. Our lab has designed two therapies to further our understanding of disease pathogenesis and
treatment strategies in T1D and will be explored in the subsequent chapters, TLR4-Ab (Chapter 2) and sCD137 treatment (Chapter 3).


Chapter 2: TLR4 monoclonal antibody reverses new onset T1D and mediates APC tolerance
Reversal of New Onset Type 1 Diabetes by Engaging TLR4/MD-2 with an Agonistic Antibody

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Running title: TLR4 Agonistic Antibody Reverses New Onset T1D

Reversal of New onset Type 1 Diabetes with an anti-TLR4 Antibody

Abstract

Type one diabetes (T1D) is currently an incurable disease, characterized by a silent prodrome followed, after the onset of clinical symptoms, by rapid, progressive autoimmune destruction of insulin-producing pancreatic β cells. Autoreactive T cells play a major role in the destruction of islet β cells, but it has recently become clear that innate immune cell cytokines and co-stimulatory molecules critically affect T cell functional status. Herein we show that an agonistic monoclonal antibody to TLR4/MD-2 (TLR4-Ab) reverses new onset diabetes in a high percentage of newly diabetic non-obese diabetic (NOD) mice. TLR4-Ab induces antigen presenting cell (APC) tolerance in vitro and in vivo resulting in decreased inflammatory cytokines, increased Interleukin-10, and decreased expression of co-stimulatory molecules. Downstream of these initial APC effects, T effector cells show decreased proliferation, while T regulatory cells were increased in both the periphery and in the pancreatic islet. The critical role of APC tolerization in vivo was demonstrated by direct targeting of only innate immune cells with the TLR4-Ab, which was sufficient to prevent T cell mediated disease. Specific stimulation of the innate immune system through TLR4 using an anti-TLR4 anitbody can therefore restore tolerance in the aberrant adaptive immune system and reverse new onset T1D, suggesting a novel immunological approach to treatment of T1D in humans.
Type one diabetes (T1D) is a complex disease caused by genetic and environmental factors that result in an autoimmune response to pancreatic β cells [1-5]. Innate and adaptive immune responses interact to produce soluble or contact dependent factors that cause toxicity and death of islet β cells [5-9]. The incidence of T1D has risen rapidly worldwide since the mid-20th century, increasing by 3-4% per annum in Europe and the United States [10-14]. One explanation for the rising incidence of T1D and autoimmunity in general is the “hygiene hypothesis”, which suggests that insufficient microbiological stimulation of the innate immune system skews the adaptive immune system towards autoimmunity [15, 16].

In support of this hypothesis, both NOD mice and human T1D patients demonstrate genetically controlled abnormalities in innate immunity. Macrophages have been shown to have developmental defects, the ability to promote CD8+ cytotoxic T lymphocyte differentiation, increased IL-12 production, and enhanced production of TNF-α, IL-1β and ROS in the pancreatic islets[17-19]. Similarly, dendritic cells have increased production of IL-12 and upregulated expression of co-stimulatory molecules which promotes T-cell activation[20, 21] Dysregulated innate immune signaling critically affects antigen presenting cell (APC) cytokine production and co-stimulatory capacity, producing downstream alterations in T cell immunity, and thus altering the balance between regulatory and effector adaptive immune mechanisms [22-27]. In the setting of overactive innate immune responses, any therapy that could “re-tune” the innate immune responses would have beneficial downstream effects on adaptive immunity and thus potentially restore tolerance.
Toll-Like receptors (TLRs) are innate pattern recognition receptors mediating host defense against pathogens [28]. In order for TLR4 to signal it must form a complex with MD-2 to drive downstream responses to lipopolysaccharide (LPS), an integral component of gram negative bacteria [29]. TLR4/MD-2 signaling is mediated through two main adaptor proteins, MyD88 and Trif, that direct production of inflammatory modulators such as TNF-α, IFN-β and IL-10 [28]. TLR4 has long been considered a “pro-inflammatory” molecule and abnormalities of TLR4 signaling have been shown in both mouse and human T1D [30-32]. Patients at risk for the development of T1D also have dysregulated TLR induced IL-1β and IL-6 responses compared to healthy individuals [33]. However, genetic studies on the effect of TLR4 knockout on T1D development have been inconsistent, with studies showing increased, decreased and no effect on T1D incidence [34-36]. Studies in liver immunology demonstrated that TLR4 expressing APCs can undergo “endotoxin tolerance” with repeated TLR4 stimulation [37, 38]. Endotoxin-tolerized immune cells have an altered immune response that has profound downstream effects on adaptive immunity [39]. We hypothesized that the TLR4 abnormalities in T1D could be corrected by therapeutic targeting of TLR4-expressing APCs, and that this could alter the course of established T1D. To achieve APC tolerance we used an agonistic antibody to mouse TLR4/MD-2 (UT18, hereafter referred to as “TLR4-Ab”) as well as a control non-agonistic antibody to TLR4/MD-2 (UT15, hereafter referred to as “Ctrl-Ab”) [40-42]. TLR4-Ab was enormously successful in the setting of established, clinical T1D, permanently reversing disease in over 70% of mice.

Research Design and Methods:
**Mice and Reagents**

NOD, NOD.BDC25 and NOD.scid mice were maintained as previously described[43]. Antibodies for flow cytometry were purchased from BD Bioscience and BioLegend. Anti-CD3/CD28 beads and recombinant mouse IL-2 were purchased from Invitrogen (California). CD4 immunomagnetic beads were purchased from Miltenyi biotech. Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen and the staining protocol provided by Invitrogen was used. The production and characterization of TLR4/MD-2 (Ctrl-Ab/TLR4-Ab) monoclonal antibodies was as previously described[40].

**Flow Cytometry**

Flow cytometry staining and analysis was preformed as previously described in our lab[44].

**Diabetes Incidence**

Diabetes incidence and blood glucose measurements were performed as previously described in our lab[43, 44].

**Prevention of T1D**

Female NOD mice 4-6 weeks old were treated once a week for three weeks with an intraperitoneal (IP) injection of either Ctrl-Ab (5μg) or TLR4-Ab (5μg), and then monitored for diabetes incidence along with an untreated control group. Diabetes incidence was measured as described above. At endstage diabetes (BG>500mg/dL) or
after 200 days disease free mice were euthanized and the pancreas removed for histology.

**Histological Scoring and Immunohistochemistry**

Histological scoring and immunohistochemistry was done as previously described by our lab for hematoxylin and eosin, glucagon, and insulin[43]. For the Foxp3 staining, the method of staining is as described above and individual Foxp3\textsuperscript{+} cells were counted per section.

**Macrophage tolerization in vitro**

Thioglycolate was injected IP into NOD mice. Macrophages were harvested three days after thioglycolate was injected and were cultured as follows: 3 groups were treated with media alone for 4 hours, washed and then treated with LPS, TLR4-Ab, or Ctrl-Ab. 3 other groups were used in this experiment: 1) Macrophages were treated with TLR4-Ab for 4 hours, washed and then either “re-treated” with LPS (“TLR4+LPS”) or 2) “re-treated” with TLR4-Ab (“2X TLR4-Ab”). 3) Macrophages were treated with Ctrl-Ab for 4 hours, washed then “re-treated” with Ctrl-Ab (“2X Ctrl-Ab”). After 24 hours after the final stimulation, the supernatant was removed from the culture and TNF-\(\alpha\) or IL-10 were assessed by ELISA and IFN-\(\beta\) was assessed by an interferon stimulated response element reporter cell line (invivogen).

**in vivo kinetic and functional studies of TLR4-Ab treatment**

NOD mice (4-8 weeks) were treated twice one week apart with an IP injection of PBS, Ctrl-Ab (5μg), or TLR4-Ab (5μg). 24 hours after the second injection total splenocytes
were used to assess CD11b, CD11c, CD4, CD8, CD86, CD80, CD40, and Foxp3 positive populations. Total cell numbers and frequency were each assessed. Serum was assessed for IL-10 by ELISA as above. For kinetic analysis of cell populations after TLR4-Ab treatment NOD mice (4-8 weeks old) were treated once with an IP injection of PBS, Ctrl-AB (5μg), or TLR4-AB (5μg). At various time points (24hrs to 28 days) mice were sacrificed and whole splenocytes were assessed by flow cytometry for the populations mentioned above.

**CFSE and BDC2.5 Proliferation Assays**

CD4⁺ CSFE proliferation assay was done as previously described[43] However, the TLR4-Ab (5μg) or Ctrl-Ab (5μg) was added on day 0 of the culture. CD11c⁺ cells were sorted with immunomagnetic beads and placed in culture along with a tolerogenic dose of TLR4-Ab (100ng) or Ctrl-Ab (100ng) for 1 hour prior to the addition of either BDC2.5 mimic peptide (peptide) or anti-CD3 antibody (CD3-Ab). After one hour CD11c⁺ cells were washed and co-cultured with BDC2.5 mimic peptide (1mM) or CD3-Ab (1μg) for an hour. CD4⁺ T cells were stained and purified as described above. Four hours after the initial dose of TLR4-Ab CD11c⁺ cells were re-stimulated with TLR4-Ab (1μg) or Ctrl-Ab (1μg). The co-cultured cells remained in culture for 3 days and CD4⁺ T cells CFSE dilution was measured by FACs at the end time point.

**Reversal of T1D**

Female NOD mice were randomly assigned to either Ctrl-Ab or TLR4-Ab groups before diabetes onset. Mice were assessed for diabetes as described above. After onset of polyuria and when blood glucose exceeded 200mg/dL, mice were treated twice, one
week apart with either Ctrl-Ab (5μg) or TLR4-Ab (5μg) IP. After the second treatment, BG was monitored and if the BG rose over 200mg/dL mice were retreated with Ctrl-Ab (5μg) or TLR4-Ab (5μg). At endstage diabetes (BG>500μg/dL) mice were euthanized and pancreas removed for histology. A sub-group of Ctrl-Ab and TLR4-Ab treated mice were weighed prior to and after onset of diabetes. A control group of NOD mice were left untreated and used to measure normal disease incidence after BG 200mg/dL was achieved.

**in vivo T1D transfer studies**

NOD.scid mice (4-6 weeks) were treated once a week for three weeks with an IP injection of either TLR4-Ab (5μg, N=13), or untreated (N=10). Two hours after the final treatment pre-diabetic NOD CD4+ and CD8+ T cells were transferred to all groups. Diabetes was assessed for up to 75 days post transfer; mice were sacrificed at 75 days or at endstage diabetes (BG>500mg/dL). Islet pathology was assessed by histology as above and splenic cell populations were assessed using flow cytometry as indicated in the figure.

**Data analysis**

All statistical analysis was performed using GraphPad Prism 5 and the tests indicated in the figure legend.
Results:

Prevention of T1D by engaging TLR4

To test the *in vivo* efficacy of TLR4-Ab, we first assessed whether antibody treatment could prevent the onset of T1D in NOD mice. To this end, we treated young (4-6 weeks old), pre-diabetic female NOD mice with TLR4-Ab or Ctrl-Ab once a week for three weeks, and then monitored for the development of T1D up to 200 days. TLR4-Ab significantly protected NOD mice from T1D, while Ctrl-Ab and untreated (UnTx) mice were statistically indistinguishable and had normal T1D incidence (Fig 1a). Normal incidence of disease in our colony is ~70% of female NOD mice become diabetic at an average of ~170 days. TLR4-Ab treated mice had decreased overall insulitis as well as significantly fewer islets in advanced stages of insulitis (Fig 1b,c).

Reversal of T1D using an agonistic TLR4/MD-2 monoclonal antibody

Due to the current inability to accurately predict T1D[45], although we have the ability to identify high-risk individuals if screened properly, most patients present clinically with elevated blood glucose levels, polyuria and weight loss, and a clinical cure would require reversal of the autoimmune component of the established disease (rather than prevention as shown in Fig 1). We therefore assessed whether TLR4-Ab could reverse new onset T1D in NOD mice. We randomly assigned female NOD mice to Ctrl-Ab, TLR4-Ab, or untreated groups, and then aged them until they developed polyuria (excessive expenditure of urine) the earliest clinical sign of T1D, at this time point in our colony the average BG was 208.3±16.1 mg/dL, (Table 1). Further, after polyuria was established T1D-induced weight loss had already begun (Fig 2e). Mice were then
treated twice, one week apart, with either Ctrl-Ab or TLR4-Ab, or left untreated. Ctrl-Ab treated and untreated mice rapidly (17.6 ± 2.9 days and 16.7±3.2, respectively) progressed to endstage T1D (BG >500, Fig 2a, b, c). In striking contrast, TLR4-Ab reversed new onset T1D in a majority of mice (15 of 21 mice) (Fig 2a, d). Detailed examination of the BG levels over time showed that a majority of TLR4-Ab treated mice (19 of 21 mice) had a clinical response to TLR4-Ab (Fig 2a, d and Table 1) as defined by either permanent reversal of T1D (n=15) or a significant delay to endstage T1D (n=4). In contrast, both the Ctrl-Ab and untreated groups showed no clinical response, and the BG rapidly rose past 500 mg/dl (Fig 2b, c, Table 1). We observed that TLR4-Ab treated mice fell into one of two groups: mice that received only the initial two treatments with TLR4-Ab (11 of 21 mice in the TLR4-Ab group) and mice that had to be “retreated” (the red time point indicates first date of retreatment (10 of 21 mice from the TLR4-Ab treated group)). Mice were retreated if the blood glucose exceeded 200mg/dL after the initial two treatments. Remarkably 9 of 11 mice receiving only the initial treatments had permanent reversal of T1D (Fig 2, d1). In the “re-treated” group, 6 of 10 mice had persistent reversal of T1D, while 4 of 10 eventually progressed to endstage T1D (Fig. 2, d2) TLR4-Ab retreatment successfully reversed recurrent T1D in mice with BG as high as 463 mg/dL (Fig 2, d2). Thus 71% of TLR4-Ab treated mice had permanent reversal of T1D and 90% had a clinical response, compared to Ctrl-Ab treated mice that had no disease reversal nor clinical response (Fig 2a-d). New onset T1D reversal with TLR4-Ab was further associated with significantly decreased BG (from 205.1 to 160.8, Table 1) with corresponding recovery from weight loss (Fig 2e). The decline in blood glucose level for an extended period of time (87.7 ± 8.8 days)
without giving exogenous insulin suggests stable and long lasting control of the disease. Moreover, islets showed a marked reduction in cellular infiltration (insulitis) in the mice treated with TLR4-Ab compared to Ctrl-Ab (Fig 2f).

**TLR4-Ab decreases the percentage of severely inflamed islets and preserves insulin production in diabetic mice**

To further understand the effects of TLR4-Ab in reversal of new onset diabetes, we examined islet histology at several time points in the disease course: in prediabetic mice (“DM(-)”), untreated endstage diabetic mice (BG > 500 mg/dl, “(DM(+))”, untreated new onset diabetic mice at the initial treatment point (BG ~200 mg/dl, “BG200”), and Ctrl-Ab vs. TLR4-Ab -treated new onset diabetic mice. Islet severity scores in Ctrl-Ab treated mice were no different than diabetic mice (Fig 3a). Both the BG200 and TLR4-Ab groups had more islet infiltration than non-diabetic mice (Fig 3a). Of great interest, however, TLR4-Ab treated mice showed decreased numbers of severely infiltrated (stage 3 and 4) islets, and an increase in minimally affected islets (stages 0-2) compared to BG200 mice (Fig 3a). The reduction in the number of pancreatic islets experiencing cellular infiltration between the TLR4-Ab and BG200 suggests that effective reversal of new onset diabetes by the TLR4-Ab was associated with the ability to reduce penetrating insulitis.

Since ending BG was significantly reduced in TLR4-Ab treated mice, we assessed insulin and glucagon staining within the islets using immunohistochemistry (representative images shown in Fig 3b). The TLR4-Ab treated group had total insulin staining comparable to the non-diabetic control and slightly elevated compared to
BG200 (Fig 3c). The diabetic and Ctrl-Ab groups had minimal insulin-staining islet cells remaining, as expected (Fig 2c). Since the total insulin level was slightly increased in the TLR4-Ab treated group compared to BG200, we assessed the total number of insulin positive islets. The non-diabetic group, as expected, had more islets than any other group (Fig 2d). The BG200 and TLR4-Ab had similar numbers of insulin positive islets, indicating preservation of insulin positive islets by TLR4-Ab treatment. The amount of insulin per islet, however, was significantly increased in the TLR4-Ab treated group; raising the possibility of islet hyperplasia or proliferation as previously reported in the setting disease reversal (Fig 3e) [46]. Overall, the increased insulin positive area of the TLR4-Ab-treated islets compared to untreated BG200 mice is consistent with the decreased numbers of severely infiltrated islets (Fig 3a), and provides further evidence of a therapeutic effect of TLR4-Ab by preserving pancreatic islet β cell insulin production.

**TLR4-Ab targets innate immune antigen presenting cells and induces antigen presenting cell tolerance**

To determine the cellular targets of TLR4-Ab acted we measured the kinetic and dynamic changes in cellular subsets after treatment with TLR4-Ab. TLR4-Ab treatment initially resulted in significant increases of CD11b+ cells at 24 hours, with an increase in CD11b+CD11c- and CD11b+CD11c+ cell subsets (Fig 4a). There was a small increase in CD19+ B cells, but it wasn’t statistically significant (Fig 4a). It has been suggested that TLR4 may act directly on CD4+ T-cells [47, 48], however we showed that direct treatment of CD4+ cells with TLR4-Ab caused neither proliferation (Fig 4c) nor upregulation of CD69 expression (data not shown). Despite this, at 14 days after
TLR4-Ab treatment there was a significant increase Foxp3+ Tregs (data not shown), which persisted at day 28 post-TLR4-Ab treatment (Fig 4b). The early kinetic study showing an increase in CD11b+ subsets suggested that the initial cellular target of TLR4-Ab is the innate immune system, while the increase in Foxp3+ cells suggested a downstream effect on adaptive immunity. This is consistent with the known regulation of adaptive cells by innate immunity [22].

We therefore tested the effect of TLR4-Ab on antigen presenting cell (APC) function in vivo by treating NOD mice with a “tolerogenic” (two treatments a week apart, identical to our reversal regimen in Fig 3)) course of TLR4-Ab. Tolerance induction is known to be associated with decreased co-stimulatory molecules on the surface of APCs as well as increased anti-inflammatory cytokines. TLR4-Ab treatment caused significant downregulation of CD11c+ CD80, CD86 and CD40 expression compared to Ctrl-Ab (Fig 4d). Under the same treatment conditions, TLR4-Ab treated mice had a significant increase in serum IL-10 production during the first 24 hrs after the final treatment (Fig 4d). To further assess effects of TLR4-Ab on APC function, thioglycolate-elicited macrophages were tested for cytokine production after a single treatment with TLR4 ligands (activating treatment) versus two treatments 4 hours apart( tolerizing treatment). LPS is the classic TLR4 ligand that induces TLR4 mediated inflammatory responses. We tested the amount of pro-inflammatory (TNF-α and IFN-β) and anti-inflammatory cytokines (IL-10) produced by macrophages given these two different treatment strategies. Macrophages were cultured with a single activating dose of LPS, TLR4-Ab, or Ctrl-Ab to assess the production of inflammatory or anti-inflammatory cytokines (Fig 4e). To assess the ability of TLR4-Ab to induce macrophage tolerance, we pretreated
macrophages for four hours with TLR4-Ab followed by stimulation with LPS (“TLR4/LPS”), or TLR4-Ab (2x TLR4-Ab). Pre-treatment with TLR4-Ab resulted in decreased pro-inflammatory cytokines and increased anti-inflammatory IL-10 in both the LPS and TLR4-Ab groups, indicating macrophage tolerization (Fig 4e). Treatment or pretreatment with Ctrl-Ab had no affect on cytokine production (Fig 4e). Further, the Ctrl-Ab did not block the ability of LPS or TLR4-Ab to signal (data not shown). Collectively, these results show that two treatments with TLR4-Ab (as we used to reverse T1D) results in decreased co-stimulatory molecule expression, decreased pro-inflammatory cytokine production, and increased anti-inflammatory cytokines, thus indicating induction of APC tolerance *in vivo* and *in vitro*. Further two treatment is an important result as a single treatment did not protect from T1D and had a marginal increase in inflammatory cytokines, however much reduced from the same dose of LPS.

**TLR4-Ab treatment decreases T cell proliferation and T cell : Treg ratios while increasing peripheral and islet infiltrating T regulatory cells**

Initial cell kinetic studies showed no immediate effect of TLR4-Ab treatment on T cells, but an increased number of Tregs (Fig 4a, b). We further investigated the effect on both CD4\(^+\) and CD8\(^+\) T cell : Treg ratios; these were decreased at day 28 after TLR4-Ab treatment (Fig 5a). The increase in regulatory cells was not only found in the periphery, but also in the islets: Foxp3 immunohistochemistry showed a significant increase in Foxp3\(^+\) cells in the successfully reversed TLR4-Ab treated NOD islets compared to Ctrl-Ab or non-diabetic NOD islets (Fig 5b). Interestingly mice treated with TLR4-Ab, that eventually progressed to endstage disease (N=6), showed massive cellular infiltrates and reduced numbers of Foxp3\(^+\) cells, similar to Ctrl-Ab treatment (data not shown).
The induction of APC tolerance (Fig 4) could affect the ability of CD11c\(^+\) cells to activate or expand CD4\(^+\) T cells. To test this CD11c\(^+\) cells were treated twice with either TLR4-Ab or Ctrl-Ab, and then co-cultured with CD4\(^+\) T cells. CD11c\(^+\) cells that were treated twice with TLR4-Ab mediated significantly decreased CD4\(^+\) T cell expansion upon stimulation with CD3 (Fig 5c). This decrease in proliferation was also found in an antigen specific system. BDC2.5 transgenic T-cells plus BDC2.5 mimic peptide were co-cultured with CD11c\(^+\) cells that had been treated twice with TLR4-Ab or Ctrl-Ab. TLR4-Ab treated CD11c\(^+\) cells had a reduced ability to stimulate antigen specific proliferation compared to Ctrl-Ab or peptide alone (Fig 5d), and decreased numbers of cells progressed past four rounds of proliferation (Fig 5d). These results demonstrate TLR4-Ab mediated induction of APC tolerance can result in downstream decreased T cell proliferation and increased numbers of Treg cells.

**Targeted TLR4-Ab treatment of innate APCs alone, in the absence of B cells, is sufficient to prevent T1D in a transfer model**

To directly demonstrate that targeting of innate immune cells with TLR4-Ab was able to cause T cell tolerization and is sufficient to protect from T1D, we treated 6 week old female NOD.scid mice (lacking T or B cells) once a week for three weeks with TLR4-Ab, reconstituted them with pre-diabetic NOD CD4\(^+\) and CD8\(^+\) T cells, and assessed diabetes incidence compared to untreated NOD.scid recipients. TLR4-Ab pre-treatment of NOD.scid significantly protected transfer of T1D by pre-diabetic CD4\(^+\) and CD8\(^+\) cells (Fig 6a), contrasting sharply with rapid disease transfer in the untreated NOD.scid recipients. This decrease in disease incidence correlated with decreased islet pathology (Fig 6b). Consistent with increased Tregs in protected NOD mice (Fig 5b) an increase in
numbers and frequency of Tregs was found in the NOD.scid mice pre-treated with TLR4-Ab compared to the untreated group (Fig 6c). These results clearly demonstrate that TLR4-Ab treatment causes a downstream increase in Treg responses, likely mediated via stimulation of innate immune cells, and that B cells are not necessary for the treatment effect. Therefore stimulation of the innate immune system via TLR4 can cause downstream tolerance in adaptive immunity, sufficient to ameliorate T1D.

Discussion

It is well established that destruction of pancreatic islet β cells in T1D is caused by antigen specific CD8+ and CD4+ T cells, however clinical trials directed at dominant T cell epitopes in T1D (e.g. insulin) have failed to reverse disease [49]. This highlights the need for new therapeutic approaches and/or combination therapies. Targeting a key molecule in the innate immune system, TLR4/MD-2, we induced downstream suppression of adaptive auto-reactive immune responses and reversed new onset T1D. Prevention of diabetes using a TLR4 agonist was not surprising in the NOD mouse model; many agents, including LPS, can “prevent” diabetes but have subsequently shown no effect in treating or reversing the established disease (LPS has no effect on “prevention” beyond 10 weeks, and has no efficacy in established disease [50]). In striking contrast, TLR4-Ab was able to reverse clinical disease (polyuria, weight loss, and increased blood glucose levels) in a high percentage of mice without any other disease modulators such as islet transplantation or exogenous insulin. These results offer a new interpretation to the literature showing increased TLR4 activation in NOD and human new onset diabetes [30-33] and suggest that stimulation of TLR4, with resultant induction of APC tolerance, is the proper approach, rather than global
blockade of TLR4. The mechanisms distinguishing LPS from TLR4-Ab signaling are of interest and should be pursued in future studies, however, two obvious factors that may explain the therapeutic efficacy of the antibody are increased half-life (~15 days vs. 8 seconds) and bivalent signaling. Prolonged TLR4 signaling in APCs could produce an altered downstream signaling cascade resulting in APC tolerance with subsequent changes in adaptive immunity and restoration of adaptive immune tolerance. We have further found that maximal dosing of APCs with TLR4-Ab produces decreased inflammatory cytokines compared to LPS (data not shown), suggesting again that TLR4-Ab and LPS differ in mediating inflammatory signals.

We have clearly shown that TLR4-Ab signaling in T or B cells is not necessary for our therapeutic effect; and that TLR4-Ab induces tolerogenic macrophages and dendritic cells which then, in a complex in vivo cellular cascade extending over as much as 4 weeks, ultimately results in restoration of adaptive immune tolerance. The mechanisms of this complex cascade are difficult to determine in detail and require multiple future mechanistic cellular immunology studies. However, we have clearly shown that the outcome of this process is increased numbers of Treg cells and decreased T cell:Treg ratios as well as decreased co-stimulation of T cells. These results collectively show that stimulation via TLR4 can alter both the innate and adaptive immune response, and restore immune tolerance, even in established T1D. Our results therefore provide a strong foundation for exploring TLR4 mediated pathways as a novel means to reverse new onset, clinically apparent T1D. The mechanisms of this strong clinical effect should be pursued in depth, since the availability of FDA-approved agonistic anti-TLR4 agents [51, 52], will make it possible to test this approach in new onset human T1D.
Acknowledgements

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References


### Table One – Reversal of new onset T1D statistics

<table>
<thead>
<tr>
<th></th>
<th>UnTx (n)</th>
<th>Ctrl-Ab (n)</th>
<th>TLR4-Ab (n)</th>
<th>P value</th>
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<tr>
<td>Age of diabetes onset (days)</td>
<td>177.4 ± 11.3 (12)</td>
<td>166.8 ± 6.4 (12)</td>
<td>183.5 ± 7.0 (21)</td>
<td>n.s.</td>
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<tr>
<td>Initial blood glucose (mg/dl)</td>
<td>174.5 ± 9.2 (12)</td>
<td>214.7 ± 4.5 (12)</td>
<td>204.7 ± 3.4 (21)</td>
<td>n.s.</td>
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<tr>
<td>Final blood glucose (mg/dl)</td>
<td>583.1 ± 7.7 (12)</td>
<td>575.3 ± 11.4 (12)</td>
<td>159.7 ± 5.5 (15)</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Days non-diabetic (non-diabetic TLR4-Ab)</td>
<td>16.7 ± 3.2 (12)</td>
<td>17.6 ± 2.9 (12)</td>
<td>87.7 ± 8.8 (15)</td>
<td>P &lt; 0.0001</td>
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<tr>
<td>Days non-diabetic (diabetic TLR4-Ab)</td>
<td>16.7 ± 3.2 (12)</td>
<td>17.6 ± 2.9 (12)</td>
<td>51.0 ± 15.3 (6)</td>
<td>P = 0.009</td>
</tr>
</tbody>
</table>

TLR4-Ab initial vs. final blood glucose (mg/dl) | 204.7 ± 3.4 (21) | 159.7 ± 5.5 (15) | P < 0.0001 |

**Table One.** TLR4-Ab treatment significantly improves blood glucose, weight loss, and overall disease free time in newly diabetic NOD mice. Values expressed as Mean ± SEM.
**Figure Legends**

**Figure One. TLR4-Ab prevents T1D and reduces insulitis.** (a) 6-8 week old female NOD mice were treated three times one week apart with Ctrl-Ab (n=13), TLR4-Ab (n=13) or untreated (n=17), and diabetes assessed for 200 days. **P=<0.005, and ***P=<0.0001, Log-Rank test (b) Insulitis severity scores: at the time of diabetes onset or after 200 days the pancreas was scored for insulitis (see methods); the percentage of each stage of insulitis is shown. (c) Representative islets from each group are shown.

**Figure Two. TLR4-Ab reverses new onset T1D** (a) Female NOD mice were randomly assigned to Ctrl-Ab or TLR4-Ab groups and then monitored until the earliest signs of T1D (polyuria); at this time the average BG was 208.3±16.1mg/dL. Mice were then treated twice, one week apart, with either Ctrl-Ab (n=12) or TLR4-Ab (n=21). “Untreated” (UnTx) mice were NOD mice outside the study that were allowed to develop spontaneous T1D. Shown is the time course between the last measurement below BG 250 mg/dl and the development of endstage disease (BG >500mg). Kaplan-Meier survival curve is shown; ***P <0.0001, Log-rank test. (b, c, d) Detailed time course of blood glucose with no treatment (b), treatment with Ctrl-Ab (c) or TLR4-Ab (d). Mice were either treated twice (d1, upper right panel, n=11), or retreated (d2, lower right panel, n=10) if the BG rose above 200 mg/dl after the initial two treatments. Red time point indicates time of first retreatment (d2). (e) TLR4-Ab reverses T1D induced weight loss. Blood glucose and weight were monitored weekly in female NOD mice. Mice (n= 6 both groups) were treated with TLR4-Ab or Ctrl-Ab starting at time point 3. Time intervals equal one week except the last interval which varied from 3-7 days. *P=<0.01, unpaired T test. (f) TLR4-Ab improves insulitis in new onset T1D. Pancreatic insulitis
was assessed by a blinded observer (see methods) in all 3 groups: UnTx (n=10, endstage diabetes (BG > 500mg/dL)), Ctrl-Ab (n=9, the first 6 cohorts of mice from Fig 2a above) and TLR4-Ab (n=13, the first 6 cohorts of mice from Fig 2b above), **P=<0.008, Mann-Whitney test.

Figure Three. TLR4-Ab preserves insulin-positive pancreatic beta cells by reducing the percentage of severely infiltrated islets. (a) Percentage of islets at each stage of insulitis (scored blindly) in five groups: “non-diabetic” (n=7, 6 week old female NOD mice), “BG200” (n=5, mice at a blood glucose of ~200mg/dL when treatment is initiated), “diabetic” (n=10, endstage diabetes (blood glucose > 500mg/dL)), “Ctrl-Ab” (n=9) and “TLR4-Ab” (n=13). (b) Pancreatic sections from mice in each group were stained for insulin and glucagon by immunohistotochemistry; representative islets from each group are shown. (c) Total insulin positive area per section, quantified using ImageJ analysis (see methods). **P=<0.005, and ***P=<0.0001, unpaired T test (d) Total number of insulin positive islets per section. *P=<0.02, **P=<0.003, and ***P=<0.0001, unpaired T test. (e) Increased beta cell insulin positive area per insulin positive islet in TLR4-mAb treated mice *P=<0.02 and **P=<0.001 unpaired T test.

Figure Four. TLR4-Ab targets macrophages and dendritic cells in vivo resulting in APC tolerance induction. (a,b) Groups of female NOD mice were treated with TLR4-Ab, Ctrl-Ab or PBS and the indicated splenic cell subsets were assessed by FACS at 24 hours or 28 days (n=2 PBS, n=5 Ctrl-Ab, n= 5 TLR4-Ab) *P=<0.05, **P=<0.009. (c) TLR4-Ab does not cause CD4+ T cell proliferation. Purified CD4+ cells treated with TLR4-Ab (n=4) or Ctrl-Ab (n=4) proliferate no differently than unstimulated controls (N=4), compared to CD3/CD28 stimulation (n=4). (d) NOD mice were injected with PBS
(n=2), Ctrl-Ab (n=4) or TLR4-Ab (n=4) once a week for two weeks. 24 hours after the final treatment splenocytes were removed and the cells were assessed for costimulatory molecules on CD11b+CD11c+ dendritic cells. One representative histogram plot is shown. Serum was harvested and assessed for levels of IL-10 by ELISA. **P=0.0036. (e) Thioglycolate was injected IP into NOD mice. After three days peritoneal thioglycolated elicited macrophages were cultured as follows: 3 groups were treated with media alone for 4 hours, and then treated with either LPS (“LPS”), TLR4-Ab (“TLR4-Ab”), or Ctrl-Ab (“Ctrl-Ab”). Three other groups were first: 1) treated with TLR4-Ab for 4 hours and then either treated with LPS (“TLR4+LPS”) 2) treated again with TLR4-Ab (“2X TLR4-Ab”) or 3) treated with Ctrl-Ab for 4 hours, then treated again with Ctrl-Ab (“2X Ctrl-Ab”). 24 hours after the final stimulation the supernatant was removed from the culture and TNF-alpha or IL-10 (n=6) were assessed by ELISA and IFN-beta (n=6) was assessed by an interferon stimulated response element reporter cell line. IFN-beta: ***P=0.0001, *P=0.02; IL-10 *P=<0.02; TNF-alpha **P=<0.008, unpaired T test.

**Figure Five. TLR4-Ab treatment leads to decreased Teffector:Treg ratios, decreased T effector proliferation, and increased islet Tregs.** (a) Mice treated with Ctrl-Ab, TLR4-Ab, or UnTx were assessed 28 days after treatment for the ratio of CD4:Foxp3 and CD8:Foxp3 cells. (b) Pancreatic sections from NOD mice treated with TLR4-Ab or Ctrl-Ab, or untreated, were assessed for pancreatic islet infiltrating Foxp3+ cells by immunohistochemistry. (c,d) CD11c+ cells were immunomagnetic bead sorted and placed in culture along with a tolerogenic dose of TLR4-Ab or Ctrl-Ab for 1 hour. (c) After one hour CD11c+ cells were washed and co-cultured anti-CD3 antibody (CD3-Ab)
or (d) BDC2.5 mimic peptide (peptide). CD4+ T cells were stained and purified as previously described. 4 hours after the initial dose of TLR4-Ab cells were then re-stimulated with an activating dose of TLR4-Ab or Ctrl-Ab. The cell remained in culture for 3 days and CFSE dilution was measured at the end time point. Representative FACs plots and histograms are shown. *P=0.0137, **P=0.0041, unpaired T-test.

Figure Six. Targeted TLR4-Ab treatment of innate immune cells, in the absence of B or T cells, prevents T1D and increases downstream Treg numbers (a) 6 week old female NOD.scid mice were treated three times one week apart with either TLR4-Ab (n=15), or untreated (unTx, n=10). 2x10^6 CD4+ and 1x10^6 CD8+ T-cells from prediabetic NOD mice (6 weeks) were transferred into the NOD.scid recipients and diabetes onset was monitored for 75 days. (b) Individual islet histological scores are shown for untreated control (n=10), and TLR4-Ab treated (n=11) NOD.scid T cell recipients. Representative islet histology is shown for each group. (c) Total splenic Tregs (CD4+Foxp3+) were assessed at time of overt diabetes or at 75 days after treatment from unTx (n=8), or TLR4-Ab treated (n=7) NOD.scid recipient mice.*P < 0.04, unpaired T test. Representative FACs plots are shown.
Figure One

(a) % Diabetes Free

(b) % of Histological Score

(c) UnTx, Ctrl-Ab, TLR4-Ab

Legend:
- 0
- 1
- 2
- 3
- 4
- 5
Figure Two

(a) % End Stage Diabetic

Days

(b) Blood Glucose Level (mg/dL)

Days

(c) Blood Glucose Level (mg/dL)

Days

(d) Blood Glucose Level (mg/dL)

Days

(e) % Weight Change

Time Points

(f) Histological Score

UnTx Ctrl-Ab TLR4-Ab
Figure Six

(a) % Diabetes Free

(b) % of Histological Score

(c) Total Tregs (x10^6)
Chapter 3: Recombinant soluble CD137 prevents type one diabetes in non-obese diabetic mice
Recombinant soluble CD137 prevents type one diabetes in nonobese diabetic mice

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Running Title: sCD137 prevents type one diabetes
ABSTRACT

Nonobese diabetic (NOD) mice are genetically programmed to spontaneously develop type one diabetes (T1D). Multiple Insulin dependent diabetes (Idd) genetic loci have been identified but their functional effects are mostly poorly understood. *Tnfsfr9*, expressing the protein product CD137, is a strong candidate gene in the *Idd9.3* locus, and NOD.B10 *Idd9.3* mice are significantly protected from type one diabetes (T1D). We previously showed that nonobese diabetic (NOD) mice have a deficiency in the numbers of CD137<sup>pos</sup> T regulatory cells, that CD137<sup>pos</sup> Tregs are the source of soluble CD137 (sCD137), and that NOD mice have low serum levels of sCD137. To test the hypothesis that correcting low levels of sCD137 could affect the disease, we constructed a lentiviral vector producing recombinant sCD137; this physiologic sCD137 is glycosylated and exists primarily as a dimer. NOD mice treated with the recombinant sCD137 are protected from developing T1D. Insulitis is significantly decreased, but not eliminated in the sCD137 treated mice, however insulin producing pancreatic beta cells are reserved despite residual insulitis. To begin to understand the protective immune mechanisms of sCD137, we tested sCD137 *in vitro*. It was previously suggested that sCD137 simply blocked the interaction between CD137 (on T cells) and CD137 ligand (on antigen presenting cells (APCs)). Here however, we use an APC independent assay and demonstrate that sCD137 can actively suppress highly purified CD4 T cells in a CD137L dependent fashion. These results support the hypothesis that sCD137 acts in a negative feedback loop to actively suppress over-zealous immune responses, and that it can be used clinically to suppress autoimmunity. sCD137 is an important Treg derived natural immunosuppressive molecule that regulates effector T cells to avert diabetes *in vivo*. 
1. INTRODUCTION

Type I diabetes (T1D) is a polygenic organ specific autoimmune disease ultimately mediated by loss of T cell tolerance to pancreatic islet antigens [1]. NOD mice spontaneously develop T1D and serve as an excellent model for understanding genetic factors influencing disease. Genetic mapping studies performed over the last 25 years have identified multiple Insulin dependent diabetes (Idd) loci influencing disease in NOD mice; these loci have been incorporated into NOD congenic mice. NOD.B10 Idd9.3 congenic mice have a 1.2 Mb B10 region on chromosome four which reduces T1D by 40% (but does not eliminate insulitis) [2]. The Idd9.3 region encodes 15 different genes with CD137 being the strongest candidate gene. Sequencing has identified three exonal CD137 sequence variants between NOD and B10 alleles: a valine to alanine substitution at position 24, a leucine to proline substitution at position 211, and the insertion of an alanine in NOD allele between amino acids 174 and 175 [2]. We and others have now published several studies showing a strong effect of CD137 on diabetes and on T cell/Treg function [3-5]. Cannons et al. showed that NOD T cells, compared to NOD.B10 Idd9.3 T cells, have defective IL-2 production when stimulated specifically via CD137 [3]. Stimulation of T cells via CD137 causes T cell expansion, cytokine production and upregulation of anti-apoptotic genes (such as Bcl-xl) that prevent activated-induced cell death [6-9] [10, 11]. Our lab and others have shown that CD137 is constitutively expressed on a subset of CD4^{pos}CD25^{pos} T regulatory cells, whereas it is only upregulated after activation in other T cell subsets [12]. CD137 signaling in the presence of IL-2 promotes cell proliferation and survival of natural Tregs in vitro and in vivo, thus enhancing regulatory function [12, 13]. We showed that agonist anti-CD137 antibody treatment prevents diabetes in NOD mice, and increases the frequency of CD4^{pos}CD25^{pos} T cells in vivo [4].
Significantly more CD4<sup>pos</sup>CD25<sup>pos</sup>CD137<sup>pos</sup> Tregs accumulate in NOD.B10 ldr9.3 mice compared to NOD mice, and CD137<sup>pos</sup> Tregs expressing the B10 allele accumulate specifically in NOD:NOD.B10 ldr9.3 mixed bone marrow chimeras [5]. This accumulation is significant because CD137<sup>pos</sup> Tregs are functionally superior in vitro compared to CD137<sup>neg</sup> Tregs [5].

In addition, we showed that CD137<sup>pos</sup> Tregs are the cellular source of an alternately spliced CD137 product, soluble CD137 (sCD137), and produce large amounts of this protein [5]. Mouse CD137 is alternately spliced to produce two different isoforms; full length CD137 protein that is bound to the cell surface and a sCD137 isoform, in which the transmembrane-encoding exon 8 is spliced out [14]. The function of sCD137, and its mechanism of action, has not been extensively studied. It was previously suggested that sCD137 could decrease T cell proliferation in an APC dependent fashion, by simply blocking the interaction of CD137 and CD137L [15]. Expression of CD137L is found on many cells with the highest expression being on antigen presenting cells, specifically dendritic cells and macrophages. Michel et al. showed that the production of sCD137 lagged the active immune response and hence could be a negative feedback loop to downregulate the immune response[16]. High serum levels of sCD137 have been demonstrated in several autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (MS), but the significance of elevated sCD137 was unknown [17-19]. Significantly, we showed that NOD mice have decreased levels of serum sCD137 compared to NOD.B10 ldr9.3 mice (that had increased accumulation of CD137<sup>pos</sup> Tregs and protection from diabetes) [5]. Here we show that “correcting” the NOD serum levels of sCD137 by treating these mice with a novel recombinant form of sCD137 prevents T1D. Moreover, we demonstrate that sCD137 does not simply block CD137-CD137L interactions, but actively suppresses effector T cells. These results show that
sCD137 may arise in autoimmune states as an active attempt to regulate the immune response, and that sCD137 can act as a natural immunosuppressant to reverse autoimmunity.

2. MATERIALS AND METHODS

2.1 Mice and reagents: NOD mice were maintained under spf conditions, and in accordance with the animal care guidelines of University of Cincinnati School of Medicine. NOD female mice in our colony have a diabetes incidence of ~80% by 200 days. Monitoring for diabetes is done as follows: cages are screened weekly for evidence of polyuria. When polyuria is detected, screening for glucosuria is usually positive. Urinary glucose analysis is performed weekly using Tes-tape (Shionogi, Osaka Japan). Once the mice demonstrate glucosuria, their blood glucose (BG) is checked with a glucometer. BG is monitored until it exceeds 600 mg/dl at which point the mice are sacrificed. Agonist anti-CD137 monoclonal antibody (clone 3H3) was previously described [11]. FACS Antibodies are from BD Pharmingen. Anti-mouse CD137L (clone TSK-1) and IgG2a isotype control (clone RTK2758) antibodies are from BioLegend (California). CD3/CD28 beads and mouse IL-2 are from Invitrogen. CD137-Fc conjugated recombinant protein is from R&D Systems (Minnesota).

2.2 Treg Transwell Suppression Assay: 100,000 FACS sorted CD4\textsuperscript{pos}CD25\textsuperscript{neg}CD137\textsuperscript{neg} T cells were cultured with 50,000 CD3/CD28-coated beads (Invitrogen) in the bottom wells of a 96 well transwell plate (Corning). 25,000 CD4\textsuperscript{pos}CD25\textsuperscript{pos}CD137\textsuperscript{pos} Tregs were cultured in the top wells with 50,000 CD3/CD28-coated beads. In some wells, 20\mu g/ml of CD137 Ligand (CD137L) (TKS-1) blocking antibodies were added to the bottom wells. The transwell plates were incubated at 37°C in 5% CO\textsubscript{2} and the cells pulsed with 1 \mu Ci [\textsuperscript{3}H] thymidine on Day 3. Cells in
the bottom wells were harvested 16 hours later and counted using a beta scintillation counter as indicated.

### 2.3 Proliferation Assay with thymidine:

CD⁴⁺CD₂₅⁻CD₁₃₇⁻ cells were stained and sorted using BD Aria flow sorter (BD Bioscience) to >99% purity. 50,000 sorted CD⁴⁺CD₂₅⁻CD₁₃₇⁻ T cells were cultured with 20,000 CD3/28 beads in triplicate wells in the presence or absence of 1μg/ml of sCD137-Fc. 20μg/ml of either CD137L blocking antibodies or IgG2a isotype control antibodies were added to select wells. Cells were subsequently pulsed-labeled with 1 μCi [³H] thymidine on Day 3, and thymidine incorporation assessed 16 hours later using a beta scintillation counter.

### 2.4 Proliferation Assay with CFSE:

Magnetically-sorted NOD CD4 T cells were stained with 0.5μM of carboxyfluorescein succinimidyl ester (CFSE) before culturing 100,000 cells per well in a 96-well u-bottom plate under one of the following conditions: Unstimulated (control), stimulated (20,000 CD3/CD28 beads), or stimulated with beads plus 15μg of purified recombinant sCD137. The CFSE-labeled cells were cultured for 3 days at 37°C in 5% CO₂, then harvested, blocked with 2.4G2 Ab and stained with CD4-PCP and propidium iodine.

### 2.5 Production of sCD137 protein:

sCD137 cDNA was obtained from Open Biosystems (Clone ID 1497753). We excised the sCD137 DNA band and inserted it into the multiple cloning site (MCS) of LeGO-iG2 using standard subcloning procedures as previously described [20]. Two different cell lines (NIH3T3 and HEK293) were transduced with VSVg-enveloped lentiviral particles containing either LeGO-iG2 or LeGO-iG2-sCD137. The transduced cells were cultured, supernatant collected, and tested for the production of sCD137 by ELISA. NIH3T3 and
HEK293 cell lines exhibiting stable EGFP expression were sorted for high EGFP expression and used for large-scale protein purification.

2.6 Purification of sCD137: We constructed an anti-CD137 affinity chromatography column to purify sCD137 protein. We purified anti-CD137 antibodies from the 3H3 clone using standard methods [21]. The secreted antibody was coupled to a CNBr-activated Sepharose™ 4B column (GE Life Sciences) [22, 23]. Coupling efficiency was assessed and then sCD137 containing supernatant was filtered (0.22μm filter) and gravity fed over the column. After elution from the affinity column, sCD137 protein was dialyzed and the amount/concentration of purified sCD137 determined by spectrophotometer.

2.7 Western Blotting: For sCD137 western blotting, 15μl of sCD137 protein samples were mixed in either or non-reducing sample buffer. The samples were boiled for 5 minutes, cooled, and loaded onto 12% Tris-glycine gels (Novex) and run at 125V for 105 minutes. SDS-PAGE protein markers (BioRad, cat. no. 161-0305) were used as size markers. The protein was transferred onto nitrocellulose paper and blocked. The primary anti-CD137 antibody (Millipore, rat anti-mouse 4-1BB clone MAB3733) was added, then polyclonal secondary antibody to mouse IgG - H&L (AP). The AP proteins were stained using NBT and BCIP, then western blot performed as described [24].

2.8 Analytical ultra-centrifugation (AUC): Sedimentation velocity experiments were utilized to determine the size distribution of sCD137 at 20°C using a Beckman XL-I analytical ultracentrifuge. sCD137 in PBS was spun at 48,000 rpm and successive scans were obtained at 280 nm using the absorbance optical system. Raw sedimentation data were deconvoluted to
produce sedimentation coefficient distributions and molecular weight estimates using the c(s) analysis routine in the program SEDFIT [25].

2.9 Histology: The pancreas was fixed for 24 hrs in 10% buffered formalin, then embedded in paraffin and stained with hematoxylin and eosin. A scoring system (0 -5) was used by a blinded observer as previously described [26, 27]. (0: normal; 1: peri insulitis, with the circumventing cell depth around the islet being 5 or less and no penetration into the islet; 2: full circumferential insulitis or peri insulitis with a cell depth of 5 or greater, no penetration into the islet; 3: full circumferential insulitis or peri insulitis with a cell depth of 5 or greater, and less than 50% coverage of the islet by cell infiltrates, 4: full circumferential insulitis or peri insulitis with a cell depth of 5 or greater, and more than 50% coverage of the islet by cell infiltrates; 5: scar or complete absence of β-cells.) (minimum of 36 islets per group).

2.10 Immunohistochemistry and ImageJ quantification of insulin and glucagon.

Immunohistochemistry (IHC) was performed at the Cincinnati Children’s Hospital Medical Center pathology core lab as follows. Pancreatic tissues were fixed for 24 h in 10% buffered formalin, embedded in paraffin and stained for insulin or glucagon with insulin and glucagon antibodies (Ventana Medical). The slides were run on a Ventana BenchMark XT automated IHC slide staining system. After the slides were cut and baked, they were treated with CC1 protein block for 8 minutes. Goat serum block was added for 8 minutes, and the samples were stained with insulin antibody for 2 hours. Goat anti-rabbit antibody was placed on for 32 minutes for immunohistochemistry, then hematoxylin and bluing reagent were incubated for 4 minutes each. The same protocol was run for the glucagon stain, but staining for the goat anti-rabbit antibody was 20 minutes.
After staining, digital recreation of the slides was obtained by taking serial pictures of each slide on an Olympus BX51 scope and Olympus DP71 camera. After the pictures were taken the slide was digitalized using PhotoShop’s PhotoMerge CS5. Once the slide was digitally recreated the insulin or glucagon positive area was obtained using ImageJ analytical software. The pixilated area was measured using the measure function in ImageJ.

2.11 *In vivo* treatment with sCD137: Female NOD mice were treated with either PBS or 200ug of sCD137, once a week for four weeks. The experiment was performed twice and the results pooled; in each experiment mice from litters born the same day were used and divided equally between experimental and control groups. Diabetes progression was monitored as described in section 2.1 above.

2.12 Statistics: All statistical analysis was performed using GraphPad Prism 5.

3. RESULTS

3.1 Production of recombinant sCD137 *in vitro*

We developed a purified form of recombinant sCD137 in order to investigate its ability to treat autoimmunity and its mechanism of action. We sub-cloned a sCD137 minigene into the lentiviral expression vector, LeGO-iG2 (Fig 1a). The LeGO-iG2 vector is bicistronic, has an EGFP reporter gene, and is self-inactivating [28, 29], which allows production of sCD137 protein without viral particles. VSVg-enveloped LeGO-iG2-sCD137 viral particles were made in separate producer cells and used to transduce two eukaryote cell lines, NIH3T3 and HEK293 cells. Stably transduced lines were sorted for high EGFP expression (Fig 1b) and tested for sCD137 production by ELISA. HEK293-EGFP high expressers produced the greatest amount of
sCD137 protein (Fig 1c), so this cell line was used for large-scale purification and of the sCD137 as described in the methods section.

3.2 sCD137 prevents diabetes and reduces insulitis in NOD mice

We previously showed that NOD mice had significantly lower levels of serum sCD137 than NOD.B10 Idd9.3 mice that have increased protection from diabetes [5]. This suggested that increasing the sCD137 levels in NOD mice might prevent diabetes. We tested this by treating NOD mice with our purified recombinant sCD137. We treated seven week old female NOD mice with 200 μg of purified sCD137 vs. PBS control, once weekly for four weeks. Urinary glucose was monitored for diabetes. Control NOD mice developed diabetes (polyuria, glucosuria, and blood glucose >600mg/dL) as expected by 178 days of age (Fig 2a). In contrast, sCD137 treated NOD mice showed virtually complete protection from diabetes (Fig 2a). Pancreatic histology (H&E) of the control versus sCD137 treated NOD mice showed that sCD137 treatment significantly reduced insulitis, although it did not eliminate it, suggesting a regulatory effect of sCD137 on the autoimmune process (Fig 2b). To further understand the effect of sCD137 treatment on pancreatic beta cells, we performed insulin and glucagon immunohistochemistry of all the treated and control mice (Fig 2c, d). The sCD137 treated mice had preservation of insulin staining beta cells while the control group had near total loss of insulin staining (Fig 2c, d). The glucagon staining was preserved in both groups, consistent with the literature showing glucagon preservation in the setting of T1D in NOD mice [30-33]. The preservation of insulin staining beta-cells in the presence of significant insulitis suggests that treatment with sCD137 produced a state of “benign” insulitis [34-36] (see discussion). These results demonstrate that our recombinant sCD137 is functional in vivo and can prevent the onset of T1D in NOD mice and reduce the pathogenicity of the insulitis.
3.3 sCD137 produced by CD137$^{\text{pos}}$ Tregs directly suppresses CD4$^{\text{pos}}$ T effector cells via CD137L

To begin to understand the mechanism of sCD137 prevention of diabetes we performed in vitro studies. We previously showed that the CD137$^{\text{pos}}$ Treg subset was the major cellular source of sCD137 and that NOD CD137$^{\text{pos}}$ Tregs can suppress effector T cells in a contact independent transwell system [5]. Others have shown that sCD137 binds to CD137 Ligand (CD137L) in vitro [37], as expected given that the alternate splicing of CD137 does not change its ligand binding domain. Thus, here we used CD137L blocking antibody (TKS1.1) to test if blocking the interaction of CD137 with its ligand, CD137L, could prevent transwell mediated suppression of CD4$^{\text{pos}}$ effector T cells. The presence of CD137L blocking antibody significantly abrogated the transwell suppression by CD137$^{\text{pos}}$ Tregs (P=0.001), while isotype antibody had no effect on suppression (Fig 3). Notably, anti-CD137L antibody alone had no effect on T cell proliferation (Fig 3). These observations suggested that soluble factors produced by CD137$^{\text{pos}}$ Tregs, including sCD137, suppressed the proliferation of CD4$^{\text{pos}}$ effector T cells through CD137L.

3.4 Soluble CD137-Fc actively suppresses CD4 T cells in vitro in the absence of APCs or Tregs

When sCD137 was initially discovered, studies of effect on T cells were only performed in the presence of APCs, and it was concluded that sCD137 simply blocked the interaction of CD137L on APCs with CD137 on T cells [15]. However, the fact that we could block suppression in a transwell assay by anti-CD137L (Fig 3) suggested that sCD137 might have a direct, active effect on T cells. To test this hypothesis, we used recombinant mouse sCD137-Fc in an APC independent assay containing only non-Treg CD4 T cells. We cultured FACS sorted
CD4^{pos}CD25^{neg}CD137^{neg} T effector cells with CD3/28 beads in the presence of sCD137-Fc (Fig 4). The addition of sCD137-Fc significantly reduced the proliferation of CD3/CD28 stimulated CD4 T cells (P=0.0001), showing that sCD137-Fc protein can directly suppress CD4^{pos}CD25^{neg}CD137^{neg} T cells in vitro in an APC independent fashion.

To prove that active sCD137 suppression is mediated through CD137L, we added CD137L blocking or isotype control antibodies to the culture along with sCD137-Fc (Fig 4). The addition of CD137L blocking antibody, but not the isotype control, abrogated the suppression mediated by sCD137-Fc, confirming that CD137L is essential for suppression of CD4^{pos} effector T cells by sCD137. Notably, anti-CD137L antibody added to CD4+ cells alone had no effect on proliferation (Fig 3), showing that sCD137 does not act by disrupting T:T interactions. Our results demonstrate that the interaction of sCD137 directly with CD137L directly and actively suppresses CD4^{pos} effector T cells. This active suppression by sCD137 completely changes how we view the role of sCD137 in autoimmunity.

3.5 Purified sCD137 decreases cell division of CD4 T cells in vitro

To further understand how sCD137 could affect CD4 cell function, we assessed the effect of sCD137 on T cell division in vitro, again in the absence of APC. We cultured CFSE-labeled CD4 T cells with CD3/CD28 beads with or without purified recombinant sCD137. Addition of sCD137 significantly reduced the CFSE dilution of CD4 T cells (Fig 5a, b). The percent of cells dividing multiple times is significantly reduced in the presence of sCD137, and the number of cells not entering cell cycle at all is significantly increased (Fig 5b). These data show that our purified protein is biologically active and functionally suppressive. sCD137 did not induce increased cell death compared to untreated bead stimulated cells (Fig 5c). Hence sCD137 does
not act by inducing CD4 T cell death. More importantly, this result again confirms that sCD137 directly inhibits T cell proliferation in the absence of APC.

3.6 Recombinant sCD137 exists predominantly as a dimer

We next examined structural features of sCD137 to gain some insight into possible mechanisms of action. First, we performed western blotting on an SDS-PAGE-gel under reducing and non-reducing conditions. Unpurified sCD137 is a ~55 kDa homodimer (non-reducing) vs. a ~35 kDa monomer (reducing) (Fig 6a, lane 3 & 8). These sizes are consistent with previous studies [37, 38], however this result contradicts a previous assertion that sCD137 exists as a trimer [37]. Affinity chromatography was used to purify sCD137. Western blotting showed that the observed sizes of the purified and unpurified protein were equivalent; (Fig 6a, lane 2 & 4), indicating that affinity column processing did not affect the apparent physical structure of the protein. Under non-reducing condition, the CD137-Fc chimeric protein shows as a greater than 110 kDa protein (Fig 6a, lane 5) which is also consistent with a homodimer structure as reported by the manufacturer (R&D Systems).

sCD137 exists predominantly as a dimer under non-reducing conditions, but as a monomer under reducing conditions, strongly suggesting that the dimer is linked by a disulfide bond (Fig 6a, lane 2 & 4 vs. lane 7 & 9). This accords with sequence homology data indicating the existence of homodimerization domains in the CD137 structure (Pubmed – Protein search / NCBI Conserved Protein Domains). Other TNFR family members (e.g. CD27 and CD40) also dimerize via intermolecular disulfide bonds [39]. The CD137-Fc chimeric protein also migrates as a ~67 kDa monomer under reducing conditions due to the breaking of the disulfide bond (Fig 6a, lane 10). Three distinct monomer bands were seen on the reduced western blot, suggesting that the
purified recombinant protein is differentially glycosylated (0, 1, or 2 modifications; Fig 6a, lane 7 & 9), unlike the sCD137-Fc protein. This is consistent with the predicted protein sequence that suggests sCD137 protein harbors two canonical N-linked glycosylation sites (Pubmed – Protein search / NCBI Conserved Protein Domains).

To further confirm the structure of purified sCD137, we used sedimentation velocity analytical ultra-centrifugation (AUC). Consistent with the western blot results, and again inconsistent with a prior report [37], AUC showed that sCD137 is predominantly present as a 54.3kDa dimer, with a small amount of monomer at 26.9kDa (Fig 6b). A minor percentage of the total sCD137 formed higher order oligomers and/or aggregates (Fig 6b).

In summary, we have established that that treatment with purified sCD137 prevented the development of diabetes and reduced the number of islet infiltrates in NOD mice, consistent with our previously published work showing increased serum sCD137 in NOD.B10 Idd9.3 congenic mice protected from diabetes. We show that sCD137 acts directly on T cells to actively suppress them, in contradiction to prior reports that suggested sCD137 had only a passive, blocking mechanism of action [15]. The purified sCD137 primarily exists as a dimer, not as a trimer as previously reported [37], which may give some insight into its mechanism of action (see discussion). The data presented here strongly supports an important, active role for sCD137 in diabetes protection in NOD mice and suggests a homeostatic role for sCD137 in autoimmunity in general.

4. DISCUSSION

The novel results we report here change several prevailing ideas concerning sCD137 in autoimmunity and open several new pathways for further investigation. First and foremost, we
show that sCD137 can be therapeutic in an autoimmune disease, T1D. Previously it had been reported that several human autoimmune diseases, such as RA and MS, showed a correlation with elevated serum CD137 [17-19], but it was impossible to conclude whether the increase was associated with pathology, or with a homeostatic response of the immune system, or was simply irrelevant. We addressed this question in our NOD and NOD B10 Id9.3 congenic mice. Previously we showed that NOD had decreased numbers of CD137\textsuperscript{pos} Tregs that are the cellular source of sCD137, and had significantly less serum sCD137 than NOD B10 Id9.3 mice [5].

Here we show that treating NOD mice with sCD137 prevented the autoimmune disease. This suggests that elevated sCD137 in autoimmune diseases may be a homeostatic attempt of the immune system to correct the autoimmune process. Certainly, the fact that increased sCD137 in the B10 Id9.3 mice does not increase but decreases the autoimmune process, and increasing the serum sCD137 in NOD mice prevents the disease altogether, strongly suggests that increased sCD137 is not pathogenic but may in fact be protective.

Next, our current results change the understanding of the function of sCD137. Previous reports had suggested that sCD137 passively blocked APC:T cell CD137:CD137L interactions [15]. These earlier studies performed in the presence of APC showed decreased T cell proliferation in the presence of endogenous sCD137 \textit{in vitro}, but they attributed this T cell response to the indirect blocking effects of sCD137. Here we show the active suppressive role of sCD137 using three different systems. First soluble factors produced by CD137\textsuperscript{pos} Tregs directly mediate suppression, without contact, via CD137L in an APC independent \textit{in vitro} system (Fig 3). Second, sCD137-Fc directly suppresses CD4 T cells in the absence of APC and in CD137L dependent manner. Finally we show directly that recombinant sCD137 can actively prevent CD4 T cell entry into cell division in the absence of APCs. Thus sCD137 has an active role in immune
suppression and does not simply block CD137:CD137L interactions between APC and T cells. This suppressive effect is also not due to blockade of CD137:CD137L interactions between T cells in vitro, since CD137L antibody blocking alone has no effect on T cell proliferation (Fig 3). In addition, sCD137 decreases the number of CD4 T cells entering and progressing through the cell cycle without inducing cell death in vitro (Fig 5). These results combined with the prior observation that sCD137 is produced at a later stage during an immune response [16] suggests that sCD137 is a natural immunosuppressant that functions as a homeostatic regulator of excess immunity.

We show here that while treatment with sCD137 decreased insulitis, it did not eliminate it, and with a mean insulitis score of ~3, substantial infiltrate still remained but did not cause diabetes. Furthermore, despite prominent amounts of insulitis, functional pancreatic beta cells were preserved in the treated animals as shown by robust insulin staining. These results strongly suggest that the insulitis in the sCD137 treated animals has been converted from an “aggressive” to a “benign” state [35, 36]. NOD BDC 2.5 mice represent a good example of this phenomenon: they develop massive insulitis as early as 2 weeks of age, but this rarely progresses to diabetes in this strain despite the presence of large numbers of potentially diabetogenic T cells. Chen et al. showed that this is due to the regulatory effects of T regulatory cells in the islet, in other words the presence of immunoregulatory factors can “restrain” the insulitis and convert it to a benign state [34]. A similar process is apparent in Idd9 biology. Lyons et al. originally demonstrated that although the Idd9 interval, (including the Cd137 gene) protected from T1D, it did not prevent insulitis [2]. Similarly, our group treated NOD mice with an agonist CD137 antibody that prevented T1D but did not prevent insulitis [4]. Moreover, we showed that pathologic T effectors still existed in the anti-CD137 treated mice. These results were explained by demonstrating the
existence of a subset of CD137-expressing Tregs that produce sCD137 [4, 5]. Our results here strongly support the overall hypothesis that sCD137 enhances regulatory immunity in the islet.

The therapeutic activity of sCD137 in T1D raises the issue of its possible efficacy in other autoimmune diseases, such as SLE. We suggest it is an attractive candidate for such diseases, due to the fact that it is a natural immunosuppressive molecule that can act in a negative feedback loop to decrease immune activation [16]. The finding of efficacy of an anti-CD137 agonist antibody in T1D (as we published [4]), and in CIA (murine RA [40]), and murine SLE [41] raises the interesting possibility that these protective effects could be partly mediated by increasing the sCD137 levels (from targeting and enhancing the CD137 Treg subset). This can be tested in future studies. Another issue is how to understand the reported increased serum sCD137 seen in human RA and MS [17-19]. This could simply represent a homeostatic response of the immune system; as we have shown, increased serum sCD137 is actually protective in NOD B10 Idd9.3 mice [5]. An alternate, interesting possibility is that the increased serum levels in these diseases do not reflect sufficiently increased concentration at the anatomic site of disease. We will address this in future studies by attempting site-specific delivery of sCD137.

The fact that sCD137 can actively suppress CD4 T cells raises interesting mechanistic questions: how can membrane-bound CD137 activate T cells while soluble CD137 suppresses them? To begin to address this issue, we performed studies of sCD137 structure in its native form. We show that, prior to the previous reports suggesting sCD137 is a trimer [37], sCD137 exists primarily as a disulfide linked dimer. Shao et al. suggested that sCD137 forms a trimer in solution, based on size exclusion chromatography (SEC) [37]. However, molecular weight estimates from SEC can be skewed significantly if the protein is elongated or if it interacts with the column matrix. In contrast, we used sedimentation velocity analytical ultracentrifugation to
analyze both the size and shape of sCD137, revealing that the predominant species in solution is a dimer (Fig 6). Consistent with our results, prior studies have shown that sCD137-Fc does not induce activation of APCs; CD137L cross-linking with immobilized CD137-Fc is essential [42-44]. Cross-linking at least two trimeric CD137L was required to elicit significant stimulatory costimulation [45]. Molecular modeling of CD137L showed that a single extracellular cysteine residue could form a disulfide bond and that cross-linking CD137L in vitro formed dimers of trimers [45, 46]. By contrast, we hypothesize that dimeric sCD137 binds to CD137L on T cells and prevents CD137L trimers from dimerizing. Then, by an undetermined mechanism sCD137 initiates inhibitory signaling through CD137L on CD4 T cells, as demonstrated by suppression of CD4 T cell proliferation by both sCD137-Fc (Fig 4) and recombinant sCD137 (Fig 5). Other well-known immune signaling molecules differ in their stimulatory effects according to whether they are plate/membrane bound or soluble—the most notable being anti-CD3 antibody, which can stimulate Treg-mediated suppression in soluble form but not when plate bound. Similarly, sCD137L cannot induce proliferation in CD3 stimulated T cells, while plate bound CD137L can [45]. Our results indicate that an inhibitory signal is generated by dimeric sCD137, but further biochemical studies will be needed to find the mechanism of this effect.

In summary we have shown that sCD137 can actively regulate effector T cells, which is sufficient to prevent progression to diabetes in NOD mice. The production and secretion of sCD137 is a novel mechanism by which T regulatory cells can immunoregulate pathogenic effectors cells and avert diabetes. The adaptation and use of naturally-occurring, soluble isoforms of immune signaling molecules for the purpose of immunosuppression, is a promising alternative or adjunct to current immunosuppressive therapies.
Acknowledgements

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Author roles: KK: performed experiments and assisted in writing paper. KB: performed experiments and assisted in writing paper. DA: performed experiments and assisted in writing paper. YW: performed experiments, maintained mouse colony. RM: provided reagents, intellectual input. MJ: performed experiments and assisted in writing paper. JH: performed experiments. AH: performed experiments and assisted in writing paper. WMR: PI, planned and discussed experiments, wrote paper.
References


Figure Legends

Figure 1: Construction of a CD137 lentiviral vector and production of recombinant sCD137 protein in vitro. (a) Schematic of the lentiviral vector, LeGO-iG2-sCD137, used to express soluble protein. Mouse sCD137 cDNA was inserted between the unique restriction sites EcoRI and NotI. (b) Transduced 0.5x10^6 NIH3T3 (left) or HEK293 (right) cells were sorted based on medium or high EGFP expression. The transduced cells were initially cultured in DMEM for three weeks and stable EGFP expression was assessed using a FACSCalibur flow cytometer. (c) Mouse NIH3T3 fibroblasts and human embryonic kidney (HEK293) cells were transduced with LeGO-iG2-sCD137 lentiviral particles. The sorted cell lines were checked for sCD137 expression by ELISA. One representative example of multiple transductions.

Figure 2: Purified sCD137 prevents diabetes in vivo, reduces pancreatic islet infiltration, and preserves pancreatic beta cells.

a) 6-8 week old female NOD mice were treated with either PBS or with 200 μg of recombinant sCD137, once a week for four weeks (n=8 per group, pooled from two separate experiments; in each experiment the mice were from litters born the same day and split evenly between experimental and control groups). b) Mice were sacrificed after 225 days or at the onset of diabetes (see methods for diabetes screening and diagnosis). The pancreas was stained using hematoxylin and eosin. One representative pancreatic section for PBS treated NOD and sCD137 treated NOD (40x magnification) is shown. The slides were blindly scored as described in the methods and the mean insulitis score is shown on the right. c) Serial sections were cut from
pancreatic tissue from all of the mice in Fig 2a, and stained for insulin or glucagon as described in the methods section. Representative tissue sections from three different mice are shown from the sCD137 treated group (left) and the control group (right). d) The pancreatic islet area staining for insulin and glucagon was quantified using ImageJ software as described in the methods section. Insulin to glucagon ratio was quantified by dividing the total insulin positive area by the total glucagon positive area for each individual mouse. Control group, n=8; sCD137 group, n=7 (the one diabetic mouse from the sCD137 group was excluded). P values were calculated using the logrank statistic (a), mann-whitney test (b) or unpaired t-test (d) in GraphPad Prism.

**Figure 3: Blockade of CD137L abrogates transwell, contact independent suppression mediated by CD137<sup>pos</sup> Tregs.**

NOD CD4<sup>pos</sup>CD25<sup>neg</sup>CD137<sup>neg</sup> T cells and CD4<sup>pos</sup>CD25<sup>pos</sup>CD137<sup>pos</sup> Tregs were FACS sorted from 5-7 week old NOD mice. 100,000 CD4<sup>pos</sup>CD25<sup>neg</sup>CD137<sup>neg</sup> T cells were plated in the bottom of a 96 well transwell plate either alone (first two columns) or with 50,000 CD4<sup>pos</sup>CD25<sup>pos</sup>CD137<sup>pos</sup> T regulatory cells (1:2 Treg:Teff ratio) in the top well (last 3 columns). 50,000 CD3/CD28 beads were added to the bottom and to the top of the plate. 20μg/ml of CD137 ligand blocking antibody (n=4 experiments) or IgG2a isotype antibody (n=3 experiments) were added to the bottom wells in columns 2, 4, and 5. The cells were pulsed with 3H labeled thymidine on day 3 and harvested after 16 hours. Statistical analysis was performed using GraphPad (unpaired t-test.)

**Figure 4: Soluble CD137-Fc actively suppresses T cells in an APC and Treg independent manner.**
NOD CD4^{pos}CD25^{neg}CD137^{neg} T cells were sorted from 6-9 week old NOD female mice. 50,000 CD4^{pos}CD25^{neg}CD137^{neg} T cells were cultured with 20,000 CD3/28 beads and 1μg/ml of soluble CD137-Fc (n=6). In some wells (as indicated), 20μg/ml of either CD137L blocking antibodies or isotype control antibodies (n=4) were added. All cells were plated in triplicates, pulsed with 1 uCi ^3H/well on day 3, and harvested after 16 hours. The unpaired t-test was performed using GraphPad.

Figure 5: Purified recombinant sCD137 suppresses CD4 T cell division in vitro: (a, b) CD4 T cells were CD4 magnetic bead purified, labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated as described in the Methods. After three days culture, cells were harvested and stained with anti-CD4. The amount of cell division was assessed by FACS analysis of CFSE levels. (a) One representative of four independent experiments for CFSE dilution without sCD137 (left) and with sCD137 (right) are shown. (b) The percent cells within each cell subset was calculated and graphed as histograms (n=4 experiments). (c) The cells were cultured as above and after three days, harvested and stained with CD4 (FITC) and propidium iodide (PI) (n=3). The unpaired t-test was performed using GraphPad.

Figure 6: Recombinant sCD137 protein is glycosylated and exists primarily as a dimer.

Western blot was performed on purified and unpurified sCD137 protein (from transduced HEK293 high-EGFP sorted cells) and separated on a 12% Tris-Glycine SDS-page gel under non-reducing (-DTT, lanes 1-6) or reducing (+DTT, lanes 7-10) conditions. Purified sCD137 of different concentration (0.45μg, 0.75μg), or an unpurified aliquot of equal volume was loaded in each lane. Low molecular weight protein standards served as size markers (lanes 1 and 6). 0.2μg of CD137-Fc fusion protein served as an antibody specificity control (lanes 5 and 10). (b)
Analytical ultra-centrifugation (AUC) was performed on purified sCD137 from HEK293 high-EGFP cells. One OD of protein in PBS was used as the starting concentration. 500μL of sCD137 was sedimented overnight. The sCD137 multimers were characterized by sedimentation velocity and the data deconvoluted to produce sedimentation coefficient distribution plots and estimated molecular weights using the c(s) analysis in the program SEDFIT. The peak labeled with an asterisk corresponds to likely degradation products, and the shoulder on the main peak labeled with an arrowhead is sCD137 monomer.
Figure 1

(a) LeGO-iG2-sCD137 (8.73 kb)

(b) NIH3T3 and HEK293 cell lines expressing EGFP with different sorting levels.

(c) sCD137 levels in HEK283 and NIH3T3 cells with high, medium, and unsorted expression.
Figure 3

![Bar chart showing CPM (10^4) for CD4^posCD25^negCD137^neg and 1:2 with CD4^posCD25^posCD137^pos. The chart includes a table with CD137L block and Isotype block, indicating the presence (+) or absence (-) of an effect. The chart also shows a *P=0.001 significance note.]
Figure 4

![Graph showing CPM levels for different conditions.](image)

<table>
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<tr>
<td>sCD137-Fc</td>
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</tr>
<tr>
<td>CD137L block</td>
<td>+</td>
</tr>
<tr>
<td>Isotype block</td>
<td>+</td>
</tr>
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</table>

*P<0.0001
Figure 5

a. Without soluble CD137

b. With soluble CD137

b. Percent of cells

Number of cell divisions

C. % PI positive

Unstimulated
Stimulated
Stimulated + CD137

*P=0.0008

*P=0.02

*P=0.01

*P=0.02

*P=0.04

*P=0.01

*P=0.01
Figure 6

<table>
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<tr>
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<tr>
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<tr>
<td>2</td>
<td>0.45 µg of purified scID137 HEK293</td>
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<tr>
<td>3</td>
<td>Unpurified scID137 HEK293</td>
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<td>4</td>
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<td>0.2 µg of purified CD137-Fc</td>
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<tr>
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<td>BioRad prestained SDS-PAGE standards, Low Range</td>
</tr>
<tr>
<td>7</td>
<td>0.45 µg of purified scID137 HEK293 + DTT</td>
</tr>
<tr>
<td>8</td>
<td>Unpurified scID137 HEK293 + DTT</td>
</tr>
<tr>
<td>9</td>
<td>0.75 µg of purified scID137 HEK293 + DTT</td>
</tr>
<tr>
<td>10</td>
<td>0.2 µg of purified CD137-Fc + DTT</td>
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Figure 6b

- Homodimer (54.3 kDa)
- Higher Order Oligomers
- Aggregates
Chapter 4: Summary of Research, Discussion, and Future Directions
Chapter 4: Summary of Research, Discussion and Future Directions

Type 1 diabetes (T1D) has no cure to date. Better technologies are becoming available to manage the disease symptoms, however it is unclear if these technologies will influence the fatal complications linked to T1D. Further, T1D is a huge burden on the health care system. Therefore, better therapeutics for the treatment of T1D that result in halting of disease progression, preserving the remaining islets, and increasing naturally produced insulin are needed. Two therapeutics, agonistic anti-TLR4/MD-2 monoclonal antibody (TLR4-Ab) and sCD137, which have the potential to reverse T1D, are currently being evaluated in our lab (described above in Chapters 2 and 3). TLR4-Ab treatment has shown great promise in not only prevention of disease, but in halting disease progression of new onset T1D and reversing the disease course, as manifest in significantly decreased BG after treatment. TLR4 antibody treatment allowed the islets to go through a “recovery” phase as evidenced by hypertrophy and increased insulin. Further, TLR4-Ab decreased the number of severely infiltrated islets, and potentially converted destructive insulitis to benign insulitis. Evidence from these studies suggests that the TLR4-Ab causes immune cell tolerization through the reduction of inflammatory cytokines (TNF-α and IFN-β) and expression of co-stimulatory molecules (CD80, CD86 and CD40), while increasing anti-inflammatory cytokines (IL-10). Further, the ratio of effector T-cells (CD4+ and CD8+) to Tregs is significantly reduced in TLR4-Ab treated mice. The increase in Tregs occurred in both the periphery and in the pancreatic islets as shown by Foxp3 immunohistochemistry. T-cell proliferation to both antigen specific (BDC2.5 transgenic T cells) and non-antigen specific activation were diminished with TLR4-Ab treatment. This further suggests that APCs (CD11c+) have a reduced capacity
to cause activation of T-cells and this was clearly shown by transferring pre-diabetic T-cells into NOD mice lacking T and B cells (NOD.scid). Pre-treatment of NOD.scid mice with the TLR4-Ab therapy targeted only to APCs, significantly protected mice from induction of T1D, while decreasing insulitis and increasing Tregs. These results clearly demonstrate that therapy directed at innate immune stimulation has a profound effect on disease and the adaptive immune response.

In the second therapeutic approach, soluble CD137 (sCD137), originally shown to be an immunosuppressive molecule secreted by Tregs, is an important negative feedback loop that could possibly control disease. We found that there is decreased serum sCD137 in NOD mice compared to disease resistant NOD.\textit{Idd9.3} congenic mice. Therefore, our lab aimed to correct this decrease by creating a purified recombinant version of mouse sCD137, through lenti-viral transduction of HEK293 cells which
produced large amounts of secreted sCD137 protein. The recombinant protein was affinity column purified using the 3H3 (anti-CD137) antibody. Purified sCD137 showed biological activity by preventing disease in NOD mice, decreasing insulitis, and preserving insulin positive islets. sCD137 reduced CD4+ T-cell proliferation in a CD137L dependent and APC independent manner. It has been previously reported the sCD137 induced activated induced cell death[1], however we did not see any increased cell death after sCD137 treatment. Finally, it was previously suggested that sCD137 was a trimer[2]. However, we showed that purified sCD137 is a disulfide linked dimer through both western blotting and analytical ultra-centrifugation (AUC). Therefore, sCD137 can play an important regulatory role in decreasing CD4+ T-cell responses in the setting of T1D. In the next two sections we will discuss the implications of TLR-4 Ab and sCD137 treatments respectively.
I. Discussion and Future Directions: TLR4-Ab therapy.

(I) TLR4-Ab prevents T1D when given to young NOD mice (4-6 weeks old)

This result was not surprising as other TLR4 agonists have shown prevention in T1D as previously discussed above (e.g. CFA, LPS). However, an interesting observation was that while TLR4-Ab induced a significant delay in T1D when only given once, it did not offer significant protection from end-stage disease (data not shown). On the other hand, two treatments one week apart caused significant disease protection, further suggesting that TLR4-Ab must be administered repeatedly in order to induce immune cell tolerization. It has been published that stimulation through TLR4 causes specific expansion or deletion of different TCR repertoires based on the magnitude of stimulation. Vaccine adjuvants revealed the dynamics of a unique mechanism of antigen-specific Th cell selection[3]. “Although all adjuvants induced clonal dominance in the helper T cell compartment, the antigen-specific TCR repertoire was differentially skewed across the different formulations. Adjuvants CpG (TLR-9) and monophosphoryl lipid A (MPL, TLR4) reset the TCR-based selection threshold to recruit and propagate clonotypes with higher peptide MHCII binding”. The clonotypes were thus skewed to specific TCRα and TCRβ chains. To this same regard different adjuvants showed preferential expansion of clonotypic T-cells and each adjuvant thus could change the overall adaptive immune system. It was further shown that this is independent of antigen dose. Altering or skewing the TCRs with different vaccine adjuvants or inflammatory mediators may thus have beneficial regulatory capacity in adaptive immune responses[3]. In the setting of T1D, TLR4-Ab may also induce skewing of TCRs to specific clonotypes, which could cause deletion or anergy of specific
diabetogenic TCRs that are involved in the destruction of islet cells. This could happen in a variety of ways: 1) The activation of TLR4 could cause differential cytokines to be produced (IL-10) which skews the adaptive immune response or recruitment of specific cells to the sites of inflammation to become expanded 2) differential presentation of antigens (CD80/CD86/CD40) and interactions between T-cells and DCs determines TCR clonotype expansion[4] 3) TLR4 is expressed on bone marrow cells which could cause a change in hematopoietic skewing or exhaustion[5]. These differences have been shown to be important in clonal expansion of particular immune responses. Another possibility is that, since TLR directed adjuvants skew cells towards those that have high peptide MHC class II binding, TLR4-Ab’s agonisitic properties could drive increased Treg production by this mechanism. In other words, since in the thymus, as previously discussed, high TCR binding affinity to self antigen causes T-cells to develop into Tregs, TLR4-Ab could increase the number of Tregs by driving up the number of high affinity T cells.

**Future Directions:** In order to determine if TLR4-Ab causes skewing of specific TCR selections, it would be possible to treat NOD mice with PBS, TLR4-Ab, or Ctrl-Ab and do clonotypic analysis on CD4$^+$ and CD8$^+$ T-cells. This could determine if there is a skewing of TCRs that could be potentially harmful in disease pathogenesis compared to “protective” TCR selections when treated with TLR4-Ab. Although this does not seem like the most likely explanation for disease prevention, it may shed light into how different TCR skewing / or deletion could affect disease outcome. The more likely scenario is that the TLR4-Ab is inducing Tregs. In this regard, the pancreatic sections from our prevention studies have been preserved and performing immunohistochemistry
for Foxp3 on these sections may show increased Tregs with treatment. This would not be unique to our TLR4-Ab as multiple treatments with low dose LPS has been shown to cause an induction of Tregs and prevention of T1D[6]. However, it has been reported that LPS cannot reverse established disease which will be discussed more in depth later[2]. Experiments to determine if treatment with the TLR4-Ab causes induction of Tregs (iTregs) or proliferation of natural Tregs (nTregs) can be performed as will be discussed later. Finally, one experiment that is currently underway in our lab is to determine if Tregs are necessary for the prevention of disease. NOD mice were first treated with PC61 antibody (Treg depletion), followed by 2x TLR4-Ab treatments one week apart. The PC61 antibody was given every 4-5 days to ensure proper deletion throughout treatment with our TLR4-Ab. If Tregs are necessary for the prevention of disease, we should observe increased disease incidence in mice treated with PC61. Further, the TLR4-Ab and Ctrl-Ab may cause cross-linking with other receptors due to their ability to bind FcRγ, as discussed in the introduction chapter. Therefore, to remove this complication of the Fc region causing cross-linking, Fab or F(ab)₂ regions will be purified. To create these regions, TLR4-Ab and Ctrl-Ab will be enzymatically digested using papain (Fab) or pepsin (F(ab)₂) and purified in accordance with Cheung et. al[7] and be tested for diabetes prevention and reversal.

(II) TLR4-Ab Reverses New Onset T1D and Decreases Insulitis

The ability of TLR4-Ab to reverse established disease was a rather striking result considering LPS given later in disease onset could not cause reversal of T1D. This was demonstrated in Caramalho et. al “LPS has no effect on “prevention” beyond 10 weeks, and has no efficacy in reversing established disease”[6]. One possible explanation
could that LPS has an extremely short half life in vivo. This suggests that LPS has a limited time to cause tolerization of cells that are expressing TLR4. In sharp contrast to this TLR4-Ab (UT12) was able to cause long term protection from septic shock lasting at least 9 days[8]. This induction of long term tolerance could then significantly impact the stimulatory capacity of APCs to cause proliferation or activation of effector T-cells.

TLR4-Ab has a lower “plateau” of activation in comparison to LPS (data not shown). This suggests that TLR4-Ab is not as stimulatory or pro-inflammatory as LPS.

However, interestingly enough, both pathways seem to be involved (MyD88 dependent and MyD88 independent (TRIF)) by the secretion of both TNF-α and IFN-β upon TLR4-Ab stimulation. LPS and TLR4-Ab may mediate differential signaling for several reasons: 1) Affinity of binding to TLR4/MD-2, 2) involvement of CD14, 3) binding site difference (shown to be important for TLR4-Ab and Ctrl-Ab stimulation of TLR4/MD-2), and 4) internalization of the receptor. These differences would cause changes in their signal “strength”, recruitment of adaptor molecules, and downstream gene activation.

The first area that we explored was if TLR4-Abs (TLR4-Ab and Ctrl-Ab) can block LPS signaling or compete for the same binding site. Our results show that Ctrl-Ab binding to TLR4/MD-2 does not inhibit LPS signaling (data not shown). However, it is much more difficult to determine if the TLR4-Ab binding to TLR4/MD-2 inhibits signaling for multiple reasons. One, the TLR4-Ab causes tolerization of cells upon exposure, therefore it is hard to distinguish if the TLR4-Ab is blocking LPS signaling or acting through different negative regulators of TLR4. This in turn makes it difficult to determine the binding sites of LPS and TLR4-Ab; it is possible that the TLR4-Ab and LPS bind to the same region of TLR4/MD-2. Another issue is whether TLR4-Ab gets internalized. For example, MPL
has impaired induction of CD14-dependent TLR4/MD-2 dimerization compared with lipid A, which resulted in decreased TNF-α production and decreased internalization of TLR4. In contrast, MPL was comparable to lipid A in CD14-independent MyD88-dependent TNF-α production and TRIF-dependent responses including cell surface CD86 up-regulation and IFN-β induction. These results suggest that internalization is important for signaling and CD14 may be a driving force, although these issues need to be explored with TLR4-Ab [9]. We have further gone on to show that the TLR4-Ab has efficacy at reversing disease at a blood glucose level ~250mg/dL (data not shown). Also, to this same effect we have shown that TLR4-Ab has the ability to lower blood glucose with some success up to 400mg/dL (data not shown). These two pieces of evidence suggests that TLR4-Ab has a profound effect on changing the adaptive immune response in regards to destruction of insulin producing cells. Further, severe insulitis is reduced in mice treated at the time of diabetes (BG~200mg/dL) and showed reversal of T1D. This suggests that immunoregulatory mechanisms are likely at play in the reversal of disease. Interestingly, data suggests that there are distinct differences in disease reversal between mice that have received multiple re-treatments with TLR4-Ab and those that only needed two treatments. We have data suggesting that mice receiving only the initial two treatments of TLR4-Ab may go through T-cell anergy and/or increases in regulatory T-cells; while mice that receive multiple re-treatments of TLR4-Ab, but never progress to endstage disease, still have active disease and the reduction in blood glucose seen may be through a different unidentified process. This was demonstrated by mice only receiving the initial two treatments were unable to transfer disease into NOD.scid recipients through CD4+/CD8+ T-cell transfers, while CD4+/CD8+
T-cells from mice that did not reach endstage diabetes but received multiple re-treatments can transfer disease into NOD.scid recipients (data not shown). There are some published reports suggesting that TLR4 agonists independently affect blood glucose metabolism. Frisard et. al demonstrated that activation of TLR4 with low (metabolic endotoxemia) and high (septic conditions) doses of LPS results in increased glucose utilization and reduced fatty acid oxidation in skeletal muscle[10]. It is possible that TLR4-Ab allows muscle cells to uptake glucose. Therefore, the quick drop in blood glucose we see in our TLR4-Ab treatment could be a result of this phenomenon, but this would not explain the long term effects on T1D disease reversal. It is possible that this sort of initial relief of hyperglycemia could reduce the stress on islet cells. This relief could then cause islet cell hypertrophy. However, this is probably not likely, due to the fact long term reversal of disease is found and there is immunological regulation at play. Future metabolic studies could investigate this mechanism in TLR4-Ab therapy.

**Future Directions:** Many experiments can be done to distinguish the differences between LPS and TLR4-Ab. One approach, which could be performed in collaboration with Andy Herr’s lab, is using surface plasma resonance to distinguish the difference in binding affinity between TLR4-Ab and LPS. However, difficulties may arise due to the fact TLR4 and MD-2 must be in complex for the TLR4-Ab and LPS to bind. To complicate matters further LPS needs to bind to LBP and requires CD14 to shuttle it to its binding site (see TLR4 signaling pathway above). Therefore, testing the difference in binding affinity would not be trivial. In order to determine if internalization is a key to differences in LPS and TLR4-Ab, we will use fluorescent LPS and label our TLR4-Abs with fluorochromes (TLR4-Ab and Ctrl-Ab). Further, fluorochromes that are sensitive to
pH changes can be attached to the TLR4-Ab and Ctrl-Ab to determine the amount of internalization. If these techniques do not work, other techniques to determine internalization will be used and the techniques are reviewed elsewhere[11]. This will allow us to detect internalization differences which in turn could lead to the explanation of the decreased activation plateau. Further, this can be done using Imagestream to see co-localization with specific endosomal markers. Another technique to see if internalization affects signaling capacity is to couple TLR4-Ab to different sized nanoparticles. The different sized particles will either be readily internalized or will be too large to be internalized. This would thus determine if internalization is important for the differences in cell signaling between TLR4-Ab and Ctrl-Ab. Finally, TLR4 signaling differences could be detected by using thioglycollate elicited macrophages, stimulating them with TLR4-Ab or LPS and looking by western blot or flow cytometry at the activation of different key adaptors and molecules (see TLR4 signaling pathway figure for molecules of interest). Further it may be that the difference in signaling or internalization/recycling of the TLR4/MD-2 receptor could affect the amount of TLR4/MD-2 on the surface of the cells. This will be tested by identifying surface expression in vivo before, during, and after two TLR4-Ab treatments one week apart along with the proper controls. The difference in TLR4/MD-2 expression would then affect the ability of the cell to signal.

Another interesting aspect of reversal of disease is the induction of Tregs and/or T-cell anergy. We can test whether CD4+ and CD8+ T effectors (Teffs) are rendered tolerogenic or anergic, or whether T regulatory cells (Tregs) are increased in function. Infectious tolerance can be tested by mixing titrated ratios of CD4+ and CD8+ cells from
Ctrl-Ab and TLR4-Ab treated mice, and determining whether the TLR4-Ab treated cells can protect from disease transferred by Ctrl-Ab treated cells. This experiment will demonstrate whether TLR4-Ab acts via transferable immune regulation[12]. To test whether Tregs are involved in TLR4-Ab mediated protection, FACS purified T effectors (Teffs) from TLR4-Ab or Ctrl-Ab treated NOD.Foxp3-GFP mice could be transferred into NOD-scid mice along with sorted CD8^+ cells. If TLR4-Ab treated CD4^+ Teff recipients become diabetic similar to Ctrl-Ab recipients, and in contrast to lack of disease transferred by the TLR4-Ab treated unfractionated CD4^+ cell population, this would implicate Tregs (CD4^+CD25^+Foxp3^+) in the mechanism of TLR4-Ab mediated transfer protection. If Treg subsets are not implicated in disease protection, which based on data from Chapter 2 we believe is the likely case, Teff cell subsets could be assessed to determine whether CD4^+ and/or CD8^+ cells are “tolerized” by TLR4-Ab treatment.

(III) TLR4-Ab reduces insulitis and improves insulin production in islets

TLR4-Ab mediated control of islet inflammation led to decreased insulitis and improved islet histological scores. Furthermore, as previously discussed it has been shown upon acute reversal of disease or relieving stress on the islets, the islets had a slight ability to proliferate and decrease insulin dependency, but in most cases this was transient[13]. We deduced that because there was no difference in the islet α cells content (indicating an islet was present before disease development because α cells are key features of islets and do not get destroyed upon T1D induction) between the mice this endogenous increase is not due to the “regeneration or de novo” synthesis of islet, but in fact hypertrophy of β-cells. In our model we could be preserving islets that enter the “honeymoon” phase during disease development. Finally, there is also recent evidence
that certain soluble factors can affect the amount of β-cell proliferation, which has been reviewed elsewhere[14, 15]. Some of these soluble factors have been found to be expressed after adjuvant stimulation and increase Reg genes, which are responsible for islet hypertrophy. However, in our hands TLR4-Ab does not stimulate the growth of β cells directly (data not shown) when given before disease onset. We therefore believe that the reduction in islet inflammation is due to increased regulation of immune cells and possibly a secondary factor such as cytokines. We have shown that IL-10 and Tregs are upregulated after stimulation with TLR4-Ab. Both IL-10 and Tregs are known inflammatory mediators, but it has also been shown that IL-10 can increase β cell proliferation along with other cytokines (e.g. IL-2, IL-4, IL-6, IL-22, IL-33)[16, 17].

**Future Directions:** We have recently shown that upon disease reversal IL-4, IL-6, IL-22, and IL-33 are all increased in TLR4-Ab treated mice reversed of T1D at the end of our experimental time point. Although, the cytokines mentioned above have been shown to be protective in T1D disease prevention, more tests need to be done to confirm these results as well as their role on β cells and Reg gene upregulation (data not shown). Due to the ability of TLR4-Ab to reduce insulitis in NOD mice, it would be interesting to see what the functional differences are between TLR4-Ab treated (mice reversed of T1D) cellular infiltrates compared to Ctrl-Ab treated cellular infiltrates in the islets. To do this a technique called laser capture microdissection will be utilized. This technique allows specific sections of frozen tissue samples to be taken with great precision and “boundaries”. Therefore, it would be possible to dissect out only the cellular infiltrates surrounding the islets as well as the islets themselves. This technique further allows the sections to immediately be processed for RNA to be isolated and
would give us the ability to run RNA-sequencing on the samples mentioned above. This would also lead into insights in what is different in the infiltrating cells and islets. In line with this experimental technique it would interesting to reverse T1D using our TLR4-Ab and at various time points during reversal harvest islet β cells and isolate RNA to see which genes are dysregualted between reversed mice and the following groups: pre-diabetic, control untreated, blood glucose ~200mg/dL, and Ctrl-Ab treated NOD mice. Although it has been shown previously that Reg genes are important for islet β cell proliferation, this may lead to discovery of new genes involved in β cell response and proliferation in an inflammatory environment. Another key issue that has not been resolved in the cellular infiltrates is to determine if there are differences in cell types between the TLR4-Ab and Ctrl-Ab treated mice. To determine if there are differences in cellular infiltrates, flow cytometry of the pancreas and/or immunohistomechistry can be preformed checking for distinct cellular subsets. We have shown differences in the Foxp3 regulatory subsets in the infiltrated islets, but there may be differences in or cell populations or subsets. Finally, to determine the differential effects on islet cell proliferation the cytokines shown above will be cultured with either an islet cell line or primary islets and islet cell increases or death will be determined by counting, Ki67, and PI.

(IV) Kinetics of TLR4-Ab treatments shows increased innate immune cells early on after treatments with a subsequent increase in Tregs. These innate cells were further shown to have decreased co-stimulation molecules, inflammatory cytokines and increased IL-10 when treated with TLR4-Ab.
To start this section, we have seen no adverse effects with TLR4-Ab stimulation or multiple rounds of re-treatment (observational). There is production of inflammatory cytokines early on in the response, but it is reduced in comparison to LPS and further decreased upon tolerization (data not shown). However, applying this to humans is more difficult as TLR4 is known to cause cytokine storms[18]. Therefore, fine mapping the activation site of TLR4/MD-2 where our antibody binds will be useful to translate this therapy into the clinic. It has been previously demonstrated by many groups that low signaling in the TLR4/MD-2 complex can cause induction of regulatory responses instead of activating responses[19]. The induction of tolerance in these innate immune cells can also cause a shift in the balance of regulatory cells compared to effector cells. As discussed above, TLR4-Ab may cause long-term tolerance in innate immune cells which enhances their ability to produce Tregs. In our kinetic studies this increase in innate immune cells could be derived from the bone marrow. In other studies it has been shown that activation with TLR4 can causes a flux of cells to exit the bone marrow which in turn has a great affect on the ability to induce Tregs as well as immunosuppressive activity[20]. Most notable, of all cells that can be initiated by TLR4 ligation in the bone marrow, is the subset designated “myeloid derived suppressor cells” (MDSCs)[21]. MDSCs have been most studied in cancer biology, but have been shown to have potent effects on decreasing antigen specific T-cell responses. Therefore, the increase we see in the CD11b+ subset may be due to increased MDSCs, which could then in turn act to generate Tregs and/or inhibit immune activation of T-cells. Monocytic and granulocytic are two subsets of MDSCs, both can prevent T-cell activation[21]. These cellular subsets found in the kinetic studies could then account for the differences
in not only disease progression, but also the amount of Tregs. However, in order to classify these cells as MDSCs their function must be tested \textit{in vitro} (see future directions). If these cells do turn out to be phenotypically similar to MDSCs it may account for the acute disease reversal within a matter of a few days as well as the long lasting increase in Treg cells which could control the disease once MDSCs have disappeared. Another interesting aspect of our kinetic study showed that the increase in Tregs was not due to direct activation or proliferation of T-cells. This was followed up by showing that there are no differences in the number of Foxp3$^+$ cells after TLR4-stimulation (data not shown). There is however, evidence that highly purified CD4$^+$CD25$^+$Foxp3$^+$ cells can proliferate and have enhanced effector functions when stimulated with TLR4 (LPS). In our hands we do not see this effect with bulk CD4$^+$ cells, however more in depth studies may need to be done (see below). This led us to believe that TLR4-Ab was causing tolerization of innate immune cells and led to downstream effects on the adaptive immune response. It has been shown that gut leakiness occurs during the onset of T1D and this allows the bacteria in the gut to increase systemically. This suggests that there is also in increase in total levels of LPS during onset of T1D. Therefore, the changes in gut microbiota with TLR4-Ab treatment may influence disease susceptibility. Our treatment with TLR4-Ab might affect the ability of LPS from the gut to cause pro-inflammatory signals and increase anti-inflammatory cytokines.

\textbf{Future Directions:} The induction of MDSCs is an interesting topic that has been relatively untouched in the T1D literature. It will be important to show phenotypically if these cells exist \textit{in vivo} and can control antigen specific responses. In order to test this
NOD mice will be injected 2x with PBS, TLR4-Ab, or Ctrl-Ab. 24 hours after the final injection spleens will be harvested and stained for the markers listed above for MDSCs. These cells will then be sorted based on those markers and once sorted multiple tests will be run to determine their function to control antigen specific T-cell responses. The first test will be antigen presentation to BDC2.5 TCR transgenic T-cells. The various MDSCs will be co-cultured with BDC2.5 mimetic peptide and CSFE-labeled BDC2.5 T-cells. This will determine whether the cells that are leaving the bone marrow are in fact regulatory in antigen presentation. The second experiment will be to determine if this regulation is contact dependent or independent. MDSCs will be placed on the top well in the transwell assay and CD4+ CFSE labeled T-cells in the bottom along with stimulation using CD3/CD28 beads. If MDSCs are able to prevent proliferation of the CD4+ this indicates that soluble factors are involved and being secreted by MDSCs. The next step will be to determine which soluble factors are causing regulation of these cells (e.g. IL-10, TGF-β). Finally, if contact dependent suppression is indicated, MDSCs will be assessed for surface markers indicated in decreased proliferation or anergy (e.g. PDL-1, CD80). If MDSCs from TLR4-Ab treated mice can control proliferation of CD4+/CD8+ T-cells it may have in vivo implications. Transfer of MDSCs into either NOD.scid recipient mice along with CD4+/CD8+ pre-diabetic T-cells or adoptive transfer into NOD mice will be used to assess if these cells can reverse established disease. MDSCs will be targeted using other approaches in vivo. NOD.CD11b.DTR mice will be obtained and depletion of CD11b+ cells will take place before and after stimulation with TLR4-Ab. Our prevention model will be used to assess the outcome on disease pathogenesis. It has been shown that depletion of CD11b+ cells early in disease does
not affect incidence of T1D. Complications and caveats arise in this experiment because CD11b\(^+\) cells contain not only MDSCs, but macrophages, granulocytes, and some DCs (although DCs have been shown to be minimally affected by DT treatment). Further, if CD11b\(^+\) and MDSCs are not implicated CD11c\(^+\) cells may be the key cellular target of TLR4-Ab to decrease antigen specific responses, which will be discussed in an upcoming section. Finally, the role of the gut microbiome of TLR4-Ab treated mice needs to be evaluated. This could be tested with co-housing studies and antibiotic therapy during TLR4-Ab treatment. However, antibiotic studies would be confounded by the fact that antibiotics themselves have been shown to prevent T1D under certain conditions or exacerbate T1D\[22\]. The nature of the gut microbiome could be assessed before and after treatment as well as against diabetic controls. Another cell type that has been under studied in our model is NK cells. NK cells can produce large amounts of IFN-\(\gamma\) upon stimulation and modulate DC function\[23, 24\]. It has been shown that NK cell activation can lead to the maturation of DCs through various soluble factors such as IFN-\(\gamma\) and TNF-\(\alpha\) as well as NK cell cytolysis of immature DCs\[25\]. Those NK cells allow for effective priming of Th1 cells\[26, 27\]. Experiments on NK cell numbers and function need to be assessed. Exhaustion of these cells from the bone marrow because of the increase in CD11b\(^+\) cells (possibly MDSCs) is possible and will be experimentally determined. In some cases NK cells have been classified as subsets: tolerant, cytotoxic, or regulatory\[28\]. These subsets will be assessed in our 2x TLR4-Ab treated model. Further, NK T-cells can also be “regulatory” cells and secrete large amounts of IL-10 inducing antigen specific regulatory cells\[29\]. Similar
experiments to determine NK-T cell numbers as well as function will be determined in subsequent experiments.

**(V) TLR4-Ab treatment causes a change in effector T-cell to Treg ratios and decreases antigen specific responses.**

Tolerization in an asthma model showed T-cells have decreased antigen specific responses after TLR4-Ab treatment (Matsushita et al) [30]. Given that TLR4-Ab can stably reverse T1D, it is possible that the induction of Tregs as well as decreases in T-cell proliferative responses is a major mechanism. One question that remains open is whether the increase in Tregs seen is due to induction of iTregs or proliferation of nTregs. nTregs are known to express Helios, a member of the Ikaros transcription family, and found as a dominate marker of thymic-dervied Tregs. Helios is not expressed in T-cells that are derived *in vitro* through TGF-β induction or in antigen specific Foxp3 cells induced *in vivo* by antigen feeding[31]. Both of these cellular subsets express Foxp3 and both have the ability to suppress effector T-cells. However, it seems unlikely that our TLR4-Ab causes proliferation of nTregs (Helios⁺Nrp-1⁺) and reasons for this are as follows: 1) we cannot detect TLR4 on the surface of T-cells, 2) TLR4-Ab does not directly affect T-cell proliferation or activation, and 3) TLR4-Ab does not produce increases in Foxp3 *in vitro*. It is possible, however, though that a different cytokine milieu not found in our culture system could upregulate TLR4 on the surface of T-cells *in vivo*. However, it seems much more likely the iTregs are being generated. iTregs (Helios⁻Nrp1⁺) are usually generated in the presence of decreased co-stimulation and increased anti-inflammatory cytokines[32]. This then allows naïve CD4⁺ T-cells to become Tregs instead of effector cells. It is possible that Th17 cells are decreased and
Tregs are increased thus affecting the balance of regulatory cells in our model. This effect, however, would be different from what was previously seen, which showed that TLR4 signals inhibited the capacity of DCs to expand Th2 and Th17 cells without causing a phenotypic switch to Th1 cells [30]. However, it is unknown from this model if the reverse is true, ie can Th1 cells undergo phenotypic subset switch after TLR4 activation? Another key issue is it is currently unknown if mice in an inflammatory environment given TLR4-Ab would change phenotypic subsets differently compared to administering the TLR4-Ab treatment before disease onset. The decrease in the ability of DCs to cause cellular proliferation is likely due to multiple mechanisms in vivo which consequently prevents and reverses T1D. It is possible that TLR4 activation causes a decrease in the ability of DCs to present islet antigens. This decreased capacity to present islet antigens could then cause induction of islet specific Tregs. Another possible explanation is TLR4-Ab changes the DC’s ability to process antigen or uptake apoptotic islet cells. This inability to present antigens would thus affect the amount of different epitopes T-cells can respond to and lead to lower amounts of destruction in the islets.

**Future Directions:** The ability to change T-cell subsets would massively affect disease outcome as different T-cell subsets are known to hasten and prevent T1D. In order to determine if T-cells can be converted in vivo NOD mice will be injected 2x with TLR4-Ab, Ctrl-Ab, or PBS. A kinetic study (Day 0-Day28) will be used to follow the T-cell subsets and subsets will be assessed ex vivo from the production of cytokines using phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation along with Brefeldin A. Day 0 will give us a baseline reading for Th1(T-bet, IFN-γ), Th2(GATA-3, IL-4),
Th17 (RORγT, IL-17) and Tregs (Foxp3, IL-10) and these markers and cytokines will be followed throughout the time course. Along with this same experiment the differences in the Treg subsets, iTreg and nTreg will be assessed through the markers Nrp1 and Helios. From this experiment we will be able to assess if treatment with TLR4-Ab changes T-cell subset phenotypes and/or increases a specific subset of Tregs. Besides CD4⁺ T-cell tolerization after TLR4-Ab treatments of DCs, it is important to show if a similar phenotype is seen in CD8⁺ T-cells. To determine if CD8⁺ T-cells can proliferate after TLR4-Ab tolerization, OVA-specific CD8⁺ T-cells will be used along with sorted CD8⁺DCs. CD8⁺DCs will be feed OVA peptide before tolerization and then be tolerized using TLR4-Ab or Ctrl-Ab, while OVA alone will be used as a control. The OVA-specific CD8⁺ T-cells labeled with CFSE will then be co-cultured with the CD8⁺DCs. This experiment will be done identically to the CD4⁺ in vitro tolerization of antigen specific responses using NOD.BDC2.5 CD4⁺ T-cells. In order to assess if DCs are able to phagocytose antigens or present islet antigens to T-cells, in vivo and in vitro techniques will be used. To test the ability of DCs to phagocytose materials after TLR4-Ab stimulation, DCs will be tolerized with TLR4-Ab, Ctrl-Ab or PBS in vitro as previously shown and fluorescently labeled apoptotic cells will be introduced into the culture. The ability of DCs to uptake apoptotic cells will be assessed by the amount of fluorescent dye uptaken by each cell using Imagestream (this can distinguish between surface associated and intracellular material). Furthermore, if there are differences in uptake of antigen, looking at the relative amounts of each known islet β cell TCR using tetramer staining or TCR clonotypic analysis from mice reversed of disease using TLR4-Ab
treatment compared to Ctrl-Ab, or PBS may give us insight into the presentation of epitope spread.

**(VI) TLR4-Ab prevents disease transfer to NOD.scid mice**

TLR4-Ab induced protection when given to NOD.scid mice before pre-diabetic CD4^+/CD8^+ T-cells. This protection implies that TLR4-Ab has a direct effect on innate immune cells which in turn control adaptive immune responses. Further Tregs are increased in TLR4-Ab treated mice suggesting a role for APCs to cause changes in CD4^+ subsets. While two hours is a short time frame before transfer, the tolerization effect of the TLR4-Ab would have been experienced by the APCs as they experienced multiple injections while the T-cells would have experienced the effects of just one dose of the TLR4-Ab (show not be activating). TLR4-Ab treated NOD.scid mice lack B-cells and this suggests against a role for B-cells in TLR4-Ab reversal. It is important to note that we found the TLR4-Ab can cause proliferation of B-cells, but it does not appear that B cells are involved in protection from T1D (data not shown).

**Future Directions:** Further experiments on B-cells could include detailed studies of pre-treated B cells and their effect on disease transfer, and studies of Breg cells. Bregs have been shown to be able to halt disease progression through IL-10 production[33]. DC’s are another subset that might be acting in the NOD.scid transfer. DC depletion could test their role, but is technically difficult. We could initially try depletion with anti-PDCA-1 antibody, then TLR4-Ab treatment. As a second approach, we could use NOD.CD11c.DTR mice. NOD.CD11c.DTR/NOD BM chimeric mice could be treated with DT and then treated with TLR4-Ab to assess whether DCs are critical to TLR4-Ab
effect. This approach requires NOD.CD11c.DTR chimeric NOD mice to progress to new onset diabetes. This is because depleting DCs early can itself affect disease, whereas depleting them later may worsen disease[34]. These studies will thus allow us to determine if CD11c⁺ cells are crucial for T1D. However, it remains a possibility that other DC subsets are needed for disease protection with TLR4-Ab. Therefore, a mixed bone marrow chimera of NOD.CD11b.DTR mice and NOD.CD11c.DTR will be used. This approach will again have to wait until mice reach new onset T1D before elimination using DT can begin and TLR4-Ab treatment. This will show if both or one subset is crucial for disease protection. It is also possible to tolerize DCs in vitro and transplant them back into NOD.scid recipients along with CD4⁺/CD8⁺ T-cells. If these tolerized DCs affect disease outcome it will indicate DCs as the key cellular subset to establish disease reversal.

II. Discussion and Future Directions: sCD137 therapy

Recombinant soluble CD137 prevents Type 1 diabetes in NOD mice

We have shown sCD137 is an immune regulator that affects the onset of T1D. The creation of the sCD137 was through the use of a lenti-viral vector system in HEK293 cells followed by column affinity purification using anti-CD137 (3H3 clone). We further showed that CD137⁺Tregs secrete sCD137, and that sCD137 decreases proliferation of CD4⁺ T-cells through CD137 ligand interactions. Recombinant sCD137 causes a decrease in proliferation of CD4⁺ T-cells in vitro which resulted in fewer total cell cycles and no difference in cell death compared to stimulated controls. Further
characterization of the protein showed that it is a dimeric molecule held together by disulfide bonds and has glycosylation sites which may be important for signaling.

**Lenti-viral vectors in HEK293 cells produce functional sCD137 protein**

HEK293 (Human Embryonic Kidney 293) cells are a mammalian cell line that are easily transfectable with virus. This stable transfection with sCD137 lenti-viral vector has allowed us to sort on high producers of sCD137. What is most important about this is HEK293 cell are able to create disulfide bonds and conserves glycosylation unlike other cells lines or bacteria. These two aspects may be extremely important for signaling of sCD137 and will be discussed later. There is however concern with labeling the cells with the green fluorescent protein (GFP). In recent years it has been shown that GFP can be silenced and it is also toxic to cells[35]. Therefore, better constructs with different reporters as well as non-silencing elements are currently underway in our lab. Furthermore, it has been shown that BDC2.5 T-cells congregate in the pancreas after transfer into NOD.scid recipients. It may be possible to use these BDC2.5 T-cells transfected with lenti-viral vectors of sCD137 as a therapeutic approach to the treatment of T1D because they will home to the pancreas and secrete large amounts of sCD137, this in turn would cause decreased proliferation of diabetogenic CD4⁺/CD8⁺ T-cells. However, T-cells have proven difficult to transfect; therefore in collaboration with Punam Malik’s lab we have used a TMEW vector to transfect primary T-cells. These T-cells will increase the production of sCD137 and be able to control disease pathogenesis. Finally, using this lenti-viral vector system it may be possible to transfect other cells of the immune system that are able to prevent disease pathogenesis or that specifically home to the pancreas. This could be done in macrophages or DCs that are known to
be needed for retention of T-cells in the pancreas. These cells thus come into contact with effector T-cells often and if sCD137 was produced in a quantity that could prevent activation or halt proliferation of pathogenic cells, this could be a viable cellular therapeutic in the future.

**Future Directions:** The signaling aspect of sCD137 is complex and will be discussed in detail later when talking about the protein structure. The lenti-viral vector system has opened doors to manipulate cells *ex vivo* in order to cause an *in vivo* effect. Therefore, our future plans are to clone the sCD137 vector into the TMEW vector which shows impressive transduction of CD4⁺ primary T-cells. These cells will then be re-introduced into NOD mice in order to achieve prevention of T1D. Furthermore, the time course of how long the transduced cells survive *in vivo* will need to be assessed. The first experiment will involve stimulated (untransduced), empty vector (transduced) and sCD137 vector (transduced) CD4⁺ T-cells to be transduced and transferred into NOD.scid hosts along with pre-diabetic CD4⁺/CD8⁺ T-cells (which transfer disease rapidly). The incidence and disease severity will be monitored between all groups, with the hope that the sCD137 production from the T-cells is able to prevent diabetes transfer. In a similar experiment (using the same experimental groups), mice will be aged until diabetes onset (BG~200mg/dL), then the experimental groups will be adoptively transferred into those mice to assess disease reversal. This can also be done using the BDC2.5 CD4⁺ T-cells as well in order to ensure a more targeted delivery to the pancreas.

**(II) Soluble CD137 prevention of T1D in NOD mice**
As previously mentioned, prevention in T1D has been shown to be easily achieved and may not have a strong clinical relevance to human disease. However, an interesting aspect of prevention using sCD137 was that the histological scores seemed to not differ drastically between the sCD137 and untreated controls. This result may indicate that sCD137 has subtle effects in vivo. Furthermore, we were concerned, given our TLR4-Ab work, whether our protein preps of sCD137 might have LPS contamination. However, we have found that there is an undetectable amount of LPS in the final concentrated protein (data not shown). Next, it is important to show disease reversal as this is the most clinical relevant question in T1D. Therefore, we are currently testing whether sCD137 is able to reverse T1D (data not shown). Further, many cell types express CD137L on the surface (e.g. macrophages and DCs) and sCD137 may be targeting other cells besides T cells. These off target effects need to be assessed in more details. Finally, it is unknown if this protein can be targeted by ubiquitin for protein degradation. Nothing in the protein structure specifically indicates it in being targeted for the ubiquitin pathway nor do the N-terminal residues indicate a short half life. We also infer that sCD137 has the ability to modulate disease in 1106 mice (NOD\idd9.3) since it is increased in these mice. We have previously shown that mice with 2 alleles of B10 \idd9.3 (1106) mice can produce more sCD137 than their age matched NOD counterpart. It is possible that an F1 mouse bred between an 1106 and NOD mouse would have moderate levels of sCD137 and have disease protection in between the breeding mouse strains. Another interesting question that we are exploring is whether human T1D patients have decreased serum sCD137 similar to NOD mice. CD137 has already shown some relevance in human clinical disease, it was shown that a SNP in
healthy relatives of T1D patients is differential expressed compared to healthy individuals and studying molecules in health relatives could lead to novel biomarkers[36]. Therefore sCD137 could be playing a role earlier on human T1D that is involved in maintaining levels of insulin before full onset or clinical diagnosis.

**Future Directions:** The immunological mechanisms that are ongoing in T1D are vast and still not fully understood. Interestingly, a molecule that has been relatively unstudied in disease pathogenesis, sCD137, may be playing a crucial role in disease protection. Therefore, we will assess the cellular mechanism in which sCD137 prevents and reverses new onset T1D. First it will be important to assess cell types in which sCD137 may be binding to cause an immunological change. sCD137 will be labeled with a fluorescent protein, injected into NOD mice at different concentrations and assessed for both surface and intracellular fluorescence of the protein 24 hours after initial injection. This will indicate which cell types sCD137 interacts within the initial stages of disease reversal. Further, depending on this result the effect sCD137 has on cell types implicated will be purified and will be phenotypically assessed *in vitro*. Whether our protein changes cytokine production *in vivo* will be assessed by multiplex kinetic studies. The half life of a protein is difficult to determine. For the excretion of protein in the urine, mice will be injected with sCD137 and placed in metabolic cages (collects urine and feces) overnight and in the morning urine samples will be collected and assessed for sCD137 by ELISA. The anti-CD137 antibody (3H3) has shown the ability to bind the monomer as well intact dimer; therefore we can determine how much of the original bolus given is removed in the urine. Finally, a kinetic study of sCD137 will be used to assess how much circulating protein is remaining after a short interval.
and different concentrations of sCD137 will be introduced to get multiple clearance rates. Mice will be bled at time points 0 (baseline) and then injected intravenously with sCD137 and bled 2hrs, 4hrs, 6hrs, 8hrs, 12hrs, 24hrs, and 48hrs after the initial injection. The amount of sCD137 in the serum will be assessed by ELISA, we should see an initial increase then a decrease in the protein over time and will then be able to assess the circulating half life. Another simple experiment that needs to be performed is looking at the difference in serum sCD137 levels between NOD, F1 (1106/NOD), and 1106 mice. This could give us a “threshold" of sCD137 that needs to be reached in order to get disease prevention. It would be interesting to assess the levels of sCD137 from NOD mice that do not get T1D compared to diabetic mice to detect if they had higher levels of sCD137. Finally and maybe most importantly, human sCD137 needs to be created and purified to test its function on human CD4\(^+\) T-cells. In conjunction with David Adams in our lab a lenti-viral vector expressing the human sCD137 protein will be constructed, transfected into HEK293 cells, and purified using an affinity column against human CD137. This purified protein will then be characterized by assessing its ability to cause down regulation of CD4\(^+\) T-cell proliferation in human blood samples, both of healthy individuals and T1D patients.

(III) sCD137 is secreted by CD137\(^+\)Tregs and causes decreased proliferation via CD137L signaling as assessed by CD137L blocking antibodies.

We have shown that sCD137 acts via CD137L. What is still unclear is how sCD137 affects the signaling pathway in order to cause a change in CD4\(^+\) T-cell proliferation. What is known is that CD137L has the ability to cause reverse signaling into cells[37]. sCD137 is a dimer and CD137L is known to be trimeric, could produce hexagonal
arrays and clustering[38]. CD137L could signal through an immunoreceptor tyrosine activation motif (ITAM) or clustering CKI binding sites; similar signaling cascades have been seen in other TNF-receptor family member complexes, such as TRAIL[39]. CD137L reverse signaling has been shown to have an effect on Akt/mTOR pathways. Akt/mTOR affects both cellular proliferation and cytokine production, through the removal of p27 from CyclinE/CDK2 as well as activation of CyclinA/CDK2 (allowing entry into S phase) and activation of NF-κB [40]. A T-cell-specific mTOR blockade causes CD4+ T-cell anergy[40], further suggesting mTOR involvement in proliferation and activation. This could also be due to the fact PI3K activates the signaling cascade by phosphorylating Akt. Akt then phosphorylates mTOR to continue the signaling cascade. PTEN is the main inhibitor of PI3K activation and subsequent activation events[40]. These molecules could possibly play a role in the ability of sCD137 to down-regulate Akt/mTOR activation. These molecules will be assessed in future directions. These pathways are essential and downstream of TCR signaling. Another key aspect of anergy of CD4⁺ T-cells is their inability to produce specific cytokines such as IL-2. We have shown that cells accumulate in the G0 phase of the cell cycle, however what is still unknown was how this affected secretion of IL-2 production. IL-2 is a potent survival and proliferative cytokine for CD4+ T-cell expansion and activation *in vitro* and *in vivo*. IL-2 production may be a key in determining the effects sCD137 has on CD4⁺ T-cells. Another key aspect to this signaling molecule is the structure of the protein as previously mentioned. Interestingly, there seems to be a difference in the ability of CD137-Fc protein to cause decreased proliferation depending on if it is plate bound or soluble (data not shown). This then could possibly mean that a soluble
molecule has differential signaling effects when plate bound compared to its soluble form. This effect has been previously shown with anti-CD3. It is still not entirely understood why when CD3 is plate bound it causes activation and in the soluble form it does not. However, a similar effect may be seen with sCD137 compared to membrane bound CD137. Membrane bound CD137 is known to cause activation of T-cells in the absence of CD28 signaling. It is possible that due to the structure of sCD137 that it can bind in hexagonal arrays as previously mentioned, while the plate bound form can only bind as a dimer of trimers due to the limited mobility of the molecule. It is also possible that internalization of the receptor, which is prevented by plate binding, is important for sCD137 suppressive effect and the prolonged signaling given by the plate bound form causes proliferation instead of suppression. Similarly on the membrane surface, clustering/synapse formation may exclude other CD137 or CD137L to be involved in signaling and therefore only a certain proportion of CD137:CD137L is able to be induced which causes activation rather than suppression. It is also possible that during the synapse formation involved on the membrane surface, CD137L has a binding partner that it can oligomerize with which causes differences in signaling. Non-membrane bound forms usually do not cause synapses and recruitment of other signaling molecules. These both could allow for the differences in soluble vs. membrane/plate bound signaling cascades involved in decreased proliferation of CD4+ T-cells.

**Future Directions:** The role of individual signaling molecules of CD4+ T-cells will be assessed through multiple different avenues. First, due to the known effect that CD3/CD28 signaling through PI3K activates AKT and NF-κB, leading to cell cycle
progression, IL-2 and IL-2R upregulation (checkpoint 1)[40], sCD137 effects on this pathway will be first assessed. CD4⁺ T-cells will be cultured as previously described, stimulated with and without sCD137. The signaling molecules downstream of CD3/CD28 will be assessed by western blot as well as flow cytometry of these cells. It is possible that other molecules are involved in the suppression such as mTORC2 and mTORC1. These will be assessed as an alternative pathway. Furthermore, the second check point that involves IL-2R signaling may also be disrupted. IL-2R signaling activates mTOR, mTOR then forms a complex with survivin and aurora B, and together they regulate G1 to S phase progression (checkpoint 2)[40]. Therefore, sCD137 may have an early effect causing changes in IL-2 production which in turn affects progression into the G1/S phase. Alternatively, it is also possible that PTEN, a known regulator of the signaling pathways mentioned above may also be increased; therefore the balance of PTEN to AKT/NF-κB may be extremely important. PTEN will also be assessed through western blot and flow cytometry. Cell cycle arrest can be defined by measuring BrdU incorporation and staining of different cyclins. In order to monitor the difference in surface clustering between plate bound and sCD137 the following assay will be done. sCD137 will be labeled with a fluorescent marker and cells will be cultured. CD137L will be stained and co-localization on the cell surface will be determined using Imagestream. Furthermore, to determine the differences in cytoskeletal re-arrangement, actin and microtubules will be stained. The induction of cross-linking can be artificially induced by linker 3,3'-dithiobis(sulfosuccinimidylpropionate), Cytochalasin E can block F-actin formation, and pophyllotoxin can inhibit microtubule formation. These will all be added in with or
without sCD137 to stimulated CD4\(^+\) T-cells. This will give us insight into if clustering of surface receptors or creating a synapse is needed.

(IV) sCD137 decreases number of cell cycles and does not cause activated induced cell death (AICD).

As discussed in the paper is was originally believed that sCD137 down regulated T-cells by causing activated induced cell death[1], however, a purified version of sCD137 did not show this ability in \textit{in vitro} culture systems. It is interesting though that both scenarios lead to the same conclusion: that sCD137 is an immunomodulatory protein that is used to decrease immune activation. Further, T1D is known to be a T-cell mediated disease therefore it may be beneficial if sCD137 was not only able to control immune activation of CD4\(^+\) T-cells, but also CD8\(^+\) T-cells (data not shown). We hypothesize that sCD137 provides a negative feedback loop for CD4\(^+\)/CD8\(^+\) T-cells that are over activated and may also provide a negative feedback loop for many cells of the immune system. Furthermore, CD137L is upregulated upon stimulation and therefore both the soluble molecule and ligand are expressed at high levels during times of immune activation. However, it is important to note that sCD137 is also being produced and the expression of the soluble protein is approximately 24hrs after surface expression[1]. Therefore, the immune system may become overzealous and need to be toned down by the secretion of sCD137. This provides evidence that sCD137 may act as a short term immunosuppressant. This could be beneficial as we have previously shown that sCD137 does not cause cell death nor does it cause harmful side effects upon multiple treatments. We know this because mice have been treated once every other day for up to 26 treatments with no major health concerns associated with
injections and upon sacrifice no abnormal histology was seen (data not shown). This could be valuable in treatment of many autoimmune diseases and due to the transient property without deletion of cells may be an alternative to many other immunosuppressants.

**Future Directions:** One major question is whether CD8 cells are affected in similar ways as CD4 cells. We are testing this currently in the lab. Another interesting point that needs to be assessed is whether sCD137 can reduce antigen specific T-cell responses. In order for this to be assessed BDC2.5 CD4⁺ T-cells will be co-cultured with DCs given the BDC2.5 mimetic peptide with and without sCD137. Further, at different time points sCD137 will be added into the culture to see if it has an effect on the initial priming of T-cells or if it modulates their proliferation after becoming stimulated.

**(V) sCD137 is a disulfide linked dimer**

As previously discussed this result was surprising because it was originally thought that this molecule was a trimer. However, it is undoubtedly a disulfide linked dimer as shown by western blot and AUC. This protein structure may have differential ability to signal when forming hexagonal arrays with CD137L. However, another interesting result that we have seen previously, is each “batch” of sCD137 acts slightly different in the ability to suppress CD4⁺ T-cells. What has been observed when trying to figure out the difference between the “batches” is the ratio of monomer to dimer changes. If there are higher amounts of monomer compared to dimer, sCD137 is not as potent inhibitory of proliferation. To this same effect the glycosylation status may be important for the
binding and/or recognition of dimer formation. sCD137 has 3 possible glycosylation sites and one or all may be needed for signaling.

**Future Directions:** We showed that sCD137 is a known disulfide linked dimer and it is postulated that this structure is important to form hexagonal arrays and signal through CD137L. A single “batch” of sCD137 will be split into 2 equal fractions. One fraction will have the disulfide bond broken by dithiothreitol (DTT), while the other fraction will have intact disulfide linked dimers. These two fractions will then be tested for suppression of CD4^+CD8^+ along with a stimulated control and stimulated + DTT. To test the cross-linking effect of sCD137 to CD137L, CNBr-activated sepharose beads will be covalently bound to sCD137[41, 42]. Stabilization of proteins or enzymes on to the beads can significantly change the properties seen upon binding its cognate ligand[42]. Therefore, creating a stable bound form of sCD137 could alter its properties to an activating phenotype (similar to the membrane bound form) rather than a suppressive molecule. Another aspect of sCD137 biology is glycosylation sites. There are 2 N-linked glycosylation and 1 O-linked glycosylation site predicted for sCD137. N-linked glycosylation occurs when glycans are attached to asparagine residues on the core protein. O-linked glycosylation occurs when glycans are attached to serine or threonine residues. Both chemical and enzymatic methods exist for removing oligosaccharides from glycoproteins. Therefore it will be important to remove only the N-linked or O-linked to test their function on sCD137 signaling. PNGase F is the most effective enzymatic method for removing almost all N-linked oligosaccharides from glycoproteins. To remove O-linked glycans, monosaccharides must be removed by a series of exoglycosidases until only the Galβ1-3GalNAc (core 1) and/or the GlcNAcβ1-3GalNAc
(core 3) cores remain attached to the serine or threonine. The Enterococcus faecalis O-Glycosidase, also called O-Glycosidase, also called Endo-α-N-Acetylgalactosaminidase, can then remove these core structures with no modification of the serine or threonine residues. Any modification of the core structures, including sialylation, will block the action of the O-Glycosidase. Sialic acid residues are easily removed by a general α2-3,6,8 Neuraminidase. In addition, exoglycosidases such as β(1-4)Galactosidase and β-N-Acetylglucosaminidase can be included in deglycosylation reactions to remove other complex modifications often known to be present on the core structures. This combination of enzymes will not remove all O-linked oligosaccharides but should remove almost all (https://www.neb.com/products/p6039-protein-deglycosylation-mix). Therefore, it is possible to do a similar experiment using these enzymes and splitting a single “batch” of sCD137 to test the signaling components of N-linked and O-linked glycosylation difference on proliferation of T-cells.

**Summary:**  sCD137 is an immunomodulatory molecule that may have beneficial effects on T1D disease pathogenesis and progression. However, the signaling pathways, cell types, and mechanism of action needed to be tested further. Other disease models may be beneficially treated with sCD137 and this should also be tested.

**Summary Statement**

Both TLR4-Ab and sCD137, whether through inhibition of innate immune cells that causes beneficial changes in the adaptive immune system, or by direct immunosuppressive effects, can affect the clinical course of T1D. In both cases T-cells can either become anergic, deleted, or transformed into non-pathogenic regulatory
cells. These molecules show potential in reversal of T1D in NOD mice as well as possible translational applicability in humans. Furthermore, it is apparent that the two “modes” of action in T1D of our TLR4-Ab and sCD137 treatments are different. This suggests that these two therapies may be used in combination with one another to see an enhancement of disease reversal. TLR4-Ab causes tolerization and increases in Tregs while sCD137 controls or halts pathogenic T-cells directly. Finally, both projects have had their difficulties, but each one has brought about new and interesting immunological questions that remain to be answered. These results highlight key stepping stones to reversal of clinical T1D.
References


