The Importance of CD19^{+} Cells from Pre-Diabetic NOD Mice in Delaying Onset of Disease in an Adoptive Transfer Model of Type 1 Diabetes Mellitus

By

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Immunology)
In the University of Michigan
2017

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DEDICATION

The dissertation presented here is dedicated to my wonderful wife Morgan and beautiful daughter Verity. We have been through so much during this process, and the support and love you both have shown me, as well as putting up with all the late nights and early mornings at the lab, is the rock that fuels my passion for research in Type 1 Diabetes. I cannot express my thanks and love enough, and know this could not be done without you both standing in my corner cheering me on throughout the entire process.

ACKNOWLEDGMENTS

This dissertation could not be completed without the mentorship and support of Massimo Pietropaolo, who saw enough potential in me to allow me to join his lab. The version of the dissertation presented herein was completed with extensive input from him and Steven Lundy, with whom many of the early B cell experiments were completed, including Fig. 4 and Fig. 5 which were generated in his lab. His B cell expertise helped set this project on its current path and all its fruitions.

I would also like to acknowledge the fine comments and insightful criticisms by Ivan Maillard and Irina Grigorova, whose experimental expertise helped in designing complete experiments that were powerful enough to handle our hypotheses.

I would like to acknowledge the Immunology program and faculty for my depth of knowledge on the subject, and Zarinah Aquil, Beth Moore, and Keith Bishop for their help in guiding me through this program. Also I would like to give a special thanks to Thomas Moore for his help in getting me to this place where I can graduate.

Lastly I would like to acknowledge the funding sources for this work, including the Robert and Janice McNair Foundation at Baylor College of Medicine, the Michigan Diabetes Research Training Center (MDRTC), and the T32 Immunology Program Training Grant from the University of Michigan Graduate Program in Immunology.

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ABSTRACT

This dissertation describes a protective effect on autoimmune diabetes in NOD.*scid* recipients following injections of splenocytes from diabetic NOD donors in addition to purified $CD19^+$ cells taken from pre-diabetic 6-week-old NOD female donors, compared to NOD.*scid* recipients receiving injections of splenocytes from diabetic NOD donors alone. Delayed progression of T1D was associated with a remarkable reduction in IL-1β plasma levels, a reduction in the severity of insulitis, and increased levels of $CD19^+$ precursor B cells (compared to controls) likely exhibiting regulatory function upon activation and interaction with pathogenic T cells. The protective effect conferred by $CD19^+$ cells was age specific as co-transfers of $CD19^+$ cells from 6-week-old NOD mice exhibited a suppressive effect halting and/or significantly delaying the progression of diabetes and insulitis, while those from greater than 15-week-old NOD donors did not confer the same protective effect. Administration of a monoclonal antibody against IL-1β in NOD.*scid* recipients following injection of diabetic NOD splenocytes significantly delayed diabetes onset, unlike the administration of an isotype-matched antibody. In conclusion, progression to overt disease correlates with the pathogenic T cell's escape from $CD19^+$ cell–mediated regulation. These data provide evidence for a novel suppressive function of the regulatory B cell compartment in autoimmune diabetes. The expansion of regulatory $CD19^+$ B cells may have therapeutic potential for T1D.

CHAPTER I

The Importance of Type 1 Diabetes

A. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease whereupon insulin production is severely limited and even lost as a result of immune-mediated destruction of pancreatic β cells responsible for generating the insulin protein (Atkinson, 2012; Bluestone, Herold, & Eisenbarth, 2010; Eisenbarth, 1986; Todd, 2010). While T1D was traditionally referred to as juvenile diabetes due its classical presentation in children and young adults, recent discoveries of patients with late adult-onset diabetes (LADA) and no longer is a defining characteristic of T1D (Gale, 2005; Leslie, 2010). Patients with T1D commonly present with symptoms of polydipsia, polyphagia, and polyuria, and along with overt hyperglycemia, represent the diagnostic hallmarks of a new-onset T1 diabetic (Atkinson, Eisenbarth, & Michels, 2014; Siafarikas & O'Connell, 2010). Clinical diagnosis of T1D typically includes patients with a fasting blood glucose higher than 7 mmol/L, a normal glucose level higher than 11.1 mmol/L, and typically the presence of autoantibodies against insulin or another T1D autoantigen (Knip, 2011; A. Michels et al., 2015). Patients who suffer from T1D have pressing needs for insulin replacement, and are consigned to a lifelong need for continued exogenous insulin treatment (Little et al., 2014). This need is due to the insulin's central role in controlling metabolic homeostasis

and maintaining circulating blood glucose, vital for the health and well-being of everyone.

Population patterns for T1D incidence also vary around the world, and although T1D can now be diagnosed at any age, it is considered by the scientific community as one of the most common chronic autoimmune diseases seen in children (Harjutsalo, Sjoberg, & Tuomilehto, 2008). Unlike other autoimmune diseases that disproportionately affect women, T1D is found just as often in men (Ostman et al., 2008). Globally, patterns of T1D incidence emerge amid varied populations (Maahs, West, Lawrence, & Mayer-Davis, 2010). For example, the highest recorded number of cases are found in populations living in Finland and Sardinia, with between 40-60 cases per 100,000 residents being newly diagnosed each year (Patterson, Dahlquist, Gyurus, Green, & Soltesz, 2009). However, the opposite is seen in countries such as China, India, Latin America, or sub-saharan Africa with a very low estimate of 0.1 recorded cases per 100,000 residents diagnosed annually (Gong et al., 2015). The differences between these populations are thought to hinge upon a number of issues including, but not limited to, environmental stimuli that may or may not trigger autoimmune activation (Maclaren & Atkinson, 1992), gut microbiota and corresponding diets (Boerner & Sarvetnick, 2011; Knip, Virtanen, & Akerblom, 2010), viral activation of autoimmune cells or direct destruction of β cells (Stene & Rewers, 2012; Yeung, Rawlinson, & Craig, 2011), and genetic susceptibility (Morran, Vonberg, Khadra, & Pietropaolo, 2015).

A main culprit to the heightened incidence of T1D is due to its strong genetic component. T1D familiar aggregation has been described due to the increased risk of developing T1D among United States Caucasian siblings who had either parents,

siblings, or offspring with diagnosed T1D (Allen, Palta, & D'Alessio, 1986; Wagener, Sacks, LaPorte, & Macgregor, 1982). This increased risk ranges from 1% to 15% depending on the select subgroup, as compared to a less than 1% chance for individuals without any relatives with T1D, and an even smaller 1.2/1,000 when compared to the general population (Libman & LaPorte, 2005; Warram, Krolewski, Gottlieb, & Kahn, 1984). However, even with this increased risk susceptibility, over 80% of T1D occurrences happen in patients with no family history of T1D, with the remaining 20% amassing within families (Redondo & Eisenbarth, 2002). As a result of many studies, it has been projected that the overall risk of developing lifelong T1D is increased in first degree relatives of individuals with T1D. The average risk is increased by 6% in offspring of T1D patients, 5% increase in siblings, and a massive 50% increase in risk for developing T1D in the identical twin of a T1D patient (Redondo et al., 1999). For example, genes for increased diabetes susceptibility among the antigen-presenting Human Leukocyte Antigen (HLA) are believed to follow the laws of simple Mendelian transmission from parents to offspring. Therefore, offspring of parents with similar high-risk HLA alleles have the greatest risk of developing autoimmune T1D, with a risk as great as 70% for developing long-term diabetes (Aly et al., 2006; Kaprio et al., 1992).

B. The Genetic Susceptibility of the HLA Complex

Human chromosome 6 (6p21) contains a 3.5 megabase gene segment containing the vital immune genes that are described as the major histocompatibility complex (MHC) (Wucherpfennig & Eisenbarth, 2001). These localized gene sequences encode HLAs, which are made up of two cell surface glycoproteins with different structures, functions, and tissue distribution (Pos, Sethi, & Wucherpfennig, 2013). While the MHC class I molecules are generated and have surface expression on nearly all nucleated cells, MHC class II molecules are only found expressed topically on antigen-presenting cells, such as dendritic cells, B cells, macrophages, and on activated T cells (Drozina, Kohoutek, Jabrane-Ferrat, & Peterlin, 2005). The main function of both the MHC class I and II glycoproteins are to act as cell-surface presenters of small peptide sequences called antigens that can be recognized by immune-causing T cells (Pietropaolo, Surhigh, Nelson, & Eisenbarth, 2008). The difference and need for MHC class I and II is due to the fact that, MHC class I present peptide antigens that are recognized by cytotoxic $CD8^+$ T cells, while helper/inducer $CD4^+$ T cells recognize antigenic peptides in the context of MHC class II presenting cells (Neefjes, Jongsma, Paul, & Bakke, 2011).

T1D, while a traditional organ-specific disease whereupon targeted destruction of insulin-producing β cells is the root cause of clinical diagnosis, is labeled as primarily a $CD4^+$ T-cell mediated autoimmune disease. The importance of class II molecules and the antigens they present becomes vital and plays a key role in susceptibility to T1D. Class II proteins depend on the structured amino-acid compositions of their alpha and beta chains to properly present different antigens. Mutations and substitutions at even

one or two critical areas causes conformational changes leading to either increased or decreased binding of important autoantigens that lead to activation of auto-reactive T cells (Khalil et al., 1990; Rowe, Leech, Nepom, & McCulloch, 1994). A classic example in T1D exists whereupon over 90% of all patients currently diagnosed with T1D express HLA-DR3-DQ2 (HLA-DR3,DQB1*0201), or HLA-DR4-DQ8 (HLA-DR4,DQB1*0302), while only 40% of non-diabetic control patients have either one or the other haplotype (Tisch & McDevitt, 1996). What's even more important, is that nearly 30% of all diagnosed T1D have both haplotypes (DR3/DR4 heterozygotes), which has been shown to impart the highest risk to developing T1D. These patients heterozygous for DR3/DR4 versus homozygotes with either DR3 or DR4, have increased risk hypothesized to be due to the interaction with the trans-complementing DQ heterodimers that are seen only in the DR3/DR4 heterozygotes (Erlich et al., 2008). Further genetic susceptibility studies using the DR3/DR4 HLA heterodimers demonstrate that the presence of the heterozygous alleles imparts increased risk to progression towards T1D, upwards of 20-40% in first degree relatives (Aly et al., 2006). Interestingly, similar changes in HLA of DR and DQ have been seen in differing populations to impart increased risk of developing T1D in patients around the globe, showing the importance of HLA and MHC class II molecules to activate a T cell-mediated autoimmune response (Cucca et al., 2001; Katahira, Segawa, Maeda, & Yasuda, 2010; Redondo & Eisenbarth, 2002; She, 1996; Varney et al., 2010).

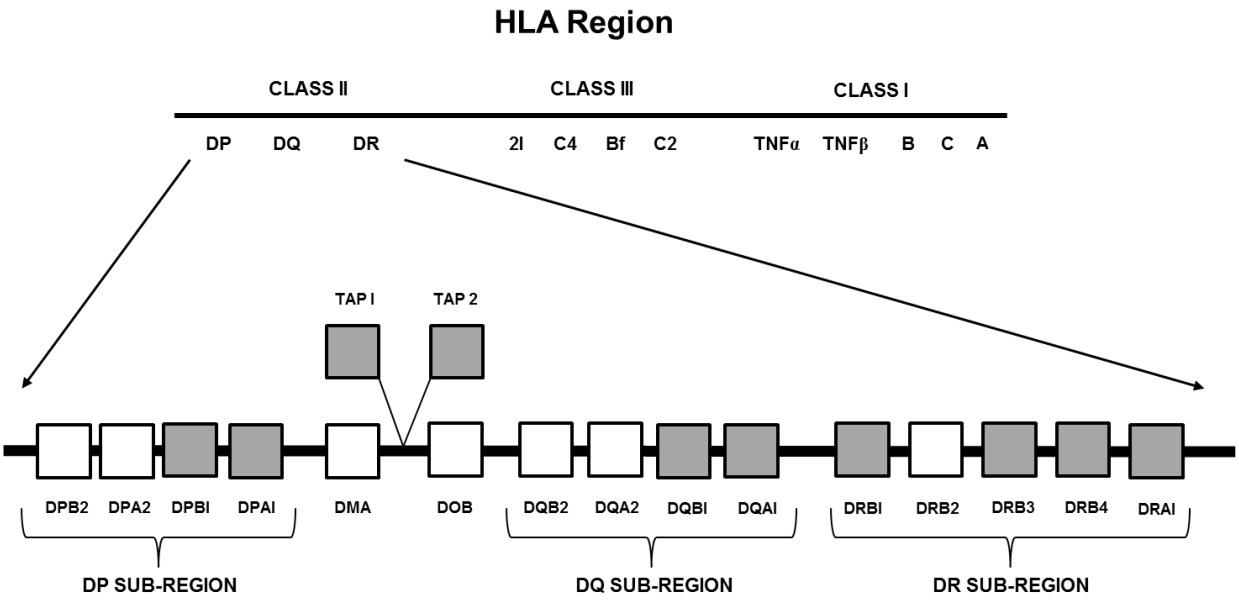

Figure 1: Schematic representation of the HLA complex contained on chromosome 6 of the human genome.

Within the MHC class II gene segment (HLA DP/DQ/DR), the genes that can capably encode a protein product are indicated using the grey color. The genes encoding non-functional or uncharacterized products remain white.

Within the non-obese diabetic (NOD) mouse model, the importance of MHC class II molecules in the pathogenesis of T1D becomes even clearer as the main activator of T cell mediated insulitis (Fig. 2). The expression of the mouse I-A β chain transgene, which finds an equivalent in the high susceptibility human class II DQB1 gene locus, with a mutation at position 57 of asparagine in lieu of serine, protects NOD mice from developing T1D (Miyazaki et al., 1990; Slattery et al., 1990). Similar mutations around the same loci, with proline 56 replacing the commonly found histidine 56 also had the same effect of preventing diabetes from occurring in NOD mice (Lund et al., 1990). Another important finding showing the importance of the class II molecules was seen in the expression of certain mutations in I-E transgenes, which mimic the human HLA-DR locus, led to protection from diabetes onset in NOD mice (Nishimoto, Kikutani, Yamamura, & Kishimoto, 1987). These results, when combined with early data showing the block of the MHC class II complex using a class II specific monoclonal antibody in NOD mice prevented them from developing diabetes, strengthen the case that T1D progression is aided immensely by the presence of specific HLA-DR/DQ molecules in human T1D (Boitard, Bendelac, Richard, Carnaud, & Bach, 1988). As a result of these and other similar studies, current research into the ability of differing therapies to block the formation of the synapse between the MHC class II molecule presenting cogent antigen and the T cell receptor (TCR), is currently underway with some limited success in the T1D mouse models (A. W. Michels, 2013; L. Zhang et al., 2014).

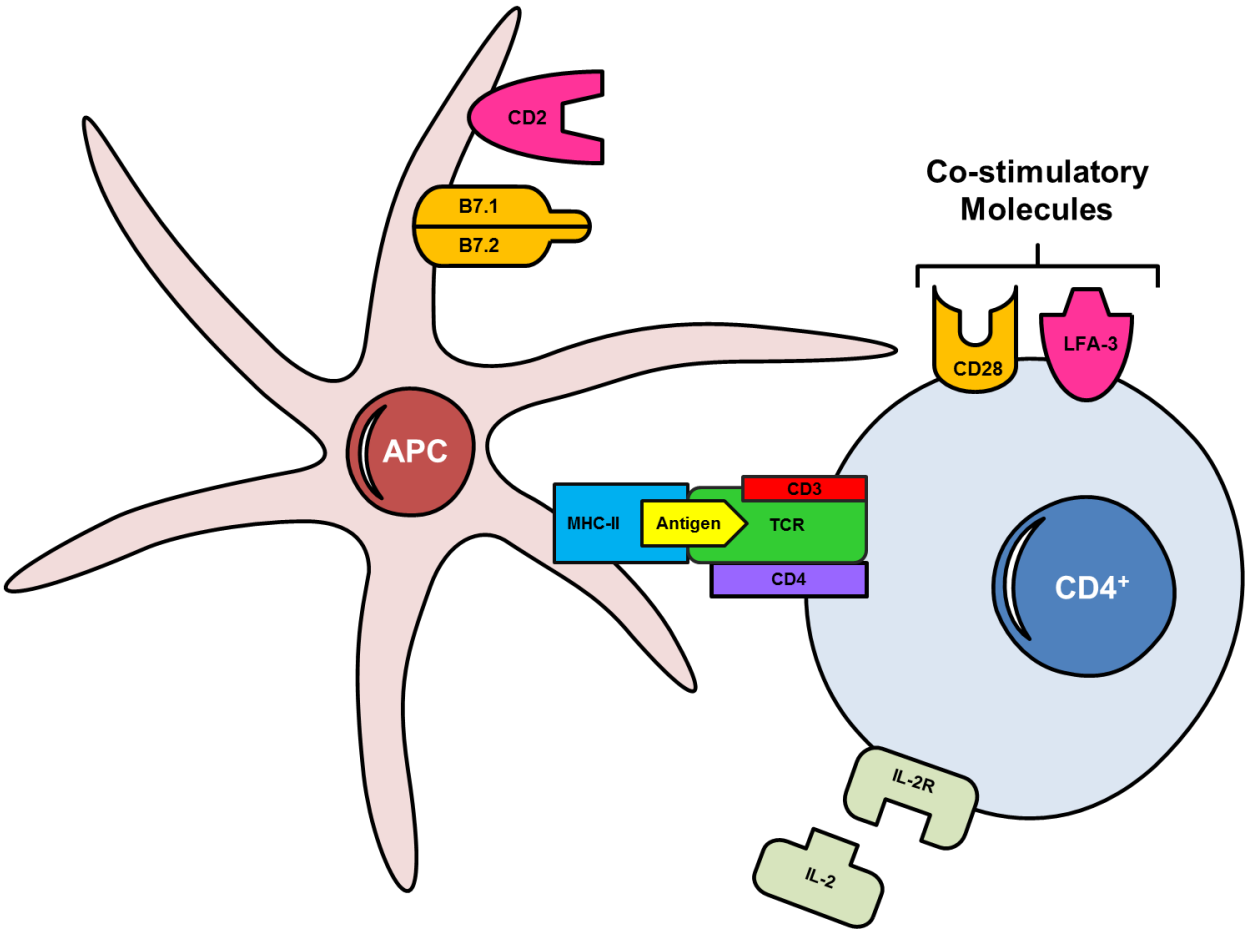

FIGURE 2: Tri-molecular Complex.
Image showing the formation of the tri-molecular complex between a $CD4^+$ helper T cell and an antigen-presenting cell expressing MHC class II presenting the T cell's cognate antigen. Expression of co-stimulatory molecules CD28, and activation of T cell proliferation cytokine IL-2 by the APC are also shown.

However, while there is understanding that the presence of certain class II molecules confers heightened risk of developing T1D, there are a number of differing hypotheses on how that conference occurs. The crystalline structure of class II molecules characterized in 1993 has led to the hypothesis that the binding site of the DQ dimer has critical residues at position 52 and 57, which are located at the opposite end of the alpha chain, and are vital to the binding potential of the DQ molecule (Brown et al., 1993). This hypothesis is supported by the previously described mutation experiments in the NOD mouse using mutations at position 57 to prevent diabetes (Lund et al., 1990). The alternative hypothesis is that substitutions at either or both of these residues leads to a conformational change within the antigen binding site of the class II molecule, thereby increasing the affinity for T1D inducing peptide binding and presentation to auto-reactive T cells. Further substantiating this hypothesis is the understanding that position 57 and 76 of the DR molecule are important for hydrogen and salt binding, and that potential mutations at either of these two sites would likely alter the antigen-binding site of the class II molecule and either decrease or increase affinity for diabetes-inducing peptides .

C. Genetic Susceptibility of Non-MHC Genes

As seen in the Type 1 Diabetes Genetics Consortium (T1DGC), there are a number of different genes encoding risk for T1D, with the presence of specific HLA haplotypes described above being the greatest (Barrett et al., 2009; Concannon, Rich, & Nepom, 2009; Cooper et al., 2012; Howson et al., 2012; Todd et al., 2007). These genome-wide association studies have so far found 55 non-MHC susceptibility loci, including insulin, the cytotoxic T lymphocyte associated antigen 4 (CTLA4), PTPN22, and others. The most important non-MHC gene discovered has been, not surprisingly, the insulin gene (*INS*), which is found on chromosome 11p15 (Bell, Karam, & Rutter, 1981; Owerbach & Nerup, 1982), and distinction as the main product of β cells, the targets of autoimmune destruction in T1D. Early on, genetic links between the presence of specific insulin genes (*IDDM2*) within populations of T1D and their families were proven (Bain et al., 1992; Julier et al., 1991). Following these studies, sequence analysis of the *INS* gene discovered a specific polymorphic locus containing a variable number of tandem repeats (VNTRs) adjacent to the insulin gene coding sequence, where the presence of specific VNTR alleles leads to either protection or increased risk of developing T1D (Bennett et al., 1995; Durinovic-Bello et al., 2014; Kennedy, German, & Rutter, 1995; Lucassen et al., 1995).

The CTLA4 gene, which encodes a key cell-surface molecule (CTLA4), is a key regulator of T cell activation. CTLA4, which is presented on activated and inactivated T cells, binds to the CD80/86 molecule on the antigen-presenting cell during MHC/TCR formation, and sends an inhibitory signal to prevent/stop T cell activation (Gough, Walker, & Sansom, 2005). CTLA4 acts in direct competition to CD28 on T cells, which

works as “signal 2” to activate a T cell that has seen its cogent antigen (Boden, Tang, Bour-Jordan, & Bluestone, 2003; Noel, Boise, & Thompson, 1996). The interplay between CTLA4 and CD28 work to control the off/on switches of T cell activation during antigen presentation. The chromosomal region of CTLA4, region 2q33, also contains the CD28 gene, and was seen to be associated with risk for developing T1D in a number of different studies (Kavvoura & Ioannidis, 2005).

A third non-MHC gene that conveyed higher risk for developing T1D was PTPN22, which encodes a lymphoid tyrosine phosphatase (Lyp), which is important in its ability to downregulate T cell receptor signaling that leads to activation and the generation of an immune response (Bottini & Peterson, 2014; Fousteri, Liossis, & Battaglia, 2013). PTPN22, which is encoded on chromosome 1p13, appears to have an important polymorphism at arginine 620 that changes it to tryptophan, and blocks Lyp from binding to c-src tyrosine kinase (Csk), thereby preventing the downregulation of the activation signal from the TCR (Bottini et al., 2004; Vang et al., 2005). Interestingly, this mutation in PTPN22 has also been observed and associated with other autoimmune disorders, including rheumatoid arthritis and systemic lupus erythrematosus (Chung & Criswell, 2007; Y. H. Lee, Bae, Choi, Ji, & Song, 2012).

CHAPTER II

Cellular Immunity in Type 1 Diabetes

A. Background Importance

Over the last 30 years, there have been numerous studies that have performed extensive histological analysis to determine the autoimmune nature of T1D (Bottazzo, Florin-Christensen, & Doniach, 1974; Conrad et al., 1994; Foulis, Liddle, Farquharson, Richmond, & Weir, 1986; Gianani et al., 2010; Pugliese et al., 2014). Patients with T1D who had died presented with β cell islets with significant lymphoid infiltration that led to cytokine destruction of β cell mass (Conrad et al., 1994; Gianani et al., 2010). Early studies initially described large numbers of activated $CD8^+$ T cells in infiltrated islets also up-regulating MHC class I molecules (Bottazzo et al., 1985). More recent studies have also show a preponderance of macrophages and B cells within the islets of patients who had been diagnosed with T1D before death (Conrad et al., 1994). In T1D, the autoimmune response is controlled by a balance between pathogenic and regulatory T cells (Bluestone & Tang, 2005), even in the presence of numerous autoantibodies generated by antibody-producing B cells (Pietropaolo & Eisenbarth, 2001).

Antigen peptides, as described above, are presented by MHC class I and II molecules presented on the cell surfaces of antigen-presenting cells that are, in the presence of CD28, activate and mobilize effector $CD4^+$ and cytotoxic $CD8^+$ T cells (Haskins, Portas, Bradley, Wegmann, & Lafferty, 1988; Mallone et al., 2007). T cells form their MHC/TCR synapse in order to recognize specific antigen peptide sequences (Basu & Huse, 2016)w. After recognition of their specific antigen, T cells traffic to specific sites and begin an immune response.

B. $CD4^+$ T Cells

$CD4^+$ T cells are vital during the cellular immune response, playing major roles in activating both $CD8^+$ T cells and B cells. It has been shown that human patients with T1D have $CD4^+$ T cells that, when taken from the nearby pancreatic lymph nodes, have TCRs that specifically respond to amino acids 1-15 of the insulin α-chain (Mannering et al., 2009). These patients containing autoreactive $CD4^+$ T cells responding to insulin are believed to have escaped methods of positive and negative thymic selection during T cell education (Kurd & Robey, 2016; von Boehmer, 2004). Also, $CD4^+$ T cells upon activation undergo production of specific transcription factors that differentiate them to secrete different cytokines, as described below. Important in T1D, $CD4^+$ T cells can differentiate into helper Th1, Th2, Th17, or regulatory T cells (Tregs).

Th1 cells differentiate upon stimulation of the IL-12 receptor during activation leading to STAT4 stimulation and production of the T-bet transcription factor. Th1 cells control cell-mediated immunity and phagocyte-dependent protective response through

activation and signaling the migration of $CD8^+$ cytotoxic T cells and macrophages. Th1 cells aid in the destruction of β cells within the islets by producing IFN-γ (a pro-inflammatory cytokine) and Interleukin-2 (proliferation signaling cytokine) (Crane & Forrester, 2005; Lan, Selmi, & Gershwin, 2008; Nelson, 2002).

Th2 cells, which are commonly believed to have the opposite effect as Th1 cells, which work to exacerbate an immune response, while Th2 cell cytokines protect against T1D. Specifically, Th2 cells secrete the cytokines IL-4 and IL-10, which have been shown to be control antibody production, eosinophil activation, and prevent macrophage function (M. S. Lin et al., 2011; Romagnani, 1999). This leads to the idea that Th2 cells mediate humoral and allergic immune response (Zhang, Zhang, Gu, & Sun, 2014). Interestingly, the activation and proliferation of Th2 cells has been shown in the NOD mouse to help prevent T1D onset (Ruffner & Robbins, 2010; Sharif, Arreaza, Zucker, & Delovitch, 2002). IL-4 expression within pancreatic islets of transgenic NOD mice were protected from T1D pathogenesis as well. IL-10 is an anti-inflammatory cytokine that has been shown to be important in establishing immune tolerance in NOD mice and preventing diabetes onset (Tai et al., 2011).

Th17 cells are a subset of $CD4^+$ helper T cells that have, as their distinction, the ability to secrete IL-17 to induce an immune response. While Th17 cells have been shown to be important in similar autoimmune disease models, such as rheumatoid arthritis and multiple sclerosis, they have not been shown as important in T1D as yet (Crome, Wang, & Levings, 2010; Marwaha et al., 2010; C. K. Wong et al., 2008). However, recent reports have observed that anti-IL-17 therapeutic compounds can somewhat regulate T1D progression in NOD mice, suggesting a role for IL-17 in

diabetes progression (I. F. Lee et al., 2013; M. Wang et al., 2011). Also, transfer of IL-17 differentiated NOD T cells *in vitro* led to diabetes progression in NOD.*scid* immunocompromised recipient mice, further validating the potential importance of Th17 cells in T1D pathogenesis (Honkanen et al., 2010).

C. Regulatory T Cells (Tregs)

Tregs are $CD4^+$ T cell subsets that act as suppressors of antigen-activated immunological responses to antigens (Kilshaw, Brent, & Pinto, 1975; Petzold et al., 2013). Tregs suppress excessive immune responses and preventing autoimmune activation by secreting cytokines such as IL-10, TGF-β, and IL-35 (Haseda, Imagawa, Murase-Mishiba, Terasaki, & Hanafusa, 2013; Sakaguchi, Yamaguchi, Nomura, & Ono, 2008). Requiring the unique transcription factor forkhead box P3 (FoxP3) to differentiate, Tregs can either be induced in the periphery upon sensing cognate antigen and activating (inducible Tregs), or derived during thymic development specifically as immune regulators (natural Tregs) (X. Lin et al., 2013). Within T1D, recent work has shown that new onset patients have been found to have increased apoptosis and decreased functionality of Tregs (Glisic et al., 2010; Rajagopalan et al., 2006; Tonkin & Haskins, 2009). Also, autologous Treg infusions in newly diagnosed children led to a delay in diabetes progression (Marek-Trzonkowska, Mysliwec, Siebert, & Trzonkowski, 2013).

D. $CD8^+$ T Cells

Cytotoxic $CD8^+$ T cells recognize antigenic peptides presented by MHC class I molecules and become activated. They are capable inducers of apoptosis through either perforin and granzyme release that induces a terminal caspase cascade, or through upregulation of the FasL receptor, which binds to Fas receptors on the target cell and induces cellular apoptosis (Bulek et al., 2012; Kreuwel & Sherman, 2001; Rasche, Busick, & Quinn, 2009; Smyth et al., 2001). The presence of $CD8^+$ T cells within the islets of T1D patients happens because β cells can present MHC class I molecules on their surfaces, but not MHC class II (Katz, Benoist, & Mathis, 1993; B. Wang, Gonzalez, Benoist, & Mathis, 1996). Also, NOD mice genetically modified to not generate capable class I presentation were protected from T1D progression (Fierabracci, 2011; Rasche et al., 2009). These studies demonstrate that $CD8^+$ cytotoxic T cells are an important component of cell-mediated destruction of β cells during the autoimmune response.

CHAPTER III

Islet Autoantigens and B cell Autoimmunity

A. Autoantibodies in T1D

Autoantibodies generated by B cells that are reactive with multiple islet autoantigens have been detected by assay from the serum of patients with T1D and those with high risk for developing diabetes (Cox & Silveira, 2009; Hampe, 2012; F. S. Wong et al., 2004). Autoantibodies are typically able to be found before onset of diabetes, with anti-insulin autoantibodies being detected in 90% of patients 5 years of age or less who have developed T1D (Bingley, 2010). Due to their nature as early predictors, and the use of these humoral immunological markers to increase the predictive value for progression to T1D, the use of autoantibodies is important in discovering new islet protein autoantigens, as well as disease-inducing epitopes (Pietropaolo, Towns, & Eisenbarth, 2012). Specific autoantibodies against numerous antigens include insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated antigen 2 (IA-2), islet cell autoantigen 69 kDA (ICA69), zinc transporter 8, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and chromogranin A (ChgA) to name but a few (Pietropaolo et al., 1993; Stadinski et al., 2010). Detection assays to

find these and other newly described epitopes present a unique chance to develop biomarkers capable of more accurately predicting the risk to developing diabetes. Within the NOD mouse model of T1D, anti-insulin autoantibodies have been detected, and can be detected from the earliest stages of insulitis (Arvan, Pietropaolo, Ostrov, & Rhodes, 2012; Lampasona & Liberati, 2016).

Antibodies, including autoantibodies, are generated by B cells in order to aid in antigen uptake by antigen presenting cells. Antibodies form complexes with specific antigens and are taken up by Fc receptors present on antigen presenting cells including B cells and monocytes consisting largely of dendritic cells (Amigorena & Bonnerot, 1999). Due to antibody binding, antigen uptake and presentation is nearly 100 fold more efficient than pinocytosis and more capable of processing and presenting bound antigen to T cells (Celis, Zurawski, & Chang, 1984; Lanzavecchia, 1985; Takai, 2002, 2005). As a result, the ability of B cells to generate autoantibodies is very important to antigen recognition and uptake by antigen presenting cells.

B. B Cell Development

B cell development begins in the bone marrow, where stem cells present in the bone marrow will receive stimuli to generate a B cell. B cell are derived from a common early lymphoid progenitor, which can differentiate into either a natural killer (NK) cell, dendritic cell, or common lymphoid-2 progenitor (LCA-2) which is considered the first stage of an immature B cell (Treml, Hao, Stadanlick, & Cancro, 2009). B cell lineage development is beholden to bone marrow stromal cells which mainly secrete IL-7, the

prime cytokine in driving B cell pathways (Clark, Mandal, Ochiai, & Singh, 2014). However, bone marrow stromal cells also produce a number of other molecules important in B cell development including Fms-like tyrosine kinase 3 (Flt3-L), and transcription factors such as PU.1, IKAROS, E2A, EBF (Early B cell factor 1), PAX5, and IRF8 (interferon regulatory factor 8) (Fuxa & Skok, 2007; LeBien & Tedder, 2008). During this period B cells undergo sequential heavy chain and light chain gene rearrangements or V(D)J recombination, generation of a B cell receptor (BCR) which is also an Immunoglobulin (Ig) molecule, and then undergo central tolerance (Oltz, 2001).

Immunoglobulin molecules are made up of 2 identical 50 kDa heavy chains and 2 identical 25 kDa light chains (Chaplin, 2010). The genes that make up the heavy chain chain repeats are a series of variable (V) genes, then diversity (D) segments, followed by a constant (c) region. The light chain contains only the V and J regions. The V portion of the heavy chain and light chain are in "juxtaposition," and each pair make up a hypervariable binding site for potential antigens. The heavy chain constant region forms the Fc domain and the heavy chain encodes for the 9 different immunoglobulins that can be generated by the B cell: IgM, IgD, IgG_{1-4}, IgA_{1-2}, and IgE (Huston, 1997; Kracker & Durandy, 2011).

During central tolerance within the bone marrow, B cells that have high affinity for specific self-antigens presented within the bone marrow and become activated are deleted or undergo another round of V(D)J recombination to generate a viable B cell (Gupta & Louis, 2013). Other B cells that did not generate BCRs that recognize antigen are deleted due to unusable heavy and light chain rearrangements. Those B cells that pass selection traffic from the bone marrow to the spleen and pass through two more

states of B cells as either transitional 1 (T1) as they traffic to the spleen, or transitional 2 (T2) B cells as they migrate into the spleen (Palanichamy et al., 2009). The transition from T1 to T2 B cells requires the presence of BAFF-R and TNF receptor family and B cells that transition from T1 to T2 reside within the follicles of the spleen (Schiemann et al., 2001). T2 B cells continue to transition toward either the marginal zones or germinal centers of the spleen, and there they continue to develop and differentiate into either marginal zone (MZ) B cells or follicular (FO) B cells. Currently, different surface markers are established in the literature to help identify these different subsets of developing and differentiated B cells (Table 1).

Developing B cells are placed within the red pulp MZ of the spleen whereby they can sample many blood-borne antigens and rapidly differentiate into antibody-producing cells. B cells that have encountered antigen move to the border of the cortex to present that antigen to waiting T cells that express CD40L. Upon T cell recognition, CD40L on the T cell binds to CD40 receptor on the B cell, thereby activating the B cell to undergo proliferation and differentiation in the germinal center, where further somatic hypermutation can occur (Aversa, Punnonen, Carballido, Cocks, & de Vries, 1994; Klaus, Berberich, Shu, & Clark, 1994). Within the germinal center, there is a “dark zone” of rapidly dividing B cells and a “light zone” where B cell selection is occurring by BCR-bound antigenic interactions with primed follicular helper $CD4^+$ T cells (Craft, 2012). Long lived plasma cells generated in the germinal center traffic out and are retained within the bone marrow under the care of IL-6, B cell activating factor (BAFF), and a proliferation-inducing ligand (APRIL) (Hart, Wang, Hogquist, & Jameson, 2011; Hoek et al., 2010).

TABLE 1: Mouse B cell subsets

Mouse B cell subset	Distinguishing Surface Marker Expression
Marginal zone precursor cells	$CD19^{+}IgM^{hi}CD21^{hi}CD23^{-}CD1d^{hi}$
Marginal zone B cells	$CD19^{+}IgM^{hi}CD21^{hi}CD23^{low}CD1d^{hi}$
Transitional 1 cells	$CD19^{+}CD93^{+}IgM^{hi}CD21^{-}CD23^{-}$
Transitional 2 cells	$CD19^{+}CD93^{+}IgM^{hi}CD21^{hi}CD23^{hi}$
B1 B cells	$CD19^{+}CD11b^{+}CD5^{+}$
Follicular B cells	$CD19^{+}IgD^{hi}IgM^{low}CD21^{+}CD23^{hi}CD1d^{low}$
Plasma cells	$CD19^{low/-}CD138^{+}CD93^{+}$
Plasmablasts	$CD19^{low/-}CD138^{+}CD93^{+}MHCII^{+}Ki67^{+}$

Table 1: Mouse B cell subsets.
Table showing currently described B cell subsets found within the NOD mouse and the corresponding surface marker expression patterns used to distinguish them (Lundy, 2009; Shen & Fillatreau, 2015).

CHAPTER IV

B Cells in Type 1 Diabetes

A. Importance of B Cells in Diabetes Progression

B cells are one of the major cell types to infiltrate the pancreatic islets in NOD mice (Fox & Danska, 1998; Pearson, Wong, & Wen, 2016; Willcox, Richardson, Bone, Foulis, & Morgan, 2009). The importance of B cell functions in the pathogenesis of T1D was first shown using the NOD mouse model of diabetes (Henry & Kendall, 2010; Kendall, Yu, Woodward, & Thomas, 2007; Silveira & Grey, 2006). In an elegant study, transgenic IgM null NOD mice, which due to the importance of IgM as the first stage of BCR formation a lack of IgM causes the deletion of all B cells, were completely resistant to T1D development (Serreze et al., 1996). However, upon successful reconstitution of the polyclonal B cell compartment, these same IgM null NOD mice went on to develop over diabetes (Vong et al., 2011). In order to understand the effects of different B cell functions on T1D progression, B cell deficient IgM null NOD mice were injected with purified antibodies from a diabetic NOD donor, but did not develop diabetes (Serreze et al., 1998). The opposite experiment generating an IgM-bound B cell transgenic mouse unable to undergo class switching showed that preserved antigen presentation

capabilities led to robust diabetes (F. S. Wong et al., 2004). Even using an anti-IgM antibody to deplete the B cell pool was effective at blocking diabetes onset in NOD mice (Noorchashm et al., 1997). Another study found that biasing the B cell repertoire with a higher frequency of insulin-specific BCR formations by only a small percentage (1-3%) was capable of rapidly increasing the time to diabetes onset in NOD mice (Hulbert, Riseili, Rojas, & Thomas, 2001). However, biasing the BCR repertoire away from insulin-specificity and toward the innocuous hen egg lysozyme (HEL) had the opposite effect of significantly delaying diabetes onset (Silveira et al., 2002). B cells play two distinct roles in disease progression, and these experiments showed that it was through receptor presentation of autoantigens that B cells were having an effect on the pathogenesis of diabetes, and not through autoantibody production. More recently, experiments measuring the presence of T cell activating co-stimulatory molecules present on antigen presenting cells showed that MZ B cells have higher percentages of co-stimulatory molecules, thus making them even more capable presenters (Falcone, Lee, Patstone, Yeung, & Sarvetnick, 1998; Marino et al., 2008).

Recently, using surface staining CD markers described in Table 1, we were able to show that there is an increase in the number of MZ and MZ Precursor B cells in 6-week-old NOD female mice as compared to control strains. The control strains chosen were the outbred C57Bl/6, the inbred Balb/c mice which are the backbone strain for the NOD mutation, and the NOR mouse, which has approximately 95% gene homology with the NOD female, but due to gene splicing with C57Bl/6, are insulitis resistant and diabetes free (Fig. 3).

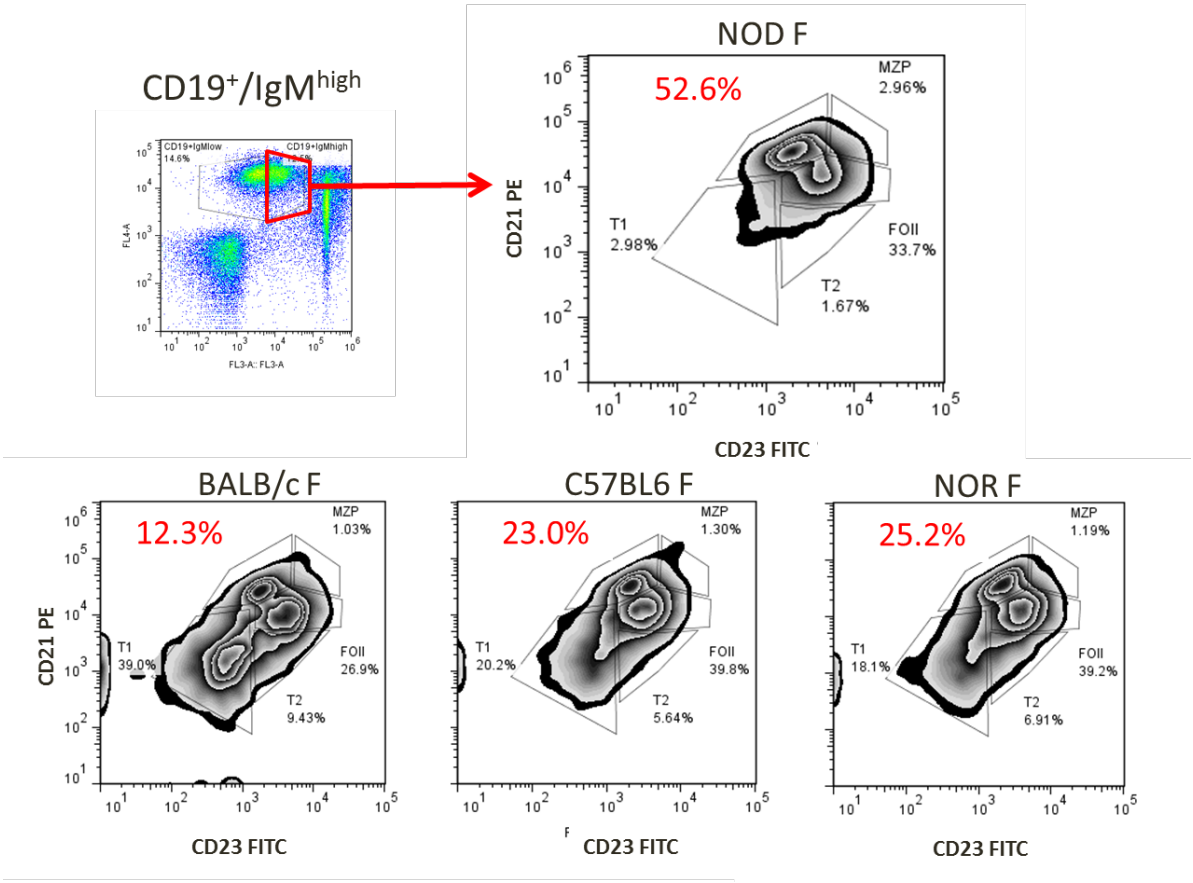

Figure 3. NOD mice have higher percentage of Marginal Zone and Precursor B Cells.

Flow cytometry results from single cell suspensions of splenocytes taken from different mouse strains. Cells stained with fluorescently labeled antibodies against CD19, IgM, CD21/35, and CD23. **(A)** Splenocytes from 6 week old NOD, Balb/c, C57Bl/6, and NOR female mice initially gated on $CD19^{+}$, then separated into IgM^{hi} and IgM^{int}. IgM^{hi} cells were further gated on levels of CD21/35 and CD23 to determine percentage of population that is Marginal Zone (MZB), Marginal Zone Precursor (MZP), Follicular II (FO II), Transitional 2 (T2), and Transitional 1 (T1).

B. B Cell Depletion Strategies

Due to the overwhelming evidence that B cells are capable antigen presenters able to effectively activate T cells and induce cellular immune responses against β cells within the pancreatic islets, recent work to develop and test B cell depleting antibodies were performed. Initial targets were the B cell surface proteins CD20 and CD22, which are found starting at the late-pre B cell stage of maturation within the bone marrow and maintained at high levels on all older B cells in the periphery (Cyster & Goodnow, 1997; Nitschke, 2005; Poe, Hasegawa, & Tedder, 2001). These surface proteins were also chosen because they are highly conserved between mice and humans (Tedder & Engel, 1994). The generation of the anti-CD20 monoclonal antibody capable of depleting was used in the NOD mouse, and was able to reverse disease in two-thirds of new-onset diabetic mice (Hu et al., 2007; Xiu et al., 2008). The use of the anti-CD22/cal monoclonal antibody was somewhat capable of reversing diabetes in newly diagnosed NOD mice, although the results showed that earlier depletion on Day 3 was more effective than treatment even on Day 5 after onset of hyperglycemia (Fiorina et al., 2008). The proposed mechanisms for why these B cell depleting antibodies worked in the NOD mouse was two-fold. Firstly, it was suggested that this was due to the decreased antigen presentation capacity as a result of losing the B cell repertoire. All of the studies performed using anti-CD20 and anti-CD22/cal in NOD mice showed resultant decreases in self-reactive $CD4^+$ T cell responses, and even in $CD8^+$ T cells. These results lend credit to the proposal that B cells not only support $CD4^+$ T cell activation, but that they are also important for $CD8^+$ T cell survival (Marino et al., 2009). The treatments also showed increases in circulating Treg levels after B cell depletion,

leading to the second method of preventing further diabetes progression by activating Tregs capable of secreting IL-10 and downregulating activated autoimmune $CD4^+$ and $CD8^+$ T cell responses. However, depletion with either antibody did not confer long-term disease protection when given before onset had even occurred, leading to the idea that B cell involvement is different before onset than stages of chronic hyperglycemia and manifestation of disease. Another caveat of these studies was that the treatments were more short term, with circulating B cell numbers being restored within 7-10 weeks of the completion of treatment.

Based on these results in the NOD mouse, the anti-CD20 monoclonal antibody Rituximab© had been tested in clinical trials to treat T1D in patients with new onset diabetes (Pescovitz et al., 2009). From the results of the trials, anti-CD20 had an acute effect on diabetes, allowing some patients with T1D to slow the disease, although long-term efficacy was unobtainable using anti-CD20 alone (Herold et al., 2011; Yu et al., 2011). Patients on anti-CD20 therapy presented with slowed decline in β cell function and arresting the continued loss of C-peptide. However, upon completion of treatment, patients saw reconstitution of the B cell compartments within a year (Pescovitz et al., 2014).

C. Regulatory B Cells in T1D

Initial work to characterize regulatory B cells (Bregs) in the NOD mouse was performed using LPS-stimulated *in vitro* cultures of purified B cells (Tian et al., 2001). Upon stimulation these Bregs secreted anti-inflammtory TGF-β, and when placed in

culture with diabetogenic T cells were able to induce T cell apoptosis (Spender et al., 2009; Yang, Rui, Wang, & Lu, 2013). More recently, IL-10 secreting Bregs that express $CD5^{+}$ and grown in culture with tolerizing dendritic cells were shown to be able to reverse new-onset T1D in NOD mice (Di Caro et al., 2014; Yanaba et al., 2008). Further work showing a progenitor B cell population expressing a number of B cell surface markers (c-kit^{low} Sca-1^{low} $CD127^{+}$ $B220^{+}$ $CD19^{+}$ IgM^{-} $CD1d^{int}$ $CD43^{+}$) had a similar ability to suppress diabetes progression after diagnosis. These cells went on to become B cells, but with the ability to suppress activated T cells by reducing IL-21 and inducing apoptosis (Montandon et al., 2013). Another study showed that activated Bregs were capable of differentiating T cells down a Th2 pathway instead of the canonical Th1 pathway needed for T1D progression (Hussain & Delovitch, 2007). The above study also showed that taking Bregs from NOD-IL-$10^{-/-}$ transgenic mice led to neither increases nor decreases in diabetes progression in recipient NODs, thus highlighting the importance of IL-10 as a required cytokine in the ability of Bregs to control disease. In our own hands, we've observed increased levels of $CD19^{+}CD5^{hi}CD1d^{lo}$ B cells, which are often described as "classical" Bregs, in 6-week-old pre-diabetic NOD female mice as compared to control strains including age matched C57Bl/6 and Balb/c female mice (Fig. 4).

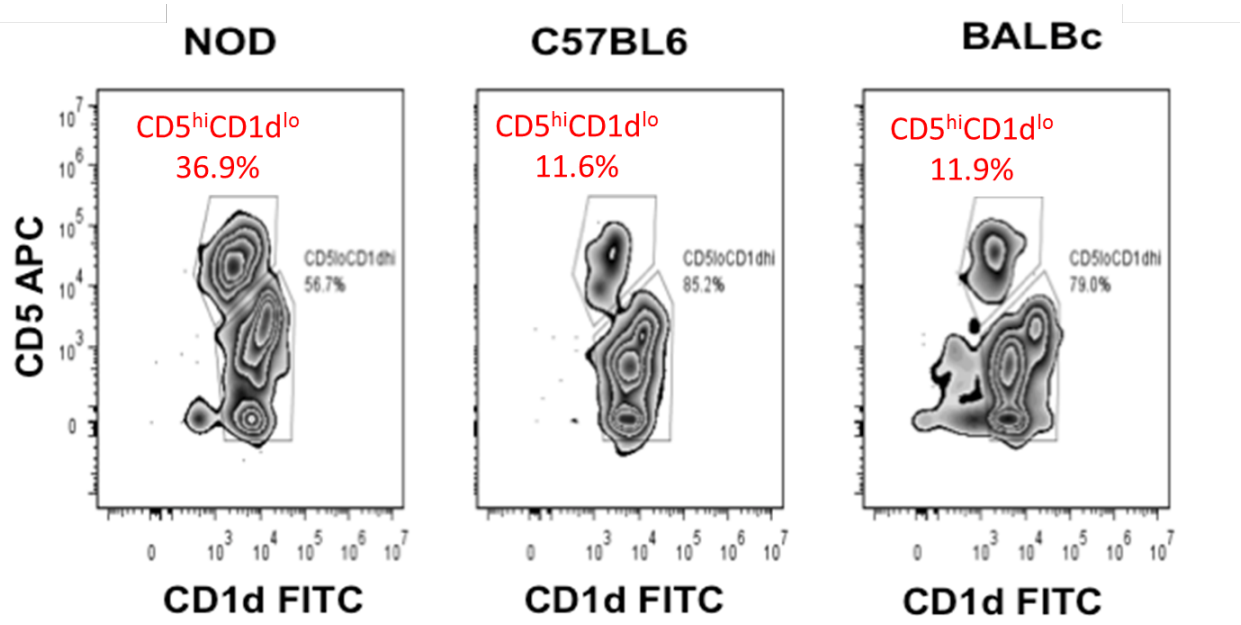

Figure 4. Increase in $CD19^{+}CD5^{hi}CD1d^{lo}$ Regulatory B cells.
Flow cytometric results of bead purified $CD19^{+}$ B cells isolated from the splenocyte pool of 6-week-old NOD, C57Bl/6, or Balb/c female mice. Sorted $CD19^{+}$ cells were stained with CD5 and CD1d to isolate classically described Bregs expressing $CD19^{+}CD5^{hi}CD1d^{lo}$ surface marker levels.

While little is known about human Bregs, recent work to characterize them has succeeded in other autoimmune diseases such as systemic lupus erythrematosus (Bouaziz et al., 2010), rheumatoid arthritis (Lundy, 2009), and multiple sclerosis (Iwata et al., 2011). Bregs with a $CD19^{+}CD24^{hi}CD38^{hi}$ phenotype in healthy controls showed robust IL-10 production upon stimulation with CD40L, as opposed to patients with long term SLE who had depleted IL-10 production (Blair et al., 2010). Work in RA showed that Bregs can be activated to secrete IL-10 and upregulate FasL expression in the presence of the stimulating IL-5 cytokine (Klinker, Reed, Fox, & Lundy, 2013). Recently in the field of MS, B cell depletion with anti-CD20 after disease onset showed slowing of relapsing symptoms, though this was shown to lose efficacy as the B cell compartment reconstituted (Hauser et al., 2008). There is also the concern that depletion of B cells may hinder the ability of Bregs to delay disease onset if given too early. For example, depletion of B cells exacerbates some diseases such as ulcerative colitis, and if given too early in MS patients, has no efficacy on reducing future symptoms (Goetz, Atreya, Ghalibafian, Galle, & Neurath, 2007). These data taken together lead to the hypothesis that Bregs may be playing a role in delaying disease onset in a number of diseases, but that once onset begins, that antigen presenting B cells are expanding the autoreactive T cell pool instead of allowing Bregs to slow autoimmune attack.

CHAPTER V

Age-Dependent Effects of Adoptively Transferred CD19^{+} Cells on Suppression of Autoimmune Diabetes through Interleukin-1β Regulation

A. INTRODUCTION

Strong evidence indicates that an imbalance between autoreactive and regulatory T cells (Tregs) plays a key role in pathogenesis of Type 1 diabetes (T1D) in animal models (D'Alise et al., 2008; Penaranda, Tang, & Bluestone, 2011; Shevach, 2009) as well as in humans (Bluestone, Buckner, et al., 2015; Bluestone, Trotta, & Xu, 2015; Daifotis, Koenig, Chatenoud, & Herold, 2013). The initiation of disease is dependent on the activity of both $CD4^{+}$ and $CD8^{+}$ T cells (Atkinson et al., 2014; Crawford et al., 2011; Eisenbarth, 1986; F. S. Wong & Janeway, 1999), and Tregs can regulate the onset of diabetes in both NOD mice and humans (Bresson et al., 2006; Brusko & Bluestone, 2008; You et al., 2005). While it is clear that T cells are the primary effectors of pathogenesis in T1D (Pietropaolo et al., 2008; Roep, 2003), solid evidence also supports an important role for B cells in disease development, both as autoantibody producers and antigen-presenters (Hinman & Cambier, 2014; Marino et

al., 2008; Morran et al., 2015; Ziegler & Nepom, 2010). In addition to a protective effect of ATG/G-CSF in NOD mice, T1D patients receiving low dose ATG/G-CSF treatment had preserved C-peptide levels after treatment, preservation of Tregs over autoreactive T cells, and significantly increased numbers of $CD19^+$ cells (Gitelman et al., 2013; Haller et al., 2016; Haller et al., 2015; Parker et al., 2009).

B cells play a fundamental role in autoimmune disorders. Antibodies specific for insulin and other pancreatic autoantigens are well-documented hallmarks of T1D (Pietropaolo & Eisenbarth, 2001). NOD mice that are deficient in B cells from birth or due to transient depletion are resistant to developing T1D, whether or not pancreatic autoantibodies are present (Noorchashm et al., 1997; Serreze et al., 1998). In humans, depletion of B cells with anti-CD20 therapy soon after onset of T1D slowed the decline of islet beta cell function and arrested the loss of C-peptide, although long term effects were less (Pescovitz et al., 2009; Yu et al., 2011). Thus, evidence of the contribution of B cells to T1D is clear, although their specific mechanisms of promoting disease pathogenesis require further investigation. As is the case in the T cell compartment, some B lymphocyte subsets exhibit immune suppressive functions and previous reports have shown that adoptively transferred B cells from pre-diabetic NOD mice were capable of regulating the development of T1D in recipient NOD mice (Kleffel et al., 2015; Montandon et al., 2013). One issue that remains unclear is how T1D develops despite the presence of regulatory B and T lymphocytes.

In the present study, we investigated age-related differences in the ability of B cells to control diabetes development in NOD mice, and potential mechanisms for these effects. Adoptive transfer experiments using splenocytes from diabetic NOD female

donors (Christianson, Shultz, & Leiter, 1993; Leiter, 2001) into immunodeficient syngeneic recipients, NOD.*scid* mice, were performed to assess whether diabetes progression was affected by the presence or absence of $CD19^+$ cells (Sarikonda et al., 2013). We provide evidence for an age-specific effect of $CD19^+$ cells in blocking pathogenic immune responses leading to overt diabetes.

B. METHODS

Mouse Models: NOD (Cat. #00196), NOD.*scid* (Cat. #001303), NOR (Cat. #002050), C57Bl/6 (Cat. #000664), and Balb/c (Cat. #000651) mice were purchased from Jackson Laboratories© or bred in-house in pathogen free cages using breeding pairs originally purchased from Jackson Laboratories©. Some diabetic NOD female cell donors were generously provided from the labs of Drs. Matthew Bettini and Maria Bettini (Baylor College of Medicine). Six-week-old NOD.*scid* female mice containing gene mutations resulting in an inability to generate mature T cells and B cells, thereby having no adaptive immune system, were used as immuno-compromised recipients for all adoptive transfer experiments described below.

Monitoring for Diabetes: All aging NOD donor mice and NOD.*scid* recipients receiving adoptive transfers of splenocytes were tested 2-3 times a week (starting one week after adoptive transfer or upon reaching 10 weeks of age) for increases in blood glucose levels. A single drop of blood would be collected from the tail through a small nick at the distal end and collected by capillary action into an Accu-Chek© Aviva Blood Glucose Monitor. Mice were considered diabetic after two consecutive blood glucose measurements above 300 mg/dL.

Cell Harvest and Preparation: Spleens were harvested from different strains of euthanized female mice at 6 weeks of age or > 15 weeks of age. Single cell suspensions were prepared using cell strainers and ACKS lysis buffer to remove red blood cells, and then suspended in phosphate-buffered saline (PBS) to be counted before being placed in culture or undergoing immediate analysis by flow cytometry. Cells being collected for adoptive transfer are described below.

Adoptive Transfer Experiments: NOD.*scid* female mice 6-8 weeks of age (Jackson Laboratories©) were used as splenocyte recipients across all treatment groups. To induce diabetes, NOD.*scid* females were intravenously injected with $5x10^6$ splenocytes from diabetic female NOD mice through a tail vein injection. For the co-transfer experiments, NOD.*scid* females received $5x10^6$ splenocytes from diabetic NOD female donor mice in addition to $5x10^6$ $CD19^+$ cells taken from 6-week-old or >15-week-old NOD female donors. Donor $CD19^+$ cells were purified using the Miltenyi© $CD19^+$ microbead system (Cat. #130-052-201) in a MACS LS magnetic sorting column (Miltenyi Biotech©).

On day 6 after initial adoptive transfer injections, a second intravenous injection of freshly purified $CD19^+$ cells obtained from the spleens of 6-week-old or >15-week-old NOD females was performed in co-transfer NOD.*scid* recipients using $5x10^6$ cell concentration in sterile PBS. On day 12 post-transfer, another boost of freshly purified splenic $CD19^+$ cells was transferred by intravenous injection to maintain a larger B cell pool within the co-transfer recipient NOD.*scid*. NOD.*scid* recipients were followed for

diabetes onset or until day 60 post-transfer of diabetic splenocytes, and euthanized upon meeting either endpoint requirement (Fig. 5).

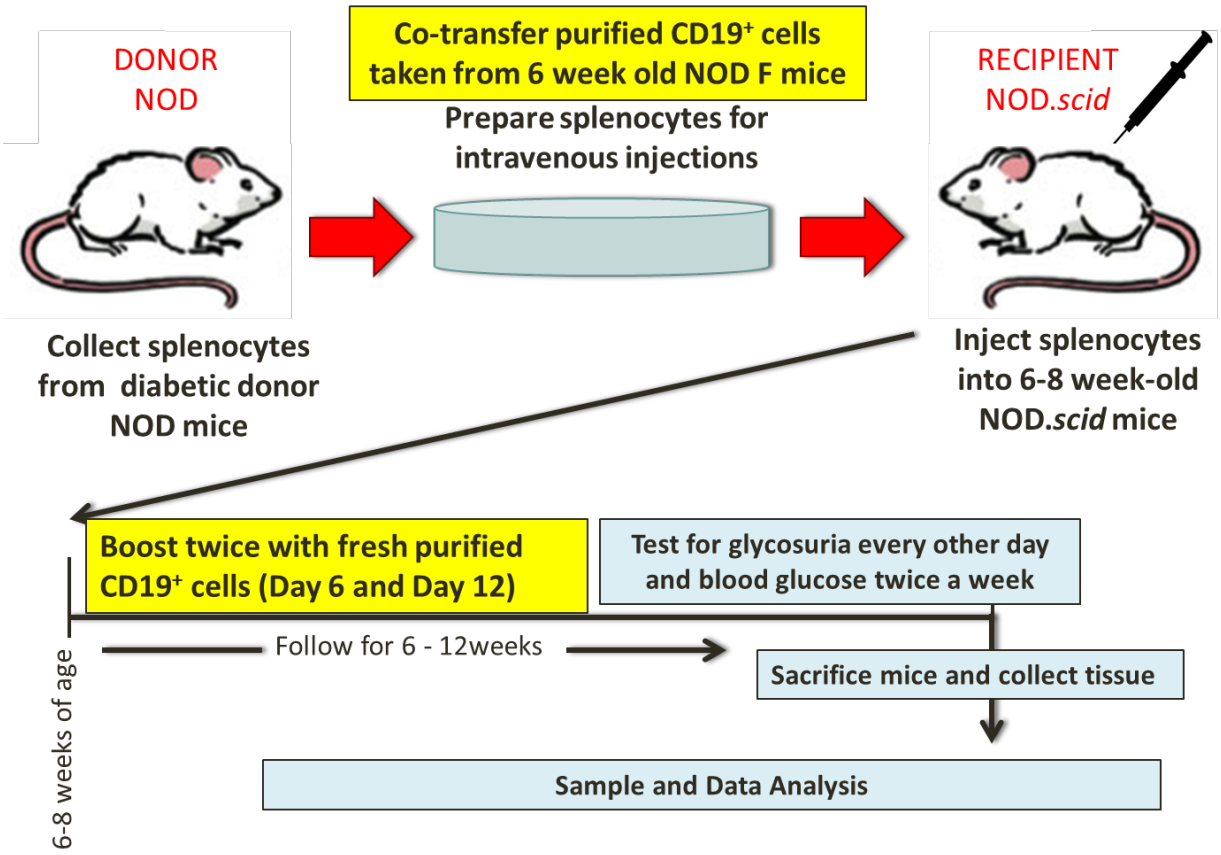

FIGURE 5: Timeline of Adoptive Co-transfer Experiments.
Diagram of adoptive co-transfer experiments including intial transfer of diabetic splenocytes from a diabetic NOD donor, and purification and co-transfer of 6-week-old CD19+ B cells from NOD female donors. Follow-up boosts of purified CD19+ B cells on Days 6 and 12 post-AT are also described.

Anti-IL-1β Antibodies: A subgroup of NOD.*scid* female recipients receiving $5x10^6$ splenocytes isolated from a diabetic female donor also received 100 ug of sterile LEAF® purified anti-mouse IL-1β antibody (BioLegend© Cat. #503504) in sterile PBS by intraperitoneal injection – see results (Cantwell, Bubeck, & Dube, 2010; Lu, Sadri, & Schneider, 2006). NOD.*scid* control mice for this antibody received whole splenocytes from the same diabetic donor, followed by a 100 ug per 100 uL intraperitoneal injection of an Armenian hamster IgG control antibody (BioLegend© Cat. #400933). NOD.*scid* recipients received the initial injection of anti-IL-1β or isotype control IgG antibodies on day 1 following adoptive transfer of splenocytes from a diabetic donor, and intraperitoneal injections on day 5 and every 5 days thereafter until day 40, and the blood glucose levels of all NOD.*scid* recipients was tested 2-3 times a week for diagnosing diabetes as described above. NOD.*scid* recipients were again euthanized upon either developing confirmed diabetes (>300 mg/dL blood glucose) or upon reaching day 60 post-transfer (Fig. 6).

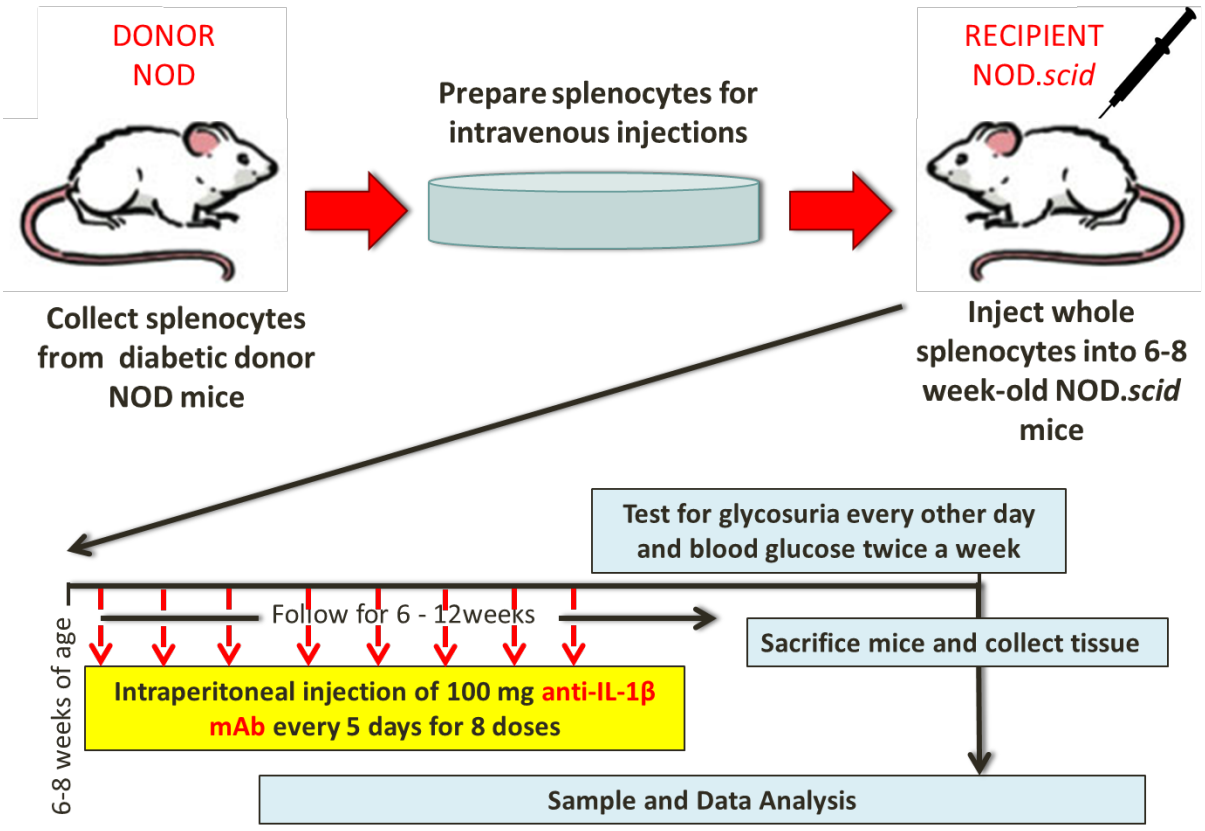

FIGURE 6: Adoptive transfer of diabetes with corresponding antibody injections.
Diagram of adoptive transfer experiments showing transfer of diabetic splenocytes from an NOD female donor into a NOD.*scid* recipient, followed by treatment with an anti-IL-1β monoclonal blocking antibody administered by intraperitoneal injection on a recurring regimen. This was also done with the matching IgG isotype control antibody in the same 100 mg dosing strategy in control NOD.*scid* recipients who also had splenocytes from a diabetic NOD donor intravenously injected.

Tissue Processing, Histology, and Insulitis Scoring: After NOD.*scid* recipients developed diabetes, they were euthanized and the pancreas was collected, fixed in 10% formalin for future Hematoxylin and Eosin Y staining to determine cellular structures. Individual islets were scored across multiple sections for each mouse for the presence of infiltrating lymphocytes to determine the level of insulitis. Individual islets were given a score of 1-4 depending on the severity of islet infiltration according to established protocols (Stumpf, Zhou, & Bluestone, 2013): a score of 1 equating to little or no insulitis, 2 being for mild insulitis covering less than 20% of the total islet, a score of 3 relating to an islet with between 20-50% moderate insulitis, and a score of 4 describing an islet with severe infiltration covering >50% of the islet. All islets were scored on an Olympus© light microscope and statistically analyzed in GraphPad© Prism 6.0 using a 2x2 contingency table with Chi-Square analysis to determine differences between treatments.

Flow Cytometry: Cells collected either directly from spleens, or after undergoing $CD19^+$ microbead purification, were placed into 96-well-plates (sterile flat-bottomed) at a preferred concentration of $5x10^5$ cells per well. Cells were washed with FACS buffer (PBS, 10% fetal calf serum (heat attenuated), and 0.5% sodium azide (NaN_3)), non-specific immunoglobulin binding was blocked with Fc-Block (BD Bioscience© Cat. #553142), and then cells were incubated with the corresponding fluorophore-conjugated antibodies (BD Bioscience©) listed below. Cells were stained with one or more of the following antibodies: CD3, CD4, CD8, CD11c, CD16/32, CD19, CD21/35, CD23, CD25, F4/80, FoxP3, IFN-γ, IL-10, IL-17, IgM, or Mac-1. After incubation with fluorescent

antibodies, cells were washed and analyzed on either a BD Accuri™ C6 or BD LSR™ II flow cytometer. Propidium iodide staining was performed to exclude dead cells from subsequent analysis. All resulting data were analyzed using Tree Star© FlowJo software.

ELISA: Serum from NOD.*scid* recipients and supernatant from cultured $CD19^+$ cells were collected for cytokine analysis. Sandwich ELISAs were performed on these collected supernatants using the BD Bioscience© kit for the following secreted cytokines: IL-1β, IL-4, IL-10, IL-17, IFN-γ, and TNF-α. All washes were done using tris-buffered saline (TBS) with 0.5% Tween20 added (TBS-T). After addition of primary antibody and biotin-conjugated secondary antibody, plates were incubated with biotin-binding streptavidin-HRP to generate enzymatic activity for the blue colorization of the BD Bioscience© Tetramethylbenzidine (TMB) substrate, followed by addition of a sulfuric acid Stop Solution to halt the enzymatic activity. Finally, plates were read on a Wallach© 1420 plate reader for light absorbance and calculated against control protein concentrations to determine the light absorbance to protein concentration ratio. All data were analyzed and graphically represented using GraphPad© Prism 6.0.

Statistical Analysis: Kaplan-Meier plots were derived to estimate the cumulative risk of developing insulin-dependent diabetes. Kaplan-Meier plots were compared using the log-rank test and computed using an exact procedure and conducted as one-sided tests (Peto & Peto, 1972).

Fisher's exact test and chi square analysis were used to compare proportions and evaluate statistically significant associations between categorical variables. The Mann Whitney *t* test for independent samples was used to compare continuous variables between two groups. Flow cytometry and ELISA comparative analyses of populations were performed using nonparametric t tests with Welch's correction. All statistical analyses were performed using GraphPad© Prism 6.0. P-values < 0.05 were considered statistically significant.

Animal Protocol Approval: All protocols involving mice were approved by the Animal Care Safety Review boards at both University of Michigan (Ann Arbor, MI) and Baylor College of Medicine (Houston, TX).

C. RESULTS

Diabetes is significantly delayed in an adoptive transfer model following injections of MHC compatible CD19$^+$ cells from young donor mice.

An adoptive transfer model in which splenocytes obtained from major histocompatibility complex (MHC) compatible diabetic NOD mice were injected into NOD.scid recipients was used to induce diabetes (Fig. 7A). Splenocytes isolated from diabetic female NOD mice were intravenously injected into 6-week-old NOD.*scid* recipient female mice. NOD.*scid* recipients receiving single transfers of diabetic splenocytes started to develop T1D at day 20 post-transfer. Co-transfer experiments were performed using CD19$^+$ cells purified from 6-week-old pre-diabetic female NOD mice to create a boosted B cell pool during the young pre-diabetes phase of the NOD donor. We observed a highly significant delay in progression to autoimmune diabetes in NOD.*scid* recipients when purified splenic CD19$^+$ cells from 6-week-old NOD mice were co-transferred (Fig. 7A). Remarkably, by day 40 post-transfer, 100% of the NOD.*scid* recipients receiving diabetic splenocytes alone had progressed to overt diabetes, while 100% of NOD.*scid* CD19$^+$ co-transfer recipients were still normoglycemic (Fig. 7A; $p < 0.0001$). CD4$^+$ and CD8$^+$ T cell populations (gated initially on CD3$^+$CD19$^-$) were not significantly different after the reconstitution process in either diabetic splenocyte only NOD.*scid* recipients or in mice receiving CD19$^+$ cell co-transfers (Fig. 7B).

Because of the observed delay in diabetes onset in the NOD mouse model, whereby occurrence of diabetes commonly manifests in mice older than 10 weeks of age, we hypothesized that the age of the co-transferred $CD19^+$ cell pool could play a role in their ability to delay diabetes progression. To test this hypothesis using similar protocols previously reported by Chatenoud et al. (You et al., 2005), we carried out adoptive co-transfer experiments injecting $CD19^+$ cells collected from either 6-week-old or aged >15-week-old NOD female donors in NOD.*scid* recipients also receiving splenocytes from NOD diabetic donors. While a similar delay in onset as the previous experiment was observed when $CD19^+$ cells from young donors were co-transferred, NOD.*scid* recipients of $CD19^+$ cells from >15-week-old NOD donors had a similar rate of diabetes progression compared to those recipients of splenocytes alone obtained from NOD diabetic donors (Fig. 7C).

In the next set of experiments, splenocytes from 6-week-old non-diabetic NOD donors were transferred into NOD.*scid* recipients, and the development of overt diabetes was significantly delayed as compared to NOD.*scid* mice receiving splenocytes from diabetic NOD donors (Fig. 7D; $p < 0.0001$). In contrast, NOD.*scid* recipients of splenocytes from non-diabetic >15-week-old NOD donors had progression rates nearly identical to that of NOD.*scid* recipients of splenocytes from diabetic NOD donors. These observations reinforced our findings indicating that there is a $CD19^+$ cellular compartment within young NOD mice that may well exhibit a robust suppressive effect towards diabetes development.

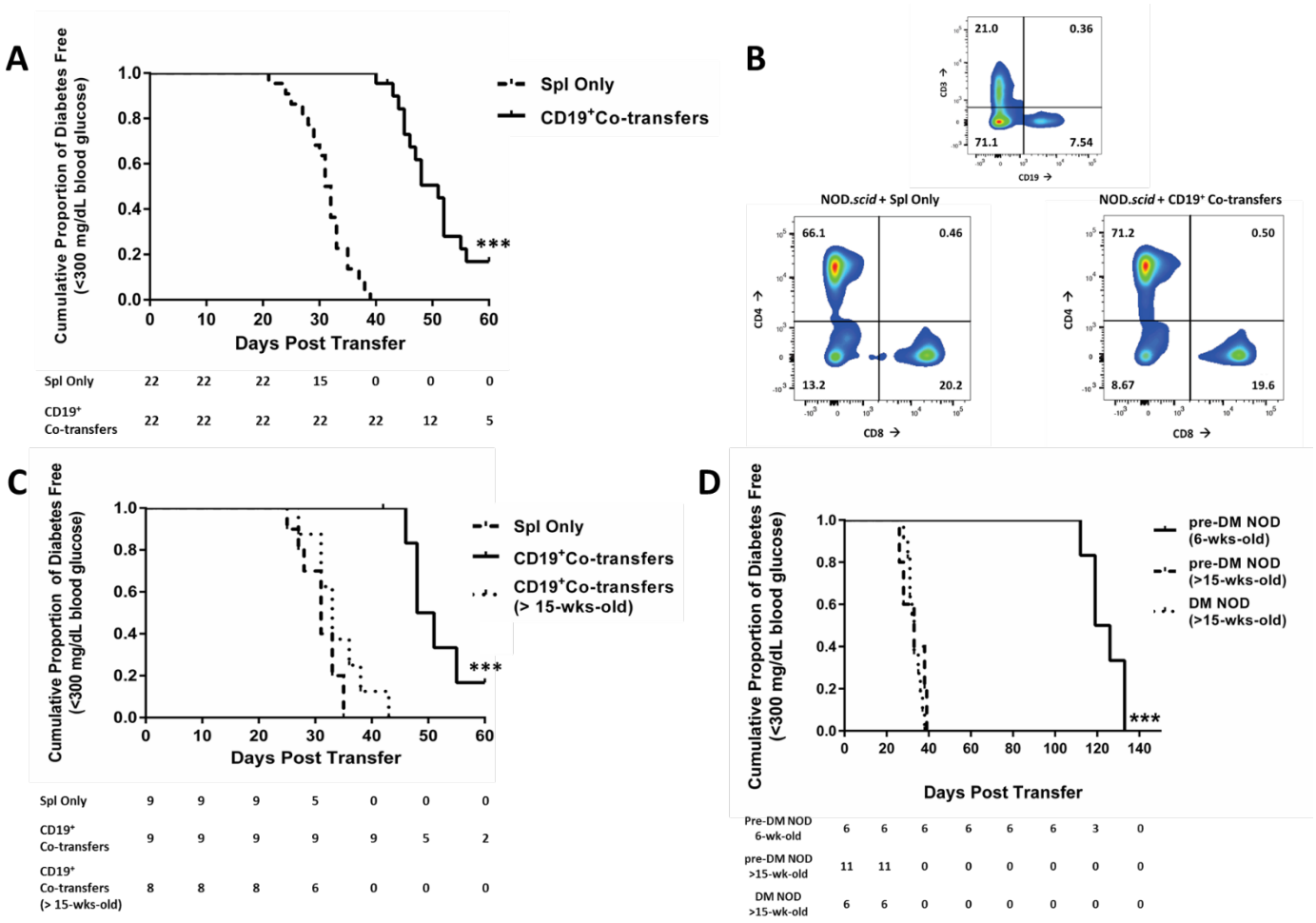

Figure 7. Adoptive transfer of diabetes is significantly delayed in the presence of 6-week-old purified CD19$^+$ cell co-transfers.

(A) Survival curve for comparison between female NOD.*scid* mice (N=44) receiving splenocytes taken from a diabetic NOD female donor (N=22, dashed line), or the same splenocytes plus bead-purified CD19$^+$ cells from 6-week-old pre-diabetic NOD female mice (N=22, solid line. Results analyzed using the Mantel-Cox Log Rank test for survivability (*** = $p < 0.0001$). **(B)** Representative staining of reconstitution experiments with CD3 and CD19 on splenocytes from NOD.*scid* transfer recipients. Cells initially gated on CD3$^+$CD19$^-$ to illustrate differences in CD4 and CD8 specific T cell populations. **(C)** Survival curve comparing female NOD.*scid* mice (N=26) receiving splenocytes from a diabetic NOD female donor (N=9, dashed line), splenocytes and purified CD19$^+$ cells from 6-week-old NOD female donors (N=9, solid line), and splenocytes plus purified CD19$^+$ cells from female NOD donors older than 15 weeks (N=8, dotted line). Group comparisons performed using the Mantel-Cox Log Rank test for survivability (p-values: DM NOD vs. 6-wk-old CD19$^+$ = 0.0002, DM NOD vs. >15-wk-old CD19$^+$ = 0.0976 n.s., and 6-wk-old CD19$^+$ vs. >15-wk-old CD19$^+$ = <0.0001). **(D)** Survival curve from transfers of either 6-week-old NOD F splenocyte donors, >15-week-old pre-diabetic NOD F, or >15-week-old diabetic NOD F splenocyte donors (>300 mg/dL blood glucose). *** denotes p-value < 0.0001.

Concurrent with the development of T1D, insulitis was significantly reduced following co-transfer of $CD19^+$ cells as compared to diabetic control transfers. The administration of splenocytes from an NOD diabetic donor in addition to $CD19^+$ cells from 6-week-old NOD donors resulted in decreased severity of insulitis as compared to adoptive transfers involving splenocytes alone from an NOD diabetic donor (Fig. 8A-E). Insulitis scoring analysis showed that there were major differences between recipient groups observed at the ends of the scoring scale (scores of 1 versus 4), with the mice receiving $CD19^+$ cells from 6-week-old donors having significantly more islets with little to no insulitis as compared to the other treatment options, and fewer statistically significant islets scoring a 4 with greater than 50% islet infiltration (Table 2).

$CD19^+$ cell co-transfers led to a decrease in IL-1β levels.

We measured serum cytokine levels including IL-1β, TNF-α, IFN-γ, IL-17, IL-4, and IL-10 (Figs. 9A-F) (Rabinovitch & Suarez-Pinzon, 2003). NOD.*scid* recipients receiving MHC compatible splenocytes and $CD19^+$ cells from a 6-week old NOD donor had no measureable IL-1β level, with strong statistical differences ($p < 0.001$) as compared to any other transfer group (Fig. 9A). In contrast, we found no significant differences in circulating IL-1β in NOD.*scid* recipients of >15-week-old $CD19^+$ cells compared to mice that received MHC compatible splenocytes only.

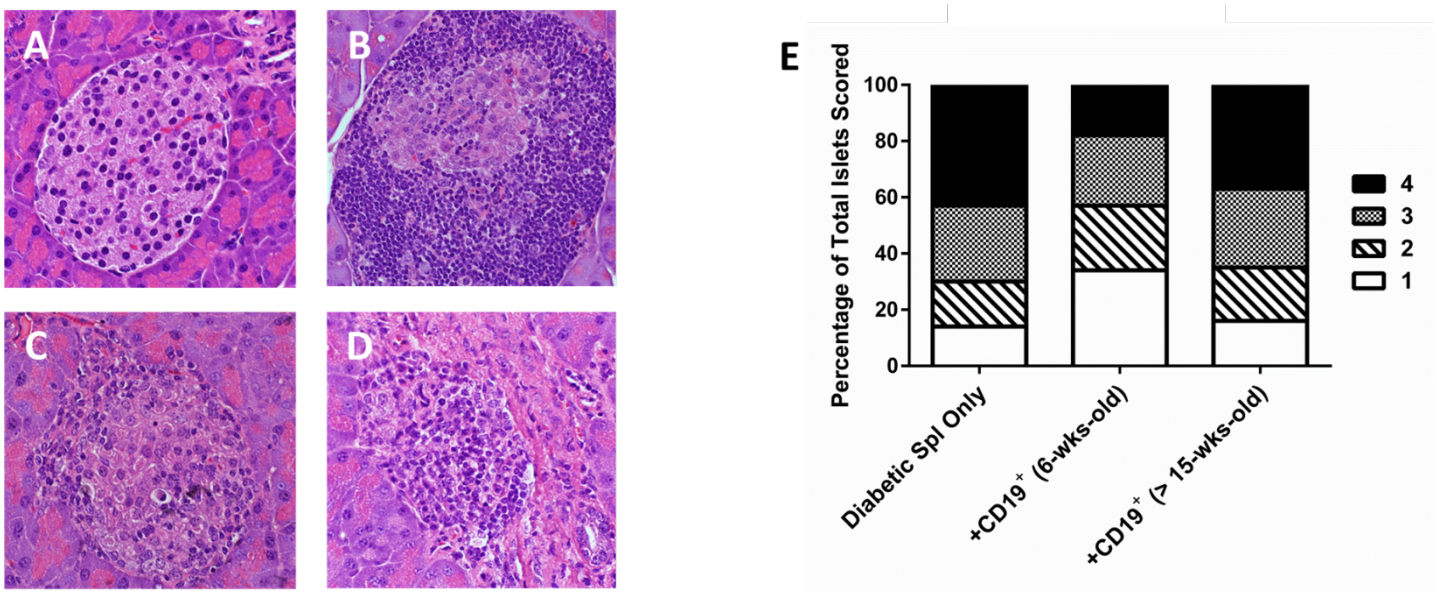

Figure 8. CD19$^+$ cells co-transferred from 6-week-old NOD donors into NOD.*scid* recipients leads to decreased islet infiltration.

Representative islets for each group after staining with hematoxylin and eosin. Images captured at 40x magnification. **(A)** Untreated 10-week-old NOD.*scid* female. **(B)** Diabetic NOD.*scid* recipient receiving only splenocytes from a diabetic NOD female donor. **(C)** Diabetic NOD.*scid* recipient receiving co-transfers of diabetic splenocytes and CD19$^+$ cells purified from a 6-week-old pre-diabetic NOD female. **(D)** Diabetic NOD.*scid* recipient receiving co-transferred diabetic splenocytes and CD19$^+$ cells from a NOD female mouse older than 15-weeks of age. **(E)** Insulitis scoring results from pancreatic sections stained with hematoxylin and eosin of NOD.*scid* recipients received either diabetic splenocytes (N=9 mice), splenocytes co-transferred with bead-purified CD19$^+$ cells from 6-week-old NOD donors (N=9 mice), or splenocytes co-transferred with CD19$^+$ cells purified from >15-week-old NOD female donors (N=8 mice). Islets individually scored on a 1-4 scale of increasing insulitis severity, and the percentage of each score as a part of the whole was graphically represented.

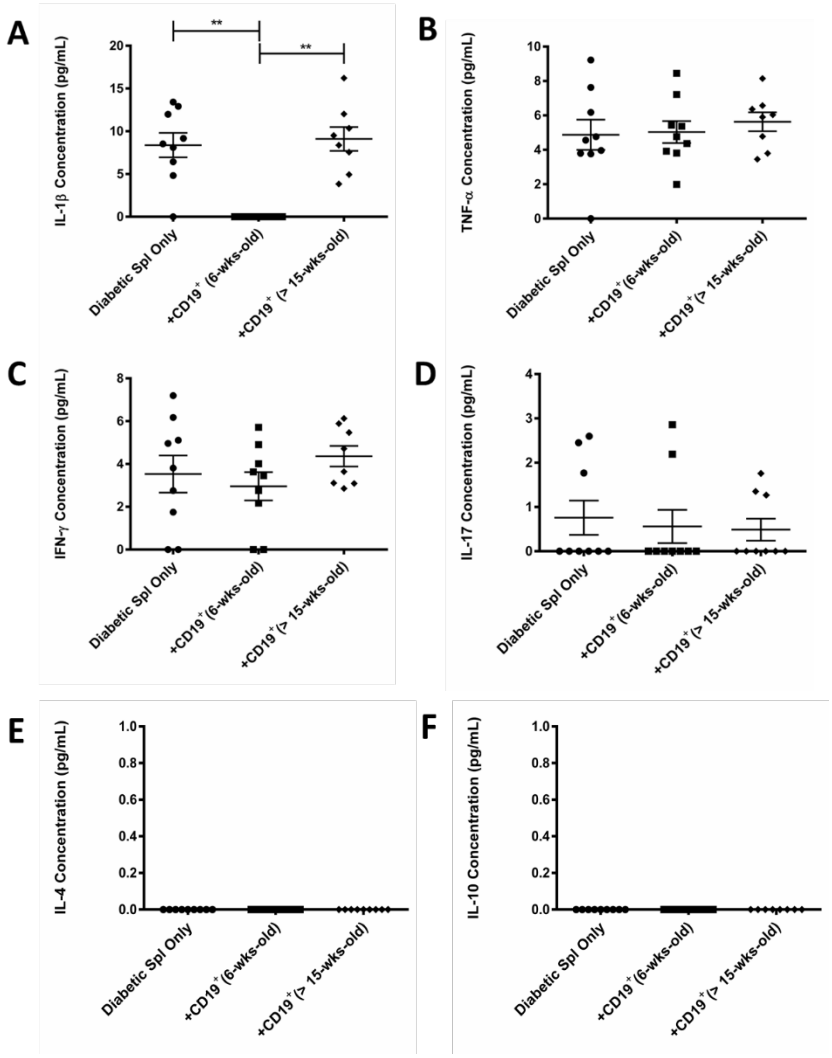

Figure 9. Adoptive co-transfer of CD19$^+$ cells purified from 6-week-old NOD donors inhibits IL-1β secretion without increasing regulatory T cells or reducing macrophage populations.

Serum was collected from NOD.*scid* recipients receiving either splenocytes from a diabetic NOD donor, diabetic splenocytes and CD19$^+$ cells from 6-wk-old NOD donors, or splenocytes from a diabetic NOD donor with CD19$^+$ cells from >15-wk-old NOD donors. The presence of **(A)** IL-1β. **(B)** TNF-α. **(C)** IFN-γ. **(D)** IL-17. **(E)** IL-4. **(F)** IL-10 was analyzed by ELISA (www** = 0.0004).

Co-transfer of CD19$^+$ cells from young mice did not affect regulatory T cell or M1 macrophage levels.

We next determined if co-transfer experiments of MHC compatible spleen cells from NOD diabetic donors and CD19$^+$ cells from 6-week-old NOD donors were capable of inducing Treg expansion, and possibly affect diabetes onset through Tregs. We performed flow cytometry analyses to look for the presence of CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$ natural and inducible Tregs (Fig. 10A and B) and found no significant differences in terms of the total number of Tregs present between any of the treatment groups (Fig. 10B).

We then assessed the presence of macrophages, which are the main source of inflammatory IL-1β secreted during the immune response (Ortis et al., 2012), to determine whether the loss of IL-1β was due to a reduced macrophage pool. M1 macrophages, which are important for islet destruction during the autoimmune attack were gated as CD11c$^-$CD16/32$^+$Mac-1$^+$F4/80$^+$ (Fig. 10C). The percentages across the different treatment groups were converted to total cell number by normalizing to the overall splenocyte counts and compared against one another. We found no statistically significant difference in the resulting M1 macrophage pool whether NOD.*scid* recipients had received 6-week-old donor CD19$^+$ cells or CD19$^+$ cells from >15-week-old NOD mice or no additional CD19$^+$ cells (Fig. 10D). These data suggest that the loss of IL-1β is not due to a lack of available macrophages in the 6-week-old CD19$^+$ cell co-transfer recipients.

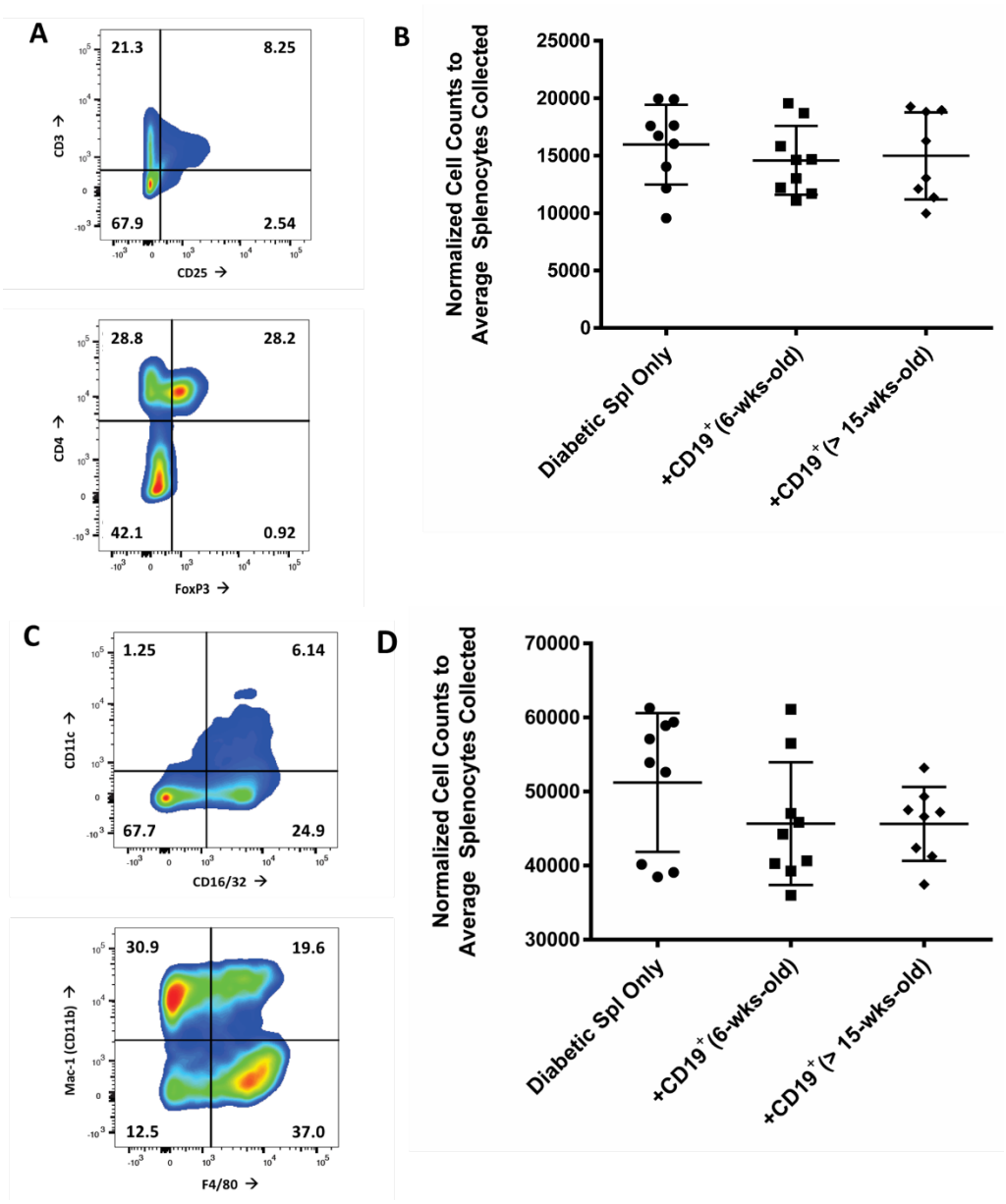

Figure 10. Analysis of regulatory T cells and M1 macrophages in NOD.*scid* recipient mice after transfers.

(A) Regulatory T cells were analyzed from splenocytes of NOD.*scid* recipients. Representative staining protocol for analyzing Tregs showing primary gating for $CD3^+CD25^+$ cells, then gating on the $CD4^+FoxP3^+$ population within the $CD3^+CD25^+$ gate. **(B)** $CD3^+CD4^+CD25^+FoxP3^+$ Tregs were quantified from 26 NOD.*scid* recipients receiving one of three stated treatment options. Total splenocytes were calculated using the percentage subgroup populations and non-significant p-values calculated as a non-parametric t-test with Welch's correction. **(C)** Representative staining for macrophages with cells initially gated on $CD11c^-CD16/32^+$, and then sub-gated on the $Mac\text{-}1^+F4/80^+$ group (upper right quadrant) of M1 macrophages. **(D)** $CD11c^-CD16/32^+Mac\text{-}1^+F4/80^+$ M1 macrophages were analyzed in 26 NOD.*scid* recipients, and total M1 macrophages were calculated using the percentage subgroup populations with non-significant p-values calculated using a non-parametric t-test with Welch's correlation.

Blocking IL-1β delays diabetes progression in NOD.*scid* recipients.

In order to further study the effects of blocking IL-1β in the model, adoptive transfers of NOD splenocytes from diabetic donors into 6-week-old NOD.*scid* recipients were evaluated following intraperitoneal injections of either an anti-IL-1β blocking monoclonal antibody (mAb) or its IgG-matched isotype control antibody (Ab). Mice were followed for onset of diabetes by blood glucose measurement and underwent the same endpoint analyses as during the other co-transfer experiments. We observed that anti-IL-1β treatment resulted in a significant delay in diabetes onset in NOD.*scid* mice that received splenocytes from a diabetic NOD donor (Fig. 11A). In contrast, administration of an isotype-matched antibody did not result in any delay in diabetes progression, as shown in more detail in the statistical analysis between different treatments (Table 3).

Moreover, anti-IL-1β mAb recipients had less severity of insulitis as compared to that of the isotype-matched Ab treated group (Fig. 11B-D), with statistically significant differences in number of islets observed having scores of 1 and 4 (Table 4).

We performed ELISAs to detect serum IL-1β and other cytokine levels in all four treatment groups (splenocyte transfers only, $CD19^+$ cell co-transfers, anti-IL-1β mAb injections, and IgG isotype-matched control Ab treatment) and observed that the mice receiving either the $CD19^+$ cell co-transfers from the 6-week-old NOD donors, or the depleting anti-IL-1β mAb, had no detectable levels of IL-1β, while the splenocytes only transfer group and the IgG-matched control Ab group had normal levels of circulating IL-1β (Fig. 11E). However, there were no significant differences in other pro-inflammatory cytokines described in Fig. 9.

We then evaluated the levels of M1 macrophages to determine if the anti-IL-1β mAb injections were impacting the M1 macrophage population after treatment with the antibody (Fig. 11F). We found no differences between any of the four treatment groups for the presence of $CD11c^{-}CD16/32^{+}Mac\text{-}1^{+}F4/80^{+}$ M1 macrophages capable of secreting IL-1β during the immune response.

Diabetes protection following injections of MHC-compatible $CD19^{+}$ cell co-transfers is associated with increased MZ Precursor cell populations.

To determine if the treatment effects were correlated with expansion by specific B cell subsets, we analyzed spleen cells of NOD.*scid* recipients following injections of MHC compatible spleen cells from diabetic NOD donors alone or in combination with additional $CD19^{+}$ cells. We gated on $CD19^{+}IgM^{hi}CD21/35^{hi}CD23^{hi}$ B cell splenocytes to distinguish the marginal zone (MZ), MZ precursor, and IgM^{hi} follicular B cell populations from other B cell subsets (Fig. 12A). We observed that NOD.*scid* mice receiving 6-week-old $CD19^{+}$ cell co-transfers had a significantly greater MZ precursor cells (p-value < 0.0001) as compared to NOD.*scid* recipients of splenocytes from a diabetic donor only transfers or mice receiving co-transfers of $CD19^{+}$ cells harvested from >15-week-old non-diabetic NOD donors (Fig. 12B).

Finally, we performed the same B cell subset analysis in mice receiving either the anti-IL-1β mAb or the IgG isotype matched control treatment. Anti-IL-1β mAb treatment did not affect the MZ precursor pool, and neither did the administration of the isotype-matched control Ab (Fig. 12C). Only in the presence of co-transferred $CD19^{+}$ cells

obtained from 6-week-old NOD donors were we able to significantly increase the MZ precursor cell population.

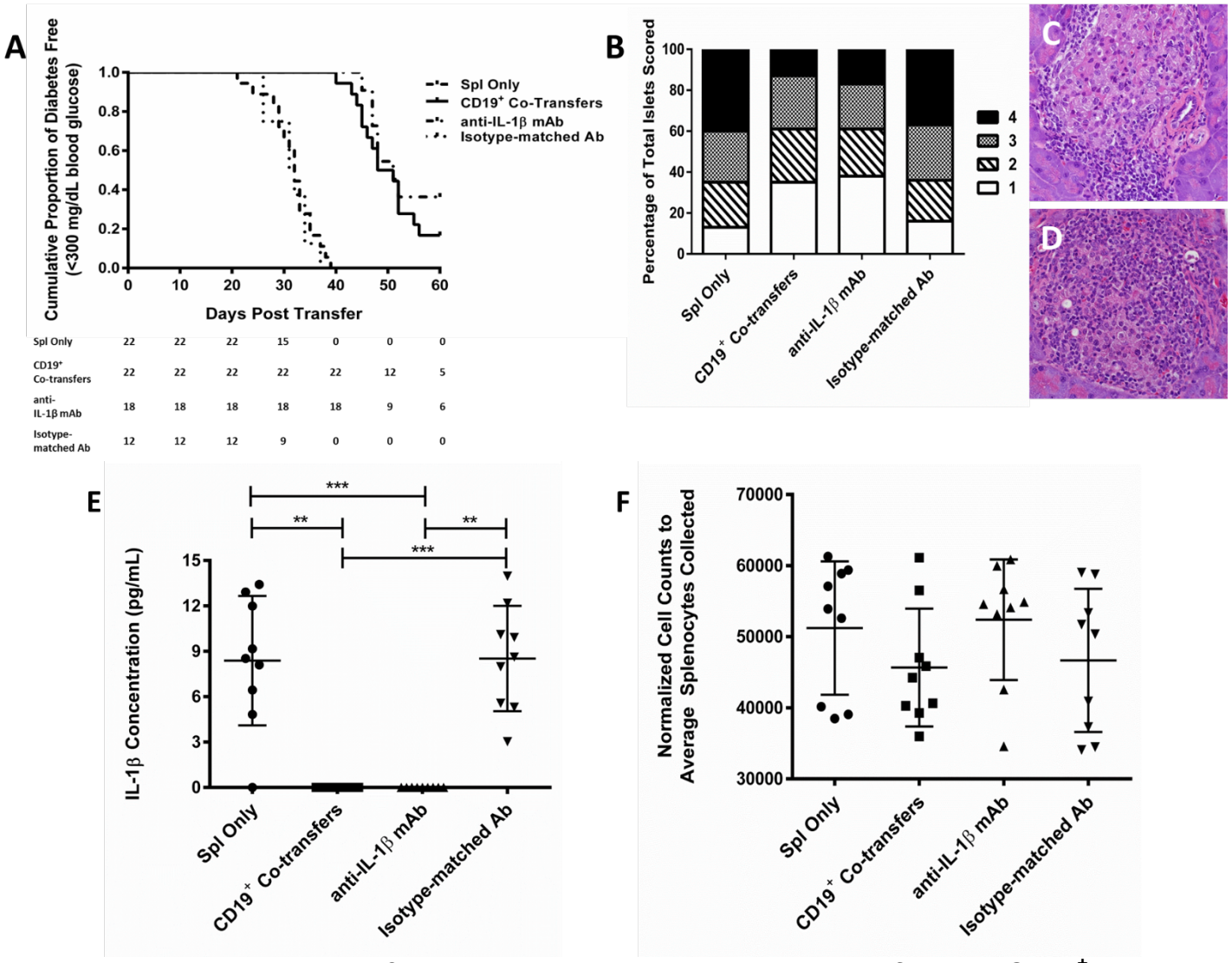

Figure 11. Blocking IL-1β using a monoclonal antibody or performing CD19$^+$ cell co-transfers from 6-wk-old NOD donors result in similar diabetes protection.

(A) Survival curve for group comparison between female NOD.*scid* mice receiving diabetic splenocytes (N=22, dashed line), splenocytes plus CD19$^+$ cells from 6-week-old NOD donors (N=22, solid line), splenocytes plus anti-IL-1β blocking monoclonal antibody injections (N=18, dot-dashed line), and splenocytes plus IgG-matched Isotype Control antibody injections (N=12, dotted line). The population table represents number of diabetes-free mice at each time-point. **(B)** Pancreatic sections scored for levels of insulitis and graphically represented as a percentage of total isles scored. **(C)** Representative hematoxylin and eosin staining of an islet from a NOD.*scid* recipient receiving diabetic splenocytes and anti-IL-1β blocking monoclonal antibody injections. **(D)** Staining of a representative islet from a diabetic NOD.*scid* after receiving splenocytes and injections of IgG isotype-matched control antibody. **(E)** Serum collected during endpoint analysis and measured by ELISA for the presence of secreted IL-1β (**= 0.0004, and ***< 0.0001). Comparisons between mice receiving either diabetic splenocytes only, splenocytes plus CD19$^+$ cells from 6-week-old NOD donors, and splenocytes with either anti-IL-1β blocking monoclonal antibody or isotype matched control antibody injections. **(F)** CD11c$^-$CD16/32$^+$Mac-1$^+$F4/80$^+$ M1 macrophages were analyzed in NOD.*scid* recipients (N=9 per group), and total macrophages were calculated using the percentage subgroup populations with non-significant p-values calculated using a non-parametric t-test with Welch's correlation.

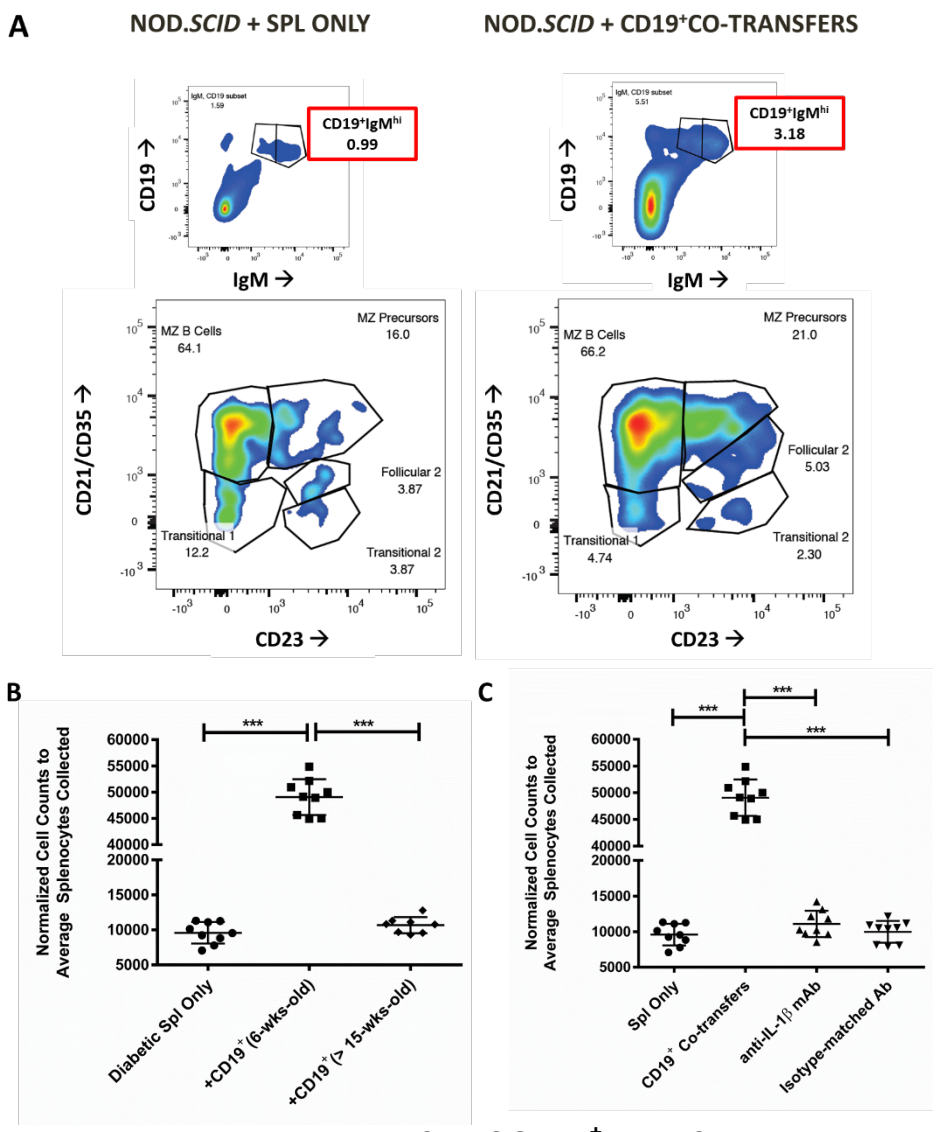

Figure 12. Adoptive co-transfer of CD19$^+$ cells from 6-week-old NOD donors leads to significantly increased pool of MZ Precursor B cells.

(A) Representative staining for CD19, IgM, CD21/35, and CD23 markers in NOD.*scid* mice receiving either splenocytes only or co-transferred CD19$^+$ cells from 6-wk-old NOD mice. Splenocytes were gated on CD19$^+$, and separated into IgM^{hi} and IgM^{low} subsets. IgM^{hi} cells were further gated on levels of CD21/35 and CD23 to determine the percentages of total population that are Marginal Zone (MZB), Marginal Zone Precursor (MZP), Follicular, or Transitional B cells as shown. **(B)** MZ Precursor population analysis from splenocytes of NOD.*scid* recipients following adoptive transfer of diabetes with either diabetic splenocytes only, splenocytes with CD19$^+$ cell co-transfers, or splenocytes with CD19$^+$ cell co-transfers from >15-wks-old NOD donors. **(C)** MZ Precursor cell populations compared between splenocytes only NOD.*scid* recipients, CD19$^+$ cell co-transfers, or injections of either anti-IL-1β mAb or IgG isotype-matched control Ab. Statistical analysis performed using a non-parametric t-test with Welch's correction (*** = $p < 0.0001$).

CHAPTER VI

DISCUSSION

Accumulating evidence suggests that B cells play a role in the pathogenesis of autoimmune disorders including T1D (Hinman & Cambier, 2014). This autoimmune disease is characterized by the generation of autoantibodies against self-antigens (Arvan et al., 2012; Fousteri, Ippolito, Ahmed, & Hamad, 2016), and the elimination of autoreactive T cells involved in the pathological immune response is a logical approach for effective therapy (Krishnamurthy, Selck, Chee, Jhala, & Kay, 2016; Vudattu & Herold, 2014; You & Chatenoud, 2016).

Our results show that diabetes is significantly delayed in an adoptive transfer model of diabetes following injections of MHC compatible $CD19^+$ cells harvested from young NOD female donors. Interestingly, the protective effect conferred by these cells appears to be age specific in that $CD19^+$ cells from 6-week-old pre-diabetic NOD mice may well have regulatory components acting to block diabetes development and insulitis, while $CD19^+$ cells from older mice were ineffective at protection. These B cells from 6-week-old pre-diabetic NOD mice may perhaps be in a honeymoon phase of the disease process, where the number of follicular B cells is decreased, but there is a relatively protected number of MZ and MZ Precursor cells in young NOD mice, but that

the number of follicular B cells increases as the mouse ages. This increased presence of MZ Precursors as a percent of the total number of B cells that an autoreactive T cell would encounter could be the missing part in understanding how using 6-week-old $CD19^+$ cells delays onset of diabetes, while using older B cell NOD donors does not slow pathogenicity. Following these observations, it would be important to look at the effects of Notch ligand stimulation on our $CD19^+$ B cell pool following reconstitution within the lymphopenic spaces of the NOD.*scid* recipients. Recently, it was shown that developing pro-B cells rely upon Delta-like ligand 1 (DLL1), which binds to Notch 2 and induces the expression of the transcription factor Fos, to stimulate the further development of pro-B cells into MZ Precursor and MZ B cells (Iwahashi et al., 2012). It was also shown that deletion of Fos increases follicular B cell development and prevents MZ B cells from being produced. Also, up-regulation of Fos (or increased activation of Notch 2) directly increased the resulting MZ B cell pool. It has also been shown that the stroma within the bone marrow of mice has strong surface expression of delta-like ligands including DLL1 (Chung et al., 2014). One hypothesis is that delta-like ligands may interact with Notch receptors (e.g. Notch 2) of B cells reconstituting the bone marrow/spleen of the NOD.*scid* recipients and, in turn, lead to increased MZ and MZ Precursor production. In a future experiment we will determine whether DLL1 expression on the stroma is affecting the injected B cell pool. We will use a Notch 2 neutralizing antibody at the same time as the adoptive co-transfer experiments. This may prevent DLL1 on stroma cells from inducing regulatory B cell up-regulation and/or expansion through Notch 2 signaling (Tran et al., 2013) and possibly dampen or lose their regulatory effect in our adoptive transfer model. The results of these neutralizing

experiments will determine if any changes occur within the B cell pool following adoptive co-transfers specifically from DLL1 activation of Notch 2. This may result in changes in the functionality of the cell population being adoptively co-transferred (from the 6-week-old NOD donor pool) that includes higher numbers/percentages of MZ Precursor B cells (Fig. 12).

Moreover, it would be helpful to look at the relative number of MZ B cells and MZ Precursors in the PBMCs of T1D, first-degree relatives (FDR) who are diabetes free with one autoantibody (low-risk subjects), and an unrelated non-diabetic control, although admittedly the yield of cells for analysis may be low in some subjects. If there were fewer of these MZ Precursor cells in the new-onset diabetic, or the FDR of a T1D, then this would strengthen our argument that the levels of MZ Precursor cells could correlate with increased risk to developing T1D. Furthermore, if the lack of MZ Precursors as a percent of the total B cell pool is associated with high risk of developing T1D, this finding could lead to a new biomarker whereupon the levels of MZ Precursor cells within collected PBMCs of patients could identify subjects with high risk of T1D progression.

Previous studies in the NOD mouse using *in vitro* stimulated IL-10 secreting B cells from older NOD donors showed that culture stimulated Bregs could delay diabetes onset in NOD.*scid* recipients through enhancing secretory IL-10 and expanding regulatory T cells (Hussain & Delovitch, 2007; Morin et al., 2003). However, our results demonstrate that the ability of 6-week-old purified $CD19^+$ cells to significantly delay diabetes progression was not dependent on *in vitro* activation, but through increasing numbers of available $CD19^+$ cells. These results suggest that the age at which $CD19^+$

cells are used as immune-modulatory therapies may play a pivotal role in their efficacy to exert their suppressive effect. Also along these lines, the importance of IL-10 secreting regulatory B cells in our system hasn't been fully elucidated. With a number of previously described papers in T1D and other autoimmune systems showing an importance for IL-10 secretion in controlling disease pathogenesis, it would be important to determine if recently described IL-10 promoter binding transcription factor Foxd3, which binds and inhibits IL-10 production in normal B cells and is actively down-regulated in Bregs, is either increased or decreased in $CD19^+$ cells (Zhang et al., 2017). Do our MZ Precursor cells act like typical Bregs in that they locally secrete IL-10, or are they actively upregulating other apoptotic surface molecules such as FasL, which binds to Fas receptors on target cells and induces the caspase cascade to cause cellular apoptosis (Lundy, Klinker, and Fox, 2015)? The answer, whether ablation of IL-10 actively prevents regulation, or if it induces other forms of regulatory B cell responses, would further validate the use of these 6-week-old $CD19^+$ cells in controlling diabetes progression.

Our findings also indicate that one differential effect of the transfer of $CD19^+$ cells from young mice was to suppress the *in vivo* production of IL-1β. The blockade of secreted IL-1β during adoptive transfer of diabetes was sufficient to cause a significant delay in diabetes onset in this model system. This suppressive effect of $CD19^+$ cells on IL-1β was lost as the donor mice aged, and was even evident prior to the onset of diabetes in the donor mice. These results suggest that control of IL-1β by $CD19^+$ cells may be a critical factor in delaying or preventing T1D development, and the mechanisms by which this occurs warrant further investigation (Cyster, 2010; Tedder,

2009). We speculate that the primary method of IL-1β depletion is done by the B cells through blocking the $CD4^+$ T cell-mediated response by infiltrating macrophages during the normal pathogenesis of diabetes. IL-1β is secreted by macrophages in conjunction with TNF-α during immune-mediated destruction of pancreatic islets, and the inactivation of the recruitment helper T cells thereby would lead to decreased macrophage activation, even in the presence of increased macrophage infiltration, and cause a noticeable lack of secreted IL-1β found in the peripheral blood. Further studies to determine whether the prevention of IL-1β is done before the internal cleavage product of IL-1β from pro-IL-1β by Caspase-1 within the golgi of trafficking macrophages, or either during secretion or afterwards are warranted to understand at what stage IL-1β is being lost during the immune response once our $CD19^+$ cells are introduced (Lopez-Castejon and Brough, 2011).

The importance of IL-1β in the destruction of β cells has been elucidated from *in vitro* observations and clinical trials in T1D (Moran et al., 2013). It has been reported that β cell destruction by IL-1β works in the NOD mouse through its induction of increased activity of NFκB, an important transcription factor that plays a crucial role in cellular apoptosis (Ortis et al., 2012). IL-1β has been observed as an important up-regulated serum cytokine in patients with active T1D β cell destruction (Perez et al., 2004). Clinical trials have attempted to control the effects of IL-1β through either using an IL-1β receptor antagonist (Anakinra©) or through binding and depleting IL-1β directly (Canakinumab©). Although Canakinumab and Anakinra were safe they were not effective as single immunomodulatory drugs in recent-onset T1D. Interleukin-1 blockade might be more effective in combination with treatments that target adaptive immunity

T1D (Cabrera et al., 2016; Moran et al., 2013). Additional observations in the NOD mouse using a combination of anti-CD3 and anti-IL-1β noted are required for such treatment to be effective (Ablamunits et al., 2012; Herold et al., 2002), and future work using such immune therapies should focus on elucidating the effects of early age B cell immunomodulation towards restoring self-tolerance and yet preserving functional β cell mass in T1D. These results using anti-CD3, anti-IL-1β, and adding MZ Precursor B cell therapy, could work as a combined immunotherapy system whereupon depleting T cell activation, inhibiting a primary disease causing cytokine, and introducing a regulatory component that prevents full macrophage activation and trafficking, as evidenced by reduced insulitis in all mice receiving co-transfers of 6-week-old $CD19^+$ cells, could become strong combined therapy approach.

The results of our NOD mouse experiments highlight the importance of using $CD19^+$ regulatory B cells, including antigen-presenting MZ precursor B cells, when analyzing cell-based therapies for treating T1D. The encouraging results of our study warrant further exploration of targeting β cell-specific T cell-mediated autoimmune destruction of insulin-producing β cells using regulatory B cells. In conclusion, our observations suggest that there is subpopulation of $CD19^+$ cells in the B cell compartment of young NOD mice that exhibits a robust suppressive effect, and may have therapeutic implications in controlling autoimmune diabetes progression.

CHAPTER VII

FUTURE DIRECTIONS

From the data presented above, the importance of pre-diabetic $CD19^+$ cells on influencing and delaying insulitis and onset of diabetes in the NOD mouse has strong therapeutic potential. The ability of these $CD19^+$ cells to ablate the secretion of IL-1β, delay insulitis, and slow the progression to disease with only minimal cell therapy treatments performed at early time points after adoptive transfer of diabetes lends itself to increased studies looking at increasing the amount of cell therapy and the number of boosts being performed. A strong first response experiment would be to understand if the continued weekly or bi-monthly transfer of purified $CD19^+$ cells from 6-week-old NOD female donors could delay onset indefinitely in our adoptive transfer model, or if there is a limit to their capacity to prevent diabetes from occurring.

An initial follow-up experiment that is currently underway to be used in future publications would be to further sort the $CD19^+$ cells, knowing that a further sorting by IgM^+ would increase the purity of the MZ and MZP B cell pool for transfer, while eliminating activated follicular B cells. By eliminating this pool using a dual-sorting protocol, sorting for $CD19^+$ and then sorting the collected population by IgM, we could

purify the B cell pool to include antigen-presenters and eliminate class-switched antibody producers. Due to the importance of B cells as APCs for controlling T1D progression being well-established in the literature (Marino et al., 2008; Serreze et al., 1998), we would hypothesize that the $CD19^{+}IgM^{+}$ dual-sorted cells would delay diabetes onset at least as well as the $CD19^{+}$ sorted cell transfers, if not more robustly than that conferred by naturally occurring IgM (Lobo, 2016). We would also hypothesize that co-transferring $CD19^{+}IgM^{-}$ cells would eliminate most of the protective effect seen in the co-transfer recipient NOD.*scid* mice, due to the elimination of most of the strong regulatory antigen-presenting B cells, which would include MZ B cells, MZP cells, and other regulatory B cells. Our preliminary data seem to confirm this hypothesis. Along these lines, it would also be important to evaluate trafficking patterns in the spleen and pancreatic islets of the different groups by comparing cells that are $CD19^{+}IgM^{+}$ versus trafficking patterns of $CD19^{+}IgM^{-}$ co-transfers.

Since there are currently no B cell labeled green fluorescent protein or red fluorescent proteins to separate the IgM expressers and non-expressers, we could employ currently used CFSE/CFDA-SE and/or PKH26 (red) labeling over the course of a shorter timeline to analyze where the B cells traffic and home. This could further be enhanced by cross-breeding a UBI-GFP or UBI-RPF mouse (available from Jackson Laboratories) with an NOD mouse to develop an NOD B cell donor expressing GFP or RFP long-term, though such mice do not currently exist for purchase. Finally, using new CRISPR-Cas9 targeted gene insertions, it might be possible to insert a GFP and tag it to be expressed in the presence of IgM, then compare co-transfers using these

cells versus mice with lower IgM to compare trafficking and ability to mediate the immune response (Lackner et al., 2015).

Evidence suggests a role of IL-10 secreting regulatory B cells in controlling T1D progression (Hussain & Delovitch, 2007; Kleffel et al., 2015; Lundy, 2009). Because of the important understanding of IL-10 secreting Bregs in controlling T1D, it would be helpful to more deeply investigate the role of IL-10 in our co-transfer system. We hypothesize that our $CD19^{+}$ co-transfers are acting directly during autoimmune T cell recruitment and activation. Using an $IL\text{-}10^{-/-}$ NOD female donor pool would allow us to transfer in $CD19^{+}$ cells incapable of secreting IL-10 in our NOD.*scid* recipients at the time of diabetes transfer, and changes in diabetes progression, insulitis, cytokine production, and other factors could be evaluated (Rajagopalan et al., 2006). Furthermore, changing IL-10 levels using *in vitro* stimulated $CD19^{+}$ cells from NOD female donors capable or incapable of secreting IL-10 would further validate the importance of IL-10 in our system (Klinker et al., 2013). If the role of IL-10 is critical in our co-transfer system, discovering new small molecules or proteins that could increase the IL-10 secretory potential of our CD19+ cells would be useful. Recent work by Dr. Steve Lundy has shed light on a new method for stimulating B cells to produce excess levels of IL-10 using IL-5 receptor stimulation. Future studies will be carried out using IL-5 stimulated $CD19^{+}$ B cells in culture conditions containing IL-5 and mCD40L expressing 3T3 NIH fibroblasts and incubated for five days to induce IL-10 production. We hypothesize that adoptive transfer of stimulated IL-10 secreting B cells from NOD mice into NOD.scid mice may result in a more significant delay of disease onset and a

strong protective effect. Enhancing and activating the regulatory B cell pool using IL-5 may be a novel therapeutic strategy for T1D.

APPENDIX I

Supplementary Tables from Results

Insulitis Score	CONTINGENCY TABLE COMPARISONS		
	Spl Only vs. $CD19^+$ Co-transfers	Spl Only vs. $CD19^+$ Co-transfers (>15-wks-old)	$CD19^+$ Co-transfers vs. $CD19^+$ Co-transfers (>15-wks-old)
4	0.0097	0.5660	0.0207
3	0.8579	0.8805	0.8534
2	0.5249	0.8844	0.6346
1	0.0357	0.8475	0.0225

Table 2. Supplementary Contingency Table P-values calculated using insulitis scores from $CD19^+$ cell co-transfer experiments described in Figure 8.

The Mantel-Cox Log Rank test was performed for each subgroup comparison described in Fig. 2E and resulting p-values shown in the Group Comparison Table. Table depicts the three different 2x2 group comparisons analyzed. Statistics were performed using 2x2 contingency tables and the Chi-Square test for each group at each level of insulitis score.

SURVIVAL CURVE COMPARISONS		
Group Comparisons	**Group n**	**P-value**
Spl Only vs. $CD19^+$ Co-transfers	22 – 22	< 0.0001
Spl Only vs. anti-IL-1β mAb	22 – 18	< 0.0001
Spl Only vs. Isotype-matched Ab	22 – 12	0.9537
Isotype-matched Ab vs. anti-IL-1β mAb	12 – 18	< 0.0001
Isotype-matched Ab vs. $CD19^+$ Co-transfers	12 – 22	< 0.0001
$CD19^+$ Co-transfers vs. anti-IL-1β mAb	22 - 18	0.6141

Table 3. Supplementary Survival Analysis calculated P-values from anti-IL-1β mAb treatment experiments in Figure 11A.

The Mantel-Cox Log Rank test for survivability differences in Figure 5A was performed for each subgroup comparison and resulting p-values shown in the Group Comparison Table. The Group n shows the number of mice in each treatment group used to compare for statistical analysis.

Insulitis Score	CONTINGENCY TABLE COMPARISONS					
	Spl Only vs. CD19⁺ Co-transfers	Spl Only vs. Isotype-matched Ab	CD19⁺ Co-transfers vs. Isotype-matched Ab	Spl Only vs. anti-IL-1β mAb	Isotype-matched Ab vs. anti-IL-1β mAb	CD19⁺ Co-transfers vs. anti-IL-1β mAb
4	0.0007	0.7806	0.0005	0.0001	< 0.0001	0.6290
3	0.9956	0.9271	0.9861	0.7018	0.3680	0.5411
2	0.6975	0.7814	0.3319	0.9637	0.4702	0.7443
1	0.0051	0.4886	0.0092	0.0001	< 0.0001	0.6466

Table 4. Supplementary Contingency Table P-values calculated using insulitis scores from anti-IL-1β mAb treatment experiments in Figure 11.

The Mantel-Cox Log Rank test was performed for each subgroup comparison described in Fig. 5C and resulting p-values shown in the Group Comparison Table. Table depicts the six different 2x2 group comparisons analyzed. Statistics were performed using 2x2 contingency tables and the Chi-Square test for each group at each level of insulitis score.

APPENDIX II

STATISTICAL ANALYSES FOR POWER

Summary of Experimental Design and Group Analysis

In this section I will describe how I separated each experiment within the CD19+ B cell co-transfer model for regulating diabetes progression. As suggested by the committee, these individual analyses will allow me to more effectively and thoroughly interpret the results I have observed. In order to have the cleanest outputs possible before analysis, I decided to separate my experiments into the 5 power Groups (A-E) that were shown in the power calculations above. I will also submit individual Excel Files for each Group (A-E) to show when I diagnosed diabetes so that the committee can see the dates used in the Log Rank tests performed and graphically represented.

- *GROUP A: DM SPLENOCYTES vs. CD19$^+$ B CELLS*

My initial hypothesis was that CD19$^+$ B cells were capable of regulating the pathogenesis of diabetes, and so the experiments are designed to show how adoptively transferring diabetes can be delayed or halted by co-transferring a number of purified CD19$^+$ B cells into a NOD.scid recipient. To validate the reproducibility of the co-transfer model, I performed the experiment four separate times with no other variables introduced and measured each experiment individually. The raw data output showing

the number of days after transfer that the NOD.*scid* was diagnosed as diabetic (blood glucose measurements > 300 mg/dL) and the value seen on the glucometer on that date. The combined Figure 5 shows the pooled values from the four previous experiments generate an overall figure for publication purposes.

- *GROUP B: DM SPLENOCYTES vs. anti-IL-1β*

This initial experiment was developed in response to our serum ELISA data showing negligible levels of IL-1β in $CD19^+$ B cell co-transferred mice. I performed a 3 group experiment using DM splenocytes versus $CD19^+$ B cell co-transfers versus DM splenocytes with intraperitoneal injections of anti-IL-1β monoclonal antibody every 5 days after initial transfer until Day 40. When performing the analysis for this experiment, I separated the groups into 3 different 2 group pairing for cross-sectional analysis. This allows me to test each group against one another and calculate its own Log Rank p-value for each pairing.

- *GROUP C: DM SPLENOCYTES vs. OLD $CD19^+$ B CELLS*

This experiment answers the importance of an age variable in the capacity of the B cells to be regulatory. This would also influence any potential clinical applications that might come from the research. These mice were also separated and individual Log Rank p-values calculated for cross-sectional analysis of groups.

- *GROUP D: DM SPLENOCYTES vs. IgG ISOTYPE*

The experiments in Group D and E were designed as follow-up to Group B after a major issue was rightly raised by the committee that I was not controlling for any non-specific influence of the blocking antibody on disease progression. To perform the

control experiment, we added the matching IgG Isotype control antibody group as the negative control, and attempt to show it cannot influence diabetes progression over the DM splenocyte only transfer group. The two groups were compared to validate any differences using the suggested Mantel-Cox Log Rank test.

- *GROUP E: IgG ISOTYPE vs. anti-IL-1β*

The experiment in Group E was designed to coordinate with Group D and show that there is a difference between the IgG Isotype antibody and the blocking anti-IL-1β mAb. This Group experiment has four different treatments for the NOD.*scid* recipients to validate differences between all four: DM splenocytes, $CD19^{+}$ B cell co-transfers, anti-IL-1β mAb, and IgG Isotype Ab. I performed 6 different paired analyses to determine the different Log Rank p-values for each.

DATA ANALYSIS AND POWER/SAMPLE SIZE

Analysis of the incidence of adoptively transferred diabetes. To determine the incidence of adoptively transferred diabetes, female NOD.*scid* recipients across all Groups were bled twice a week starting one-week post-splenocyte transfer. Diabetes was diagnosed if mice had a glycemic value >300 mg/dL, according to the literature (Christianson et al., 1993; Leiter, 2001). The different groups of mice (Group A-E; see below) were evaluated prospectively to assess diabetes development.

Survival Analysis of adoptive transfer experiments. Survival analysis was applied to estimate the cumulative risk of diabetes onset (Odd O. Aalen, 2008). The approach that I used toward the survival analysis is similar to that previously published by our group (Pietropaolo et al., 2002). All experiments were analyzed on GraphPad Prism 6.0 and p-values were calculated using the Mantel-Cox Log Rank Test for Survival. Survival curves were compared using the log-rank test (see also below). Resulting survival curves were illustrated in GraphPad Prism 6.0 and Follow Up life table were constructed using Microsoft Excel with data generated in Prism. Data was also analyzed using the Statistical Analysis System Software on Windows operating system (Release 6.12; SAS Institute Inc., Cary, N.C.) and SPSS software (SPSS Inc. Chicago, IL).

Sample size, log-rank test power analyses As a general rule, large samples to give more reliable results and small samples often leave the null hypothesis

unchallenged. Large samples may be justified and appropriate when the difference sought is small and the population variance large. Established statistical procedures help ensure appropriate sample sizes so that we reject the null hypothesis not only because of statistical significance, but also because of practical importance. These procedures must consider the size of the type I and type II errors as well as the population variance and the size of the effect. The probability of committing a type I error is the same as our level of significance, commonly, 0.05 or 0.01, called α, and represents the willingness of rejecting a true null hypothesis. We applied both α 0.05 and 0.01 in our power calculations (see tables below). Power analysis can be used to calculate the minimum sample size required so that one can be reasonably likely to detect an effect of a given size. We have applied power sample size calculations using the log-rank test. The log-rank test is one the most popular tests for comparing two survival distributions. This test compares survival across the whole spectrum of time, not just at one or two points. This module allows the sample size and power of the log-rank test to be analyzed under general conditions. Log-rank test power analyses for the planned survival analyses were determined using the Lachin and Foulkes method using the PASS 14 software (Lachin & Foulkes, 1986; Peto & Peto, 1972). Table 1 (Group A-E) presents the projected power to detect differences of proportions of diabetes free mice between the following groups: 1) The control group was estimated as a group of NOD.*scid* mice that received only splenocytes from diabetic NOD mice, in which the proportion surviving at 40 days of follow-up was estimated at 0%; We consistently see 100% of diabetes in NOD.*scid* mice, following adoptive transfer of splenocytes from NOD diabetic mice. 2) The treated group was defined as a group of NOD mice

subjected to a number of treatments detailed below. The proportion surviving at 40 days of follow-up was estimated below for different groups of mice. We then performed a power analysis considering different case scenarios with regard to diabetes incidence for the following groups:

Group A: 50-70%, Group B: 50-70%, Group C: 0-20%, Group D: 0-20%, and Group E: 50-70%.

TABLE 5: GROUP A: DM Splenocytes vs. CD19$^+$ B cells w/ DM Splenocytes

- **HYPOTHESIS:** Purified CD19$^+$ B cells from pre-diabetic NOD female donors delay the onset of diabetes in an adoptive transfer model of diabetes.

Power	Sample Size		Follow-up time (days)	Proportion Diabetes- Free		α
	Splenocytes from DM NOD mice *and* CD19$^+$ B cells (young NOD mice)	Splenocytes from DM NOD mice alone		Splenocytes from DM NOD mice *and* CD19+ B cells (young NOD mice)	Splenocytes from DM NOD mice alone	
0.9251	15	15	40	0.7	0	0.01
0.9831	15	15	40	0.7	0	0.05
0.9079	15	15	40	0.6	0	0.01
0.9778	15	15	40	0.6	0	0.05
0.8778	15	15	40	0.5	0	0.01
0.9675	15	15	40	0.5	0	0.05

Table 5. Log-rank test power to detect a difference in diabetes incidence between NOD.scid mice treated with splenocytes from diabetic NOD mice *and* CD19$^+$ B cells (young NOD mice) vs splenocytes from diabetic NOD mice alone.

Power analysis: Based on the power analysis presented in Table 1A, we have sufficient power and number of mice to test our hypothesis.

TABLE 6: GROUP B: DM Splenocytes vs. anti-IL-1β

- **HYPOTHESIS:** IL-1β secretion will be blocked using a commercially available anti-IL-1β mAb and it will delay diabetes onset in an adoptive transfer model of diabetes.

Power	Sample Size		Follow-up time (days)	Proportion Diabetes- Free		α
	Splenocytes from DM NOD mice *and* Anti-IL-1β mAb	Splenocytes from DM NOD mice alone		Splenocytes from DM NOD mice *and* Anti-IL-1β mAb	Splenocytes from DM NOD mice alone	
0.8157	11	11	40	0.7	0	0.01
0.9430	11	11	40	0.7	0	0.05
0.7890	11	11	40	0.6	0	0.01
0.9312	11	11	40	0.6	0	0.05
0.7463	11	11	40	0.5	0	0.01
0.9106	11	11	40	0.5	0	0.05

Table 6. Log-rank test power to detect a difference in diabetes incidence between NOD.scid mice treated with splenocytes from diabetic NOD mice *and* anti-IL-1β mAb vs splenocytes from diabetic NOD mice alone.

Power analysis: Based on the power analysis presented in Table 1B, we have sufficient power and number of mice to test our hypothesis.

TABLE 7: GROUP C: DM Splenocytes vs. Old CD19$^+$ B cells (>15 weeks of age)

- **HYPOTHESIS:** Purified CD19$^+$ B cells from older (>15 week old) NOD female donors loses its protective effect and does not delay the onset of diabetes in an adoptive transfer model of diabetes.

Power	Sample Size		Follow-up time (days)	Proportion Diabetes- Free		α
	Splenocytes from DM NOD mice *and* CD19$^+$ B cells (old NOD mice)	Splenocytes from DM NOD mice alone		Splenocytes from DM NOD mice *and* CD19$^+$ B cells (old NOD mice)	Splenocytes from DM NOD mice alone	
0.0100	4	4	40	0	0	0.01
0.0500	4	4	40	0	0	0.05
0.0867	4	4	40	0.1	0	0.01
0.2484	4	4	40	0.1	0	0.05
0.1565	4	4	40	0.2	0	0.01
0.3717	4	4	40	0.2	0	0.05

Table 7. Log-rank test power to detect a difference in diabetes incidence between NOD.scid mice treated with splenocytes from diabetic NOD mice *and* CD19$^+$ B cells (old NOD mice) vs splenocytes from diabetic NOD mice alone.

Power analysis: Based on the power analysis shown in Table 1C, the effect of treatment (diabetic NOD mice alone vs splenocytes from diabetic NOD mice *and* CD19$^+$ B cells from older NOD mice) supports the null hypothesis.

TABLE 8: GROUP D: DM Splenocytes vs. IgG Isotype Control

- **HYPOTHESIS:** The presence of a non-specific IgG Isotype control antibody after adoptive transfer of diabetes will not interact with progression to disease or delay the onset of diabetes.

Power	Sample Size		Follow-up time (days)	Proportion Diabetes- Free		α
	Splenocytes from DM NOD mice *and* IgG Isotype Control Ab	Splenocytes from DM NOD mice alone		Splenocytes from DM NOD mice *and* IgG Isotype Control Ab	Splenocytes from DM NOD mice alone	
0.0100	11	11	40	0	0	0.01
0.0500	11	11	40	0	0	0.05
0.2340	11	11	40	0.1	0	0.01
0.4824	11	11	40	0.1	0	0.05
0.4438	11	11	40	0.2	0	0.01
0.7054	11	11	40	0.2	0	0.05

Table 8. Log-rank test power to detect a difference in diabetes incidence between NOD.scid mice treated with splenocytes from diabetic NOD mice *and* IgG isotype Control Ab vs splenocytes from diabetic NOD mice alone.

Power analysis: Based on the power analysis presented in Table 1D, the effect of treatment (diabetic NOD mice *and* IgG Isotype Control Ab) vs a group of mice adoptively transferred with splenocytes alone obtained from diabetic NOD mice, supports the null hypothesis.

TABLE 9: GROUP E: IgG Isotype Control vs. anti-IL-1β

- **HYPOTHESIS:** The use of the anti-IL-1β blocking antibody is specific to preventing IL-1β interactions in the transfer model of diabetes and has a significant effect on the progression of disease when compared to the control IgG Isotype antibody.

Power	Sample Size		Follow-up time (days)	Proportion Diabetes- Free		α
	Splenocytes from DM NOD mice *and* Anti-IL-1β mAb	Splenocytes from DM NOD mice *and* IgG Isotype Control Ab		Splenocytes from DM NOD mice *and* Anti-IL-1β mAb	Splenocytes from DM NOD mice *and* IgG Isotype Control Ab	
0.8157	11	11	40	0.7	0	0.01
0.9430	11	11	40	0.7	0	0.05
0.5099	11	11	40	0.6	0.1	0.01
0.7600	11	11	40	0.6	0.1	0.05
0.1904	11	11	40	0.5	0.2	0.01
0.4227	11	11	40	0.5	0.2	0.05

Table 9. Log-rank test power to detect a difference in diabetes incidence between NOD.scid mice treated with splenocytes from diabetic NOD mice *and* IgG isotype control Ab vs splenocytes from diabetic NOD mice and anti-IL-1β mAb.

Power analysis: Based on the power analysis presented in Table 1E, we have sufficient power (82% and 94% power with α of 0.01 and 0.05 respectively) and a sufficient number of mice to test our hypothesis if the incidence of diabetes is 100% in the group treated with splenocytes from diabetic NOD mice *and* IgG isotype matched Ab vs splenocytes from diabetic NOD mice *and* anti-IL-1β mAb. In the first two rows of Table 1E, we predict 30% (70% diabetes-free) of diabetes incidence in the latter group vs 100% incidence in the former group. We also calculated the power considering different case scenarios with regard to diabetes incidence regarding these two groups.

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