DYSREGULATED T-B LYMPHOCYTE COLLABORATION IN AUTOIMMUNITY POSES A BARRIER TO TRANSPLANT TOLERANCE

By

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DEDICATION

To my Mother,

who gave everything for her children

and

To my wife Meredith,

my rock.

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As so concisely and eloquently stated by Peter Medawar in his 1960 Nobel Lecture, "Far too much is still uncertain." Although in reference to the scientific community's understanding of immunologic tolerance, to which I can only hope that my thesis work has contributed to in some way, Dr. Medawar's brief statement reminds us why science exists in the first place. Simply, we don't have all the answers. Luckily, there is no better time to find these answers than right now and no purer way to discover new knowledge than science. And in today's world, the scientific community is just that, a community. Gone are the days of the lone scientist. Instead, knowledge is now created through the immense and coordinated efforts of scientific teams. Accordingly, I would like to briefly thank my scientific team, as none of my work would have been possible without their unwavering support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Overview

Organ replacement via surgical transplantation represents a potentially life-saving intervention for numerous autoimmune conditions; however, the heightened immunogenicity characteristic of these autoimmune patients remains a significant barrier to long-term graft acceptance. Every year a significant number of autoimmune transplant recipients must undergo re-transplantation directly attributable to both recurrent autoimmunity and heightened allograft immunogenicity. No better is this clinical scenario modeled than the autoimmune non-obese diabetic (NOD) mouse, a model in which no therapy has ever induced permanent transplant tolerance to islet allografts or any other allograft type in the intact NOD immune system, thereby indicating the severity of this immunologic barrier. Overall, determining the immunological barriers to successful organ tolerance in the autoimmune environment would prove invaluable. Not only would developing a mechanistic understanding of how the autoimmune environment fails to tolerate foreign allografts provide new targets for restoring graft tolerance in autoimmunity, but moreover, this knowledge would enhance the scientific community's understanding of specific cellular and molecular pathways that govern immunological tolerance.

Autoimmune pathogenesis and failures to maintain graft tolerance are in part driven by insufficiently regulated, antigen-specific T and B effector cells of the adaptive immune system. Although numerous studies have so far defined specific roles for inadequate immune regulation in driving autoimmunity and perpetuating loss of graft tolerance independently, it remains to be

determined whether the failures in immune regulation that perpetuate loss of self-tolerance in autoimmunity directly contribute to an inability to later "learn" how to tolerate foreign graft tissue as self. Moreover, whether known dysregulations in T-B cell collaboration that drive different forms of autoimmunity directly contribute to an enhanced, inadequately regulated anti-graft response remains to be explored. In my dissertation, I explore the specific hypothesis that dysregulated T-B cell collaboration in autoimmunity poses a stringent barrier to transplant tolerance. Using murine models of Type 1 Diabetes (T1D) and Systemic Lupus Erythematosus (SLE), I define specific cellular and molecular T-B cell aberrancies in these autoimmune environments that lead to a generalized resistance to transplant tolerance.

In setting the stage for my findings, I have provided background information pertinent to the immunologic mechanisms that drive the transplant response. After conducting a brief historical review of clinical transplantation in the United States, I highlight the pioneering work of basic transplant immunologists and their roles in defining the mechanisms of transplant rejection and tolerance. As T and B cells possess the unique ability to recognize graft tissue in an antigen specific manner, I mechanistically address how these cells actively reject or learn to tolerate foreign graft tissue. Finally, I review the key studies in which a generalized resistance to transplant tolerance in autoimmunity was first described while highlighting the outstanding knowledge gaps generated by these bodies of work. Overall, my findings reveal that enhancing graft-protective T Regulatory Cell function is a necessary component of restoring transplantation tolerance in the autoimmune setting. Moreover, interrupting specific pathways that enhance anti-graft effector cell function may further provide a targeted means to enhance transplant tolerance in autoimmunity.

Historical Overview of Clinical Transplantation in the United States

Joseph Murray made clinical organ transplantation a reality when, on December 23rd, 1954 at the Peter Bent Brigham Hospital, he performed the first successful kidney transplant between monozygotic twins[1]. At 24 years of age, Richard Herrick was dying of renal failure secondary to chronic nephritis. Uniquely however, Richard was blessed with a monozygotic twin brother named Ronald who was willing to donate a healthy kidney to his best friend. After 17 different genetic identity tests (including a skin transplant from Ronald to Richard that showed no signs of rejection after 4 weeks), as well as extensive ethical counseling of the twins, Drs. J. Hartwell Harrison and Joseph Murray agreed to perform the donor procurement and recipient placement procedures, respectively[2]. Ronald Herrick's donated kidney, now anastomosed to his twin brother's Richard's iliac vessels, began producing urine just minutes after placement. Within days, Richard's renal function and overall health would return to normal. Richard would go on to live another 8 years after his operation, eventually succumbing to a heart attack[A]. Looking back, Dr. Murray would eloquently reflect in his 1990 Nobel Prize acceptance speech, "in a way, it was spying into the future because we had achieved our long-term goal by bypassing, but not solving the issue of biological incompatibility[3]." Technically, Joseph Murray had proven that solid organ transplantation represented a life-saving surgical intervention. However, for the majority of patients in need of a functioning organ that were not blessed with a

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[A] Ronald Herrick, aged 79, passed away in 2010 from complications after heart surgery. Although insurance data at the time suggested that the average life expectancy of those living with one kidney did not differ from those with two, Ronald still agreed to donate his kidney although no data existed for the life expectancy of a living organ donor[2].

monozygotic twin donor, there remained the immense need to regulate the intense immune reaction brought on by the introduction of a foreign graft.

Prior to Dr. Murray's landmark operation, basic immunologists had made some scientific progress in understanding the fundamental biology driving the transplant response (discussed below in detail in the *Foundational Studies in Transplantation Immunology* section*)*. From these studies, it was apparent to clinicians that successful clinical organ transplantation would require clinical interventions or immunosuppressive agents strong enough to shut down the alloreactive immune response[4]. Early clinical attempts to quell organ rejection, in part, included recipient immune cell ablation via sublethal Total Body Irradiation (TBI) prior to transplantation[5] or use of the anti-leukemic agent 6-mercaptopurine[6], its analogue Azathioprine (AZA)[7], the immunosuppressive steroid Prednisone[8], and the lymphocyte depleting agent Anti-Thymocyte Globulin (ATG)[9]. Moreover, introduction of clinical Mixed Lymphocyte Reactions (MLRs) in 1964 and Human Leukocyte Antigen (HLA) matching in 1968 helped further reduce the likelihood of the anti-graft response during transplantation[10]; the process of donor/recipient matching remains in clinical transplantation practice today[B], although this practice now creates the challenge of finding the best match for high need individuals. Although these early trials demonstrated marked improvements in allograft survival in a few instances, overall 1-year graft survival rates continued to hover around 50%. Clearly in its infancy, the medical community

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[B] Today, the required stringency of donor/recipient HLA-matching depends largely on the type of organ graft[11]. Complete HLA-matching at all 6 loci is critical during bone marrow transplantation [as to avoid Graft Versus Host Disease (GVHD)] and kidney/pancreas allograft survival rates are significantly enhanced with better matching. Due to reduced organ availability, HLA matching for heart and lung transplantation is not necessarily undertaken. HLA matching is not required for liver transplantation.

primarily viewed organ transplantation as an experimental procedure in lieu of a clinically adoptable intervention.

Despite a still insufficient arsenal of pharmacologic agents that could adequately, safely, and reproducibly control organ rejection, leading surgeons continued to push the envelope, working to technically master new methods of organ transplantation. In 1963 Dr. Thomas Starzl performed the first successful liver transplant in Denver, CO[12]; in 1966 Drs. Richard Lillehei and William Kelly performed the first successful pancreas transplant in Minnesota, MN[13]; and in 1967 Dr. Christiaan Bernard performed the first successful heart transplant in Cape Town, South Africa[14]. Unfortunately however, clinicians continued to question the ethics of clinical organ transplantation as most of the patients undergoing these pioneering surgeries succumbed to immune mediated rejection and death.

Fortunately, the discovery of Cyclosporine A in 1976 by scientists at Sandoz and the promising results of its efficacy in transplantation[15,16] ushered in the modern era of organ transplantation. Cyclosporine A, isolated from the fungus *Tolypocladium inflatum*, halts T cell proliferation via inhibition of calcineurin[17]. Spurred by this landmark discovery, research into the development of new anti-rejection drugs exploded. Discovery of FK506/Tacrolimus in 1986 by Goto[18], which similarly but more powerfully inhibits T cell activation by blocking calcineurin activity[17], further improved clinical graft survival rates. In 1995, the FDA approved Mycophenolate Mofetil for kidney transplantation, an agent that irreversibly blocks requisite purine synthesis in dividing T cells[19]. Rapamycin/Sirolimus, which blocks IL-2 mediated signaling in T cells by binding the mammalian target of rapamycin complex (mTOR)[20], was approved by the FDA in 1999 as an alternative to the often nephrotoxic calcineurin inhibitors (CNIs). Finally, the advent of biologic pharmaceutical era has led to the approval of numerous

monoclonal antibody (mAb) based therapies administered at the time of transplantation. Notable agents and their targets include Basiliximab/Simulect (anti-CD25, which blocks activated T cells[21]) and Alemtuzumab/Campath (anti-CD52, which targets and destroys mature T and B lymphocytes[22]). Ultimately, the discovery and introduction of these agents made clinical transplantation the reality that it is today: in 2015, a record 30,973 solid organ transplants were performed in the United States as documented by the Organ Procurement and Transplantation Network.

Immunologic Mechanisms of the Transplant Response

Foundational Studies in Transplant Immunology

Our modern understanding of the transplant response is commonly attributed to Peter Medawar who in 1944 demonstrated that rejection of skin allografts in rabbits was mediated by the host immune system in a process termed "actively acquired immune reactions" or host versus graft (HVG) disease[23]. In this landmark study, Dr. Medawar demonstrated that a skin autograft from the same rabbit (syngeneic) was accepted indefinitely, whereas placement of a skin homograft from a genetically dissimilar rabbit (allogeneic), was rapidly rejected within 3 weeks. In this same study, Dr. Medawar further demonstrated that placement of a second matched allograft was more quickly rejected than the first, implying, but not yet directly stating, the concept of anti-graft memory. Placement of a second allograft, not matched to allotype of the first graft, was rejected with normal kinetics. The biological principles of the allograft response gleaned from these elegant experiments still hold true today.

Today, it is well appreciated that the Major Histocompatibility Complex (MHC), or Human Leukocyte Antigen (HLA), is the primary driver of the anti-graft response. However, it

would take nearly 30 years to prove this concept. In 1948, Gorer and Snell discovered that the small loci of genes encoding the Major Histocompatibility Complex (MHC) were strong determinants of the alloresponse[24]. Using a tumor transplant model, Gorer et al demonstrated that a specific set of tumors successfully engrafted in mice that shared similar Antigen II alleles. However, when transplanted into mice possessing different Antigen II alleles, these tumors were rapidly rejected. Successive crosses and backcrosses between these strains confirmed that the genes controlling the aggressive alloresponse were limited to a small genetic loci, henceforth named the H-2 locus (H for Histocompatibility, 2 for Antigen II). Gorer and Snell named this locus the Major Histocompatibility Complex (MHC) and Snell would receive the Nobel Prize for his findings in 1980. In 1954, Mitchison would demonstrate that allograft immunity was primarily mediated by a cellular, rather than humoral, mechanism[C]; only the transfer of cells from a tumor draining lymph node from a previously immunized mouse could speed the rejection of an allogeneic sarcoma in an otherwise naïve recipient[26]. In 1969, McDevitt and colleagues would definitively prove that the MHC locus (H-2 region in mice) was the primary driver of the alloimmune antibody response via a series immunization and antibody mapping experiments among 33 inbred strains of mice possessing 8 different MHC alleles[27]. Finally, in 1974 Zinkernagel and Doherty introduced the concept of MHC Restriction, a finding that would implicate the MHC as the primary means through which a T lymphocyte is activated. This landmark finding lead to their award of the Nobel Prize in 1996. Using a Cr^{51} release cell-killing assay, Zinkernagel and Doherty demonstrated that Cytotoxic T Lymphocyte (CTL) function

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[C] At the time, researchers debated whether allograft rejection was primarily a humoral or cell mediated process[25]. Medawar would strongly contend the former, and Gorer the latter. Ultimately, researchers would come to appreciate the unique yet often overlapping roles of both humoral and cell mediated allograft rejection.

required LCMV infected targets cells to express both the viral antigen as well as an MHC haplotype that matched the CTLs[28]. In this same report however, Zinkernagel and Doherty referenced previous work by Cerottini and colleagues (1970) providing evidence that T cells alone could robustly mount a cytotoxic response against MHC mismatched lymphocytes[29]. Thus, despite Zinkernagel and Doherty's finding that MHC haplotype matching between target and effector cells was an absolute requirement during viral infection, there existed strong evidence that the alloresponse may not be confined by the principles of MHC restriction.

Direct and Indirect Allorecognition by T Lymphocytes

Today, Cerottoni's findings can be described as the "Direct Alloresponse," or the ability of a T cell to be activated by engaging with a foreign MHC protein[30] (Figure 1.1). MHC Class I expressing graft endothelium represents the primary target of directly alloreactive CD8 T cells whereas MHC Class II expressing donor-derived passenger Macrophages, Dendritic Cells (DCs) and B lymphocytes represent the primary target of directly alloreactive CD4 T cells. Strikingly, research estimates that approximately 5-10% of the polyclonal T cell repertoire is comprised of directly alloreactive T cells[31]. Two hypotheses exist as to why such a large fraction of one's T cell repertoire is comprised of directly alloreactive T cells. The High Determinant Density Hypothesis envisions that alloreactive T cells are selected for their ability to bind foreign MHCs regardless of the peptides they are loaded with[32]. As thymic selection for directly alloreactive T cells would be independent of peptide, a range of low, medium and high affinity T cells would be selected thus contributing to their high precursor frequency. Alternatively, The Multiple Binary Complex Hypothesis posits that a high number of directly alloreactive T cells could instead be selected by specific foreign MHC/peptide structures[32]. As foreign target cells express

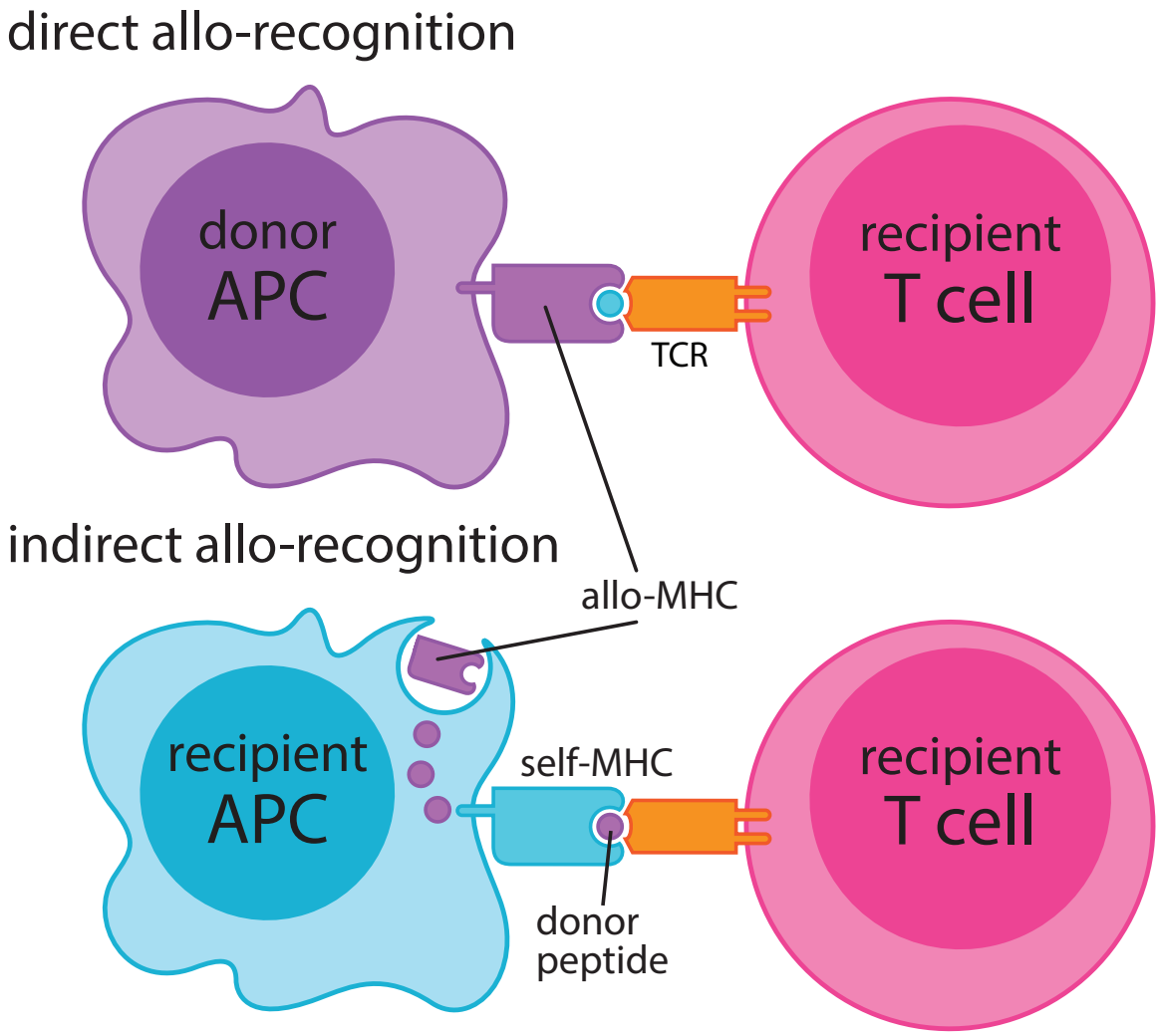

Figure 1.1. Mechanisms of direct and indirect allorecognition by recipient T cells.

a variety of different self-peptides within the context of its own MHC haplotype, a host's directly alloreactive T cell repertoire should be adequately diverse enough to engage with a variety of different foreign MHC/peptide complexes. Regardless of how the alloreactive repertoire is biologically shaped, it is generally appreciated that the acute transplant response, characterized by vascular endothelial destruction 1 to 12 weeks after transplantation, is perpetuated mainly by directly alloreactive T cells[30] (Table 1). Conclusive evidence for direct allorecognition was demonstrated in 2000[33]; transplantation of heart allografts into MHC Class II deficient / Severely Combined Immunodeficient (SCID mice, which possess a nonfunctional VDJ recombination element Protein Kinase, DNA activated, catalytic polypeptide [Prkdc]) recipients alongside adoptive transfer of CD4 T cells resulted in rejection only when the heart allografts expressed MHC Class II. Rejection was solely attributable to directly alloreactive CD4 T cells as these cells could not interact with host antigen presenting cells (APCs) to initiate graft rejection.

In addition to Direct Allorecognition, allografts can also be rejected more insidiously through a process termed the "Indirect Alloresponse" (Figure 1.1). Herein, host APCs process graft antigen and subsequently activate alloreactive T cells via presentation of foreign peptide in the context of self-MHC. In contrast to the direct alloresponse, the slowly evolving process of graft fibrosis characteristic of chronic rejection (which occurs anywhere from 3 months to decades after engraftment) is generally attributed to indirect allorecognition[30]. Initiation and perpetuation of the indirect alloresponse is generally thought to be slower for both anatomical reasons (graft antigen must travel to draining lymph nodes, be processed and presented by host APCs, which then must activate alloreactive T cells, which then must recirculate to the graft itself to initiate destruction) as well as the fact that the precursor frequency of indirectly

Type of Rejection	Onset	Immunologic Mechanism
Hyperacute	Minutes to Hours	Caused by preformed antibodies to donor tissue (against mismatched ABO blood type or MHC) Antibodies bind endothelium and activate complement Causes rapid graft intravascular coagulation, thrombosis, and occlusion
Acute	Weeks to Months	Caused by the recipient's directly and indirectly alloreactive T cells Alloreactive B cells produce alloantibody that deposits on graft endothelium Results in graft inflammation of T cell infiltration
Chronic	Months to Years	Prolonged activation and effector function of alloreactive T and B cells Results in graft vessel intimal thickening, fibrosis, and atrophy

Table 1. Onset and immunologic mechanisms of graft rejection.

alloreactive T cells is nearly undetectable within a normal polyclonal T cell repertoire[34]. Conclusive evidence of rejection mediated solely by indirect allorecognition was demonstrated in MHC Class I knockout mice transplanted with skin allografts from MHC Class II deficient mice[35]. In this setting, graft rejection was restricted to recipient CD4 T cells that could only recognize foreign graft peptides in the context of self-MHC Class II expressing APCs. Despite its delayed initiation however, emerging evidence suggests that in comparison to anti-graft effector T cells generated by direct allorecognition, anti-graft effector T cells generated by indirect allorecognition proliferate more rapidly, take on stronger effector phenotypes, more readily traffic to the allograft, and persist longer as anti-graft memory cells[30]. Regardless of the primary means through which alloreactive T cells recognize and destroy graft tissue (direct vs. indirect allorecognition), these endstage effector cells remain the primary barrier to long-term graft survival and function. The molecular means by which alloreactive T cells are activated and exert the their anti-graft effector function are reviewed below (see *Mechanisms of Transplant Rejection by Alloreactive T and B cells)*.

Allorecognition Mediated by B Cells

Complementing the immensely diverse and strongly immunogenic alloreactive T cell repertoire, B lymphocytes can detect alloantigen and perpetuate graft rejection. Similar to T lymphocytes, B lymphocytes undergo VDJ Recombination from which up to $3x10^{11}$ unique B Cell Receptors are generated, each possessing its own antigen specificity. Attempts to quantify the endogenous alloreactive B lymphocyte repertoire have proven difficult because of their low precursor frequencies even during active immunization[36,37]. However, as a single alloreactive B cell can exponentially give rise to numerous antibody-secreting plasma cells capable of secreting

thousands of alloantibodies per second, it is well appreciated that the alloreactive B cell repertoire becomes substantial after foreign MHC sensitization. In a study analyzing 15 HLA-sensitized multiparous women, precursor frequencies of HLA-alloantibody secreting B lymphocytes averaged as high as 43 per 10^6 B cells[38]. Although the pathogenic role of alloantibodies during graft rejection has yet to be fully elucidated, alloantibody can induce acute graft rejection via complement activation (C4d deposition) and neutrophil and macrophage recruitment (via Fcγ receptors), as well as chronic rejection via altering gene expression in graft endothelial cells ultimately leading to basement membrane remodeling and fibrosis[39]. Furthermore, B lymphocytes can present alloantigen indirectly to alloreactive CD4 T cells via MHC Class II[40]. A detailed description of the alloreactive B lymphocyte response during transplantation is discussed below (see *Mechanisms of Transplant Rejection by Alloreactive T and B cells)*.

Mechanisms of Transplant Rejection by Alloreactive T and B Cells

Alloreactive CD8 T Cells in Transplantation

As graft endothelium richly expresses foreign MHC Class I complexes, directly alloreactive CD8 T cells represent some of the earliest responders in the transplant response. Immediately following transplantation, acute inflammation events result in the release of a panoply of cell attractant chemokines (CCL family) as well as the upregulation of the endothelial cell adhesion molecules (Intercellular Adhesion Molecule 1 [ICAM-1], Lymphocyte Function-Associated Antigen 1 [LFA-1]) [41,42]. Subsequently, directly alloreactive CD8 T cells undergo vascular arrest and MHC/T Cell Receptor (TCR) ligation ensues. Whereas blockade of cellular integrins can delay or prevent allograft rejection[43,44], blockade of specific chemokines or their

receptors has yet to provide any evidence of graft survival prolongation[45-47], perhaps due to the often overlapping and redundant roles of these chemoattractant molecules. However, recent evidence suggests that the cognate interactions between MHCs/TCRs may in fact provide stronger signals for T cell graft arrest than traditional Ga_i-coupled chemokine receptor signaling pathways[48]. Specifically, in 2013 Walch and colleagues demonstrated that OT-I CD8 T cells (a transgenic model in which all CD8 T cells react with the Ovalbumin [OVA] derived peptide SIINFEKL loaded in the H-$2K^b$ MHC) strongly arrested in OVA-expressing heart and kidney grafts even when Ga_i-coupled chemokine receptor signaling was chemically blocked. Moreover, similarly inhibited OT-I cells remained capable of precipitating graft rejection. Elucidating the interplay of cues that guide alloreactive CD8 T cell migration to allografts remains an active area of investigation.

In the event of adequate CD28/B7.1/2 and CD40/CD40L co-stimulation, alloreactive CD8 T cells take on different effector phenotypes[49]. High avidity TCR/MHC interactions result in the generation of CD8+ Tc1 cells capable of producing IL-2 and IFNγ independent of CD4 T cell help. In contrast, low avidity TCR/MHC interactions result in the generation of CD8+ Tc2 cells skewed toward IL-4 and IL-5 production. In the former case, transfer of Tc1 cells to immunodeficient $RAG^{-/-}$ recipients (Recombination Activating Gene null) receiving cardiac allografts perpetuated graft vasculitis and arteriopathy whereas transfer of Tc2 cells perpetuated increased graft eosinophil infiltration. Despite this dichotomy, characteristic of graft CD8 T cell infiltrate is upregulation of the activation markers CD44/CD69 and downregulation of the cell homing marker CD62L and the Protein Tyrosine Phosphatase (PTP) CD45RB[50].

Direct destruction of graft tissue by alloreactive CD8 T cells is largely attributed to their release of inflammatory cytokines and cytolytic molecules. IFNγ production by alloreactive CD8

T cells not only results in graft damage, but also aids in the upregulation of graft MHC Class I expression (leading to enhanced alloantigen presentation), activation of CD4 T Helper cells, and recruitment of Macrophages and NK Cells[51,52]. In the presence of IFNγ, alloreactive CD8 T cells are capable of producing the chemokines IP-10 and Mig further augmenting neutrophil recruitment[53]. Although increased levels of granzyme B, perforin, and FasL have been reported in cases of acute kidney rejection[54,55], the pathogenic role of these CD8 T cell associated cell-killing molecules remains in question. In a murine model of islet transplantation in which islet allografts were transplanted into immunodeficient SCID recipients, transfer of *in vitro* primed alloreactive CD8 T cells rapidly led to graft rejection even if they genetically lacked perforin or FasL[51]. In contrast, CD8 derived IFNγ was absolutely essential for graft rejection[51]. Moreover, in 2014, Zimmerer and colleagues demonstrated that animals genetically deficient for CD8 T cells ($CD8\alpha^{-/-}$) generated higher IgG1 alloantibody levels when challenged with fully MHC mismatched splenocytes[56]. Specifically, CD8 expression of FasL and perforin was required to eliminate alloreactive B cells in an MHC Class I dependent manner. In a broader context, however, the extent and intensity of alloreactive CD8 T cell mediated acute rejection depends largely on the type of organ allograft. Whereas islet and skin allografts appear to be exquisitely sensitive to alloreactive CD8 T cell mediated rejection, the brunt of cardiac and liver allograft rejection is perpetuated by alloreactive CD4 T cells[57].

Alloreactive CD4 T Helper Cells in Transplantation

Since the discovery in 1986 that CD4 T cells are composed of unique "Helper Subsets"[58], a significant body of research has since described the roles of CD4 T Helper cells in the transplant response. The largest body of investigation has centered on TH1 and TH2 cells.

Whereas the former subset is characterized by its expression of the master transcription factor T-bet which is driven by STAT4 signal transduction, the latter expresses the master transcription factor GATA3 induced by STAT6 signaling[59]. Immediately following allogeneic stimulation, TH1 cells secrete IL-2 and IFNγ leading to the activation of alloreactive CD8 T cells that are further capable of producing IFNγ[60]. Alloreactive TH1 cells can also perpetuate graft damage directly through Fas/Fas-L interactions as well as activate B cells to produce alloreactive antibody[60]. Although other models have associated TH1 cells with enhanced immunogenicity and TH2 cells with protective immunity (IL-4 and IL-10 production)[61], this paradigm may not necessarily hold true during the transplant response. As informed by two complementary reports, *in vitro* differentiated TH1 and TH2 cells can independently reject skin[62] or islet[63] allografts when transferred to T cell deficient mice. IL-4 production by rejecting TH2 cells was accompanied by enhanced eosinophilic graft infiltrate, an observation consistent with acute rejection events. Further supporting the overlapping roles of CD4 T Helper Cells in allograft rejection, transfer of T-bet deficient T cells to immunodeficient skin allograft recipients failed to delay allograft rejection over the transfer T-bet sufficient cells[64]. Overall, evidence continues to suggest that both TH1 and TH2 cells are significant participants in acute and chronic rejection.

Nearly a decade prior to the initial discovery of TH17 cells in 2005[65] (characterized by the master transcription factor RORγt driven by IL-6, IL-23, HMGB-1, and TGFβ mediated STAT3 signaling), a report by Van Kooten and colleagues noted increased IL-17 staining in renal allograft biopsies taken from 6 patients experiencing acute rejection[66]. In 2007, Snell et al. found that lung transplant recipients experiencing bronchioloitis obliterans syndrome (BOS) possessed high levels of IL-17 their in bronchoalveolar lavage biopsies[67]. Transient rises in serum IL-17 were then associated with acute rejection events in liver transplant recipients

(2009)[68]. However, the direct contribution of IL-17 producing TH17 cells to the transplant response would not be revealed until 2008. In work published by Yuan and colleagues[69], the authors demonstrated that T-bet deficient animals displayed accelerated rejection of MHCII mismatched cardiac allografts with increases in IL-17 expressing CD4 TH17 infiltrate. In this setting, antibody mediated depletion of CD4 T cells restored graft tolerance while IL-17 blockade doubled the time to rejection. Further supporting the direct role of TH17 cells in allograft rejection, in 2013 Sabet-Baktach and colleagues found that only 40% of cardiac allografts were rejected when RORγt$^{-/-}$ T cells were transferred to immunodeficient recipients[64]. Transfer of RORγt sufficient (albeit T-bet deficient) T cells precipitated rejection in 100% of recipients. Although IL-17 itself is a pleotropic cytokine with numerous isoforms, its definitive role in transplantation has been linked to graft neutrophil recruitment[59]. In fact, depletion of neutrophils can delay cardiac allograft rejection in murine models[70]. Thus, although it appears that TH17 cells play a significant role in acute rejection, emerging evidence has further implicated these cells in chronic rejection. Of particular note, TH17 derived IL-17 has been noted to induce cardiac fibrosis[71] and obliterative airway lesions[72] in murine models and cardiac and lung allograft models, respectively. Research in TH17 mediated transplant rejection remains an active area of research, with recent findings implicating Platelet Factor 4 as a strong negative regulator of TH17 cells during cardiac transplantation[73].

Finally, in comparison to the vast amount of research describing the roles of TH1, TH2, and TH17 cells in the transplant response, very few reports have so far defined a role for T Follicular Helper (TFH) cells in transplantation. Since the discovery of TFH cells in 2000[74] (which express the master transcription factor Bcl-6 and produce IL-4 and IL-21) a single report has demonstrated a mechanistic role for TFH cells during the transplant response. In 2012,

Conlon and colleagues demonstrated that only indirectly-alloreactive transgenic CD4 T cells (B6.TCR75, which bind the H-2K$^{d}_{54\text{-}68}$ derived peptide loaded in self I-A^{b}) transferred to TCR$^{-/-}$ recipient hosts were capable of differentiating into CXCR5+PD-1+ TFH cells[75]. In the presence of B cells, these TFH cells perpetuated a long-lasting IgG alloantibody response. Directly-alloreactive transgenic CD4 T cells transferred to TCR deficient hosts receiving cardiac allografts failed to differentiate into TFH cells and no IgG alloantibody response was observed. Although not directly attributed to attenuation of the TFH cell response, IL-21 Receptor blockade was recently demonstrated to enhance islet allograft tolerance when used in conjugation with anti-CD40L costimulatory blockade + Donor Specific Transfusion (DST) protocols[76]. Despite a still unclear role of TFH cells in the transplant response, emerging clinical data has found that acute renal rejection episodes are associated with increases in the presence of follicular like structures in kidney graft biopsies containing Bcl-6+ TFH cells and IgG+ B cells[77].

Alloreactive B Cells in Transplantation

The pathogenic role of B cell derived alloantibody remains incompletely understood despite clinical data associating chronic rejection events with increased Donor Specific Alloantibody (DSA)[39]. A direct role for alloantibody in graft rejection was in part defined by Brandle and colleagues, who in 1998 found that when alloreactive T cells were inhibited by Cyclosporine A (CsA), passive transfer of alloantibody-rich serum from B cell sufficient mice rejecting cardiac allografts to B cell deficient hosts hastened graft rejection[78]. The necessity of CsA administration stemmed from their previous observation that B cell deficient and B cell sufficient hosts rejected cardiac allografts with similar kinetics. Only when T cell mediated rejection was attenuated via CsA did the B cell deficient hosts demonstrate delayed rejection

kinetics. In this setting, whether the absence of alloantibody was the primary determinant of delayed rejection was not directly answered. More recently, B cell rich tertiary lymphoid tissues (TLT) in chronically rejected kidney allografts were found to produce alloantibodies of diverging specificities in comparison to those alloantibodies generated in secondary lymphoid tissues[79]. The function and pathogenesis of TLT-derived alloantibody vs. secondary lymphoid tissue derived alloantibody remains unknown.

Beyond the perplexing role of DSA in transplantation, alloreactive B cells perpetuate acute and chronic rejection via presentation of alloantigen to T cells. In 2006, Noorchashm and colleagues demonstrated that in the absence B cell MHC Class II expression, median cardiac allograft survival reached > 70 days whereas the presence of MHC Class II expression on B cells rapidly precipitated rapid graft rejection (Median Survival Time 9.5 days)[80]. Mechanistically, the absence of alloantigen presentation by B cells resulted in attenuated alloreactive CD4 T cell activation and proliferation. In contrast, the absence of MHC Class II on B cells did not delay skin allograft rejection[80], thereby bringing to light the diverging roles alloreactive B cells in the context of different organ allografts. Beyond acute rejection events, in 2014 Zeng et al demonstrated that alloreactive B cells promote chronic allograft rejection independent of alloantibody[40]. Using a model in which B cells were genetically unable to secrete alloantibody, the authors found that B cell alloantigen presentation via MHC Class I and II to alloreactive T cells was required to precipitate chronic cardiac allograft rejection. In the absence of MHC Class I/II mediated alloantigen presentation by B cells, alloreactive T cell IFNγ/TNFα production was attenuated, decreased graft infiltrate was observed, and minimal graft vasculopathy was noted.

Pharmacologic targeting of the B lymphocyte compartment during transplantation represents a new opportunity to abrogate rejection[81]. However, depletion of specific B cell

subsets leads to different patterns of rejection in murine models depending on the type of organ allograft. Whereas complete B cell, plasmablast, and plasma cell depletion achieved by an anti-CD19 antibody delayed renal allograft rejection and reduced IgG alloantibody levels, partial B cell depletion achieved by an anti-CD20 antibody did not extend renal allograft survival or reduce alloantibody levels[82]. B cell depletion by either CD19 or CD20 antibodies did not alter cardiac allograft rejection kinetics; however, skin allograft rejection was hastened in the absence of B cells. In the context of murine islet allografting, B cell depletion using a novel anti-CD22 antibody conjugated to the cell toxin calicheamicin (anti-CD22/cal) doubles islet allograft survival[83]. These results mirror findings that B6 mice genetically deficient of B cells (μMT) are slower to reject islet allografts donated by fully MHC mismatched Balb/c mice[84]. Alternatively, antibody mediated blockade of the B cell survival factor BAFF/Blys resulted in permanent tolerance of islet allografts when used in conjugation with Rapamycin (Rapamycin alone did not induce permanent tolerance)[85]. Although still in clinical trials, the B cell depleting anti-CD20 agent Rituximab/Rituxan (indicated for patients receiving HLA mismatched renal transplants) has shown some efficacy in reducing the severity of acute rejection events and preventing chronic alloantibody mediated rejection in high-risk patients[86].

Immunologic Mechanisms of Transplantation Tolerance

"Immunological tolerance may be described as a state of indifference or non-reactivity towards a substance that would normally be expected to excite an immunological response."

– Peter B. Medawar, 1960, Nobel Prize Acceptance Speech[87]

Foundational Studies in Transplantation Tolerance

Overcoming the aggressive transplant response is a daunting task. In work ahead of its time, however, Billingham, Brent, and Medawar would prove that permanent allogeneic tolerance could in fact be achieved between fully MHC disparate mice. Taking advantage of inbred mouse strains generated by George Snell at the Jackson Laboratories, Medawar and colleagues found that embroynic injection of allogeneic splenocytes (A Strain; today's A/J H2-a) into d15 pregnant females (CBA Strain; H2-k) would permit permanent acceptance of A Strain skin allografts in these fetally inoculated mice after they reached adult age[88]. Moreover, CBA mice made tolerant to A-Strain mice were able to rapidly reject non-matched 3rd party skin allografts (AU Strain; today's B6 H2-b) thereby demonstrating the antigen-specific nature of tolerance. Taking a step back, it is important to reflect on these experimental findings in the context of the time (1953); the function and classification of lymphocytes was essentially unknown, the role of the thymus had yet to be identified, and the identification of the MHC locus as the primary transplant antigen had yet to be made. Justifiably, Medawar received the Nobel Prize in 1960 for his pioneering work in immunological tolerance.

Just two years later, Main and Prehn would develop a radical protocol to render adult mice tolerant to allogeneic tissue. Aptly titled, "Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow," their work demonstrated that matching graft allotype to immune system allotype could in theory permit graft tolerance[89]. Such a means to induce graft tolerance did not come without cost however, as Simonsen, Billingham, and Brent quickly recognized the lethality of Graft vs. Host Disease (GVHD) that ensued from allogeneic Bone Marrow Transplantation (1957). From a clinical standpoint, the benefits of graft tolerance did not outweigh the lethality of uncontrollable GVHD.

Thus, developing an alternative means to safely induce graft tolerance was needed; however, it would take nearly 4 decades of research to reach this goal. Researchers would first need to mechanistically understand how the immune system responded to foreign tissue antigen before they were able to control it.

Pharmacologically Induced Transplant Tolerance

In the late 1980s, T cell depletion using a monoclonal antibody directed against CD3 (OKT3) was widely used in clinical transplantation[90]. Although similar anti-CD3 agents could induce transplant tolerance[D] in the preclinical murine setting[91], the growing number of adverse events associated with total T cell depletion led clinicians to question its safety. Interest in preventing alloreactive lymphocyte homing to graft tissue gave way to the discovery in 1992 that antibody blockade of the cellular adhesion molecules ICAM-1 and LFA-1 could induce transplant tolerance to cardiac allografts in mice[92]. In 1996, Larsen and colleagues demonstrated that simultaneously blocking T cell co-stimulation using CTLA-4-Ig (which outcompetes B7.1/2 ligation to inhibit CD28 signaling in T cells) and an anti-CD40L antibody (clone MR1, which inhibits the CD40/CD40L co-stimulation axis in T cells) could induce transplant tolerance to skin and heart allografts[93]. In the same year, Lazarovits and colleagues found that an antibody directed against a splicing variant of the PTP CD45 (anti-CD45RB) could similarly induce

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[D] Reference to induction of "transplant tolerance" herein describes an experimental setting in which the following criteria are met: 1) A short course of the therapy is used around the window of transplantation. Agents given continuously that induce a state of generalized immunosuppression are excluded. 2) Prolonged allograft acceptance is achieved in the majority of animals undergoing therapy (generally accepted as graft survival > 100 days in mice). 3) Placement of a matched allograft is accepted without further treatment whereas a 3rd party non-matched allograft is rejected with normal kinetics.

transplant tolerance to renal allografts in mice and non-human primates[94]. Although the exact mechanism of tolerance induction by anti-CD45RB remains unknown, its principal mechanism of action is thought to center around the generation of antigen-specific, graft-protective T Regulatory Cells (Tregs – described in detail below)[95]. More recently, discovery of the JAK-3 specific inhibitor Tofacitinib (CP-690,550), a small molecule that inhibits IL-2 mediated signal transduction in T cells, was demonstrated to induce transplant tolerance to cardiac allografts in mice[96]. It is important to note that this list of tolerance inducing agents is in no way exhaustive. However, these selected agents each provide mechanistic insight into how transplant tolerance may be achieved: deletion of effector cells (anti-CD3), prevention of lymphocyte homing to graft tissue (anti-ICAM-1/LFA-1), interruption of T cell co-stimulation (CTLA-4Ig/anti-CD40L), induction of graft-protective T Regulatory Cells (anti-CD45RB), and inhibition of effector T cell activation (Tofacitinib) (Figure 1.2).

Transplantation Tolerance in the Clinic

Clinical Operational Tolerance (COT) is defined as an immunosuppression-free state in which a transplant recipient demonstrates prolonged allograft function in the absence of any signs of acute or chronic rejection after 1 year. It is currently predicted that up to 20% of liver allograft recipients may achieve this state after undergoing immunosuppression-withdrawal protocols[97]. Potential mechanisms driving COT in liver allograft recipients include 1) increases in peripheral blood resident γδT cells, CD4 Tregs, plasmacytoid and conventional DCs, and B Cells[98-100], and 2) evidence that liver allograft endothelium can be replaced by recipient bone-marrow derived cells due to the high vascular turnover of this organ[101]. In the latter case, the vasculature of liver allografts would be viewed as "self" and consequently protected from

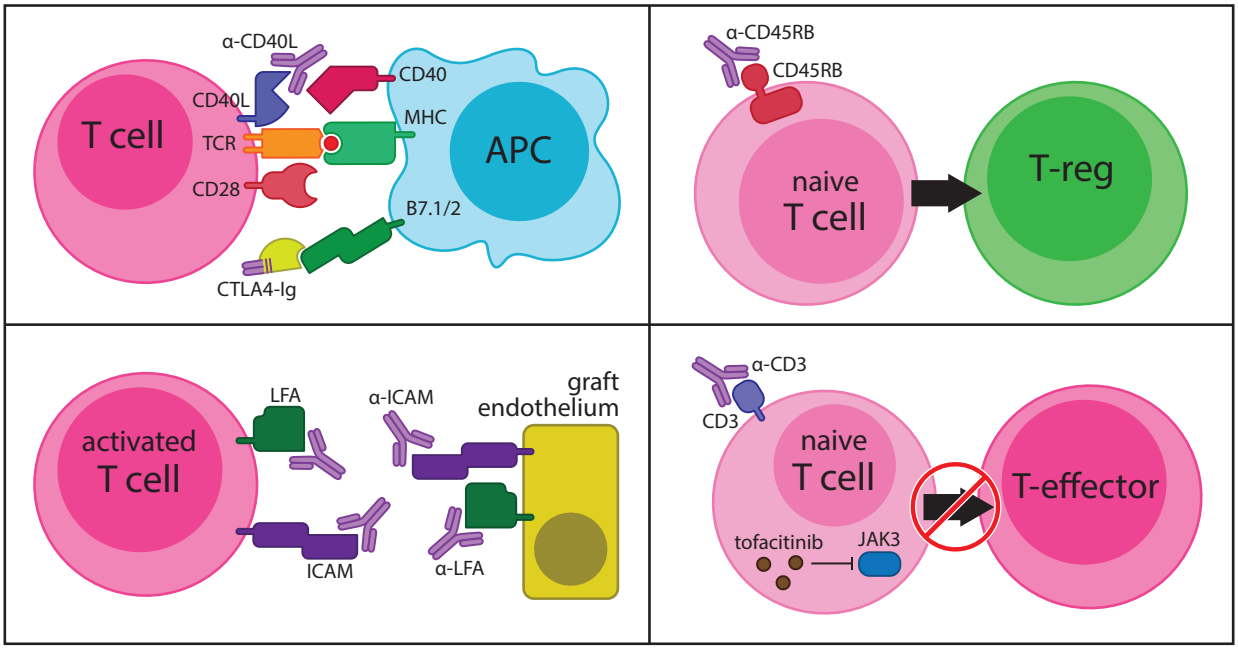


Figure 1.2. Mechanisms of pharmacologically induced transplant tolerance. A variety of preclinical agents can reliably induce permanent transplant tolerance in small animals models. Mechanistically, these agents act via T cell costimulation blockade (upper left panel – anti-CD40L, CTLA4-Ig), generation of graft-protective T Regulatory Cells (upper right panel – anti-CD45RB), inhibition of activated T cell homing to graft tissue (lower left panel – anti-ICAM-1, anti-LFA-1), or deletion/inhibition of anti-graft T effector cell activation (lower right panel – anti-CD3, Tofacitinib).

alloimmune-mediated rejection. In contrast to the tolerogenic nature of liver allografts, COT has only been observed in 1 heart transplant recipient and 1 lung transplant recipient, but never in any pancreatic or intestine transplant recipients[102]. Due to the high number of renal transplant recipients, however, there now exists a cohort of approximately 100 patients who have achieved COT due to immunosuppression non-adherence or carefully monitored immunosuppression withdrawal. Compared to immunosuppressed controls, patients who had achieved COT possessed increases in peripheral blood CD20, IGKV4-1, IGLLA, and IGKV1D-13 mRNA transcript levels as well as higher CD19+ B cell frequencies, with specific increases in Naïve (CD19+CD27-IgD+) and Transitional (CD19+CD38+CD24+IgD+) B cell subsets[97]. Although in its infancy, identification of biological signatures characteristic of COT patients represents an unprecedented opportunity to understand what immunologic factors drive transplantation tolerance.

T and B Cell Mediated Regulation of the Transplant Response

CD4 T Regulatory Cells in Transplantation

Predating the discovery of CD4 T Suppressor Cells (now termed CD4 T Regulatory Cells or CD4 Tregs) was the finding that transfer of CD4 T cells from mice made tolerant to skin allografts could instill tolerance in otherwise untreated recipients receiving matched allografts[103]. Third-party grafts placed on these same secondary recipients were rapidly rejected demonstrating the antigen-specific nature of the transferred tolerance. At this time however, the subset of CD4 T cells conferring such "infectious transplant tolerance" had yet to be identified. In 1995, Sakaguchi and colleagues were first to describe a molecular identifier of CD4 T suppressor cells capable of dampening the transplant response[104]. Using a highly stringent skin allograft model,

the authors found that transfer of CD25 depleted splenocytes into nude mice sped graft rejection compared to animals receiving CD25 replete cells. Genetic identification of Forkhead Box3 (Foxp3) as the master transcription factor of CD4 Tregs in 2001 has since permitted extensive study of the ontogeny, phenotype, of function of these cells[105].

Today, it is widely appreciated that Foxp3+ CD4 Tregs are generated either "naturally" during thymic selection (nTregs – stable Foxp3 expression) or are "induced" peripherally during ongoing immune reactions (iTregs – transient Foxp3 expression). Development of both nTregs and iTregs depends on TGFβ and IL-2 signaling, which respectively induces SMAD and STAT5 binding to the Foxp3 promoter region[106,107]. To date, there are no cell surface or intracellular markers that can be reliably used to distinguish these two cells populations. However, genetic Foxp3 fate mapping models have suggested that nTregs can be identified by extensive demethylation of their Foxp3 promoter region, which confers stable Foxp3 expression[108]. In assessing the relative contribution of nTregs vs. iTregs during transplant tolerance induction, Camirand and colleagues found that the tolerance inducing agent anti-CD45RB preferentially expands nTregs[109]. After adoptive transfer of either Foxp3+ or Foxp3- CD4 T cells to Thy-disparate mice, the authors documented extensive proliferation of Foxp3+ transferred cells and found no evidence of Foxp3- to Foxp3+ conversion. Equally important to mention is propensity of iTregs to convert from a protective to pathogenic state. When exposed to IL-6, iTregs can convert to IL-17 producing TH17 cells[110]. IL-4, IL-6, IFNγ, OX40, Tim-1, and sustained Akt-mTOR signaling each decrease the stability of Foxp3 expression[111-113]. Further defining the contribution of nTregs and iTregs in the transplant tolerance induction remains an active area of investigation.

In assessing the relative contribution of peripheral vs. centrally derived Tregs during transplant tolerance induction, Deng and colleagues posed the paradigm that peripheral Tregs are critical for the establishment of transplant tolerance whereas central Treg generation is critical for the long-term control and maintenance of this state. Specifically, thymectomized animals (thereby preventing the generation of new centrally derived thymic nTregs) demonstrated prolonged, albeit non-permanent, cardiac allograft survival when treated with the tolerance inducing agent anti-CD45RB[114]. Approximately 70% of non-thymectomized controls were permissive to permanent tolerance induction. Thus, the state of transplant tolerance can be viewed as an ongoing process in which previously reprogrammed and newly generated CD4 Tregs work in concert to actively attenuate the anti-graft response.

Beyond Foxp3, CD4 Tregs express a variety of markers including but not limited to CD25, FR4, IL-10, CTLA-4, GITR, GARP, and low levels of CD45RB[115]. A number of reports have documented that both CD25+ and $CD45RB^{LO}$ CD4 T cells actively suppress allograft rejection mediated by their CD25- and $CD45RB^{HI}$ counterparts[116]. In fact, researchers postulate that the tolerance inducing effects mediated by anti-CD45RB is underpinned by the finding that this antibody depletes $CD45RB^{HI}$ effector cells thereby permitting "anatomical space" for the expansion of $CD45RB^{LO}$ Tregs. Moreover, administration of either the CD4 Treg depleting antibody anti-CD25 (PC61) prior to antibody mediated tolerance induction is a routinely used strategy to investigate CD4 Treg mediated control of the transplant response[117]. More recently, scientists are working to determine where CD4 Tregs dampen the transplant response and promote graft tolerance. Although CD4 Tregs can be found residing within the tolerant graft itself, it is unknown whether these cells suppress the transplant response in a similar manner to lymphoid resident CD4 Tregs. In 2009, a study by Zhang and colleagues demonstrated that the

allograft tolerant state is achieved by the sequential migration of CD4 Tregs from the blood, to the graft, and finally to the graft draining renal lymph node[118]. Overall, the dynamic role of CD4 Tregs in the establishment of transplant tolerance has led to clinical trials in which the efficacy of these cells in renal (The ONE Study - NCT02091232) and liver (deLTa - NCT02188719) transplantation is currently being explored.

CD8 T Regulatory Cells in Transplantation

In comparison to their extensively studied counterparts, there exists much fewer data describing the role of CD8 T Regulatory Cells (CD8 Tregs) in the transplant response. Until the discovery of Helios in 2015 as the master transcription factor of CD8 Tregs[119], identification of murine CD8 Tregs has relied on various combinations of cell surface expression phenotypes including ($CD44^{HI}$, GITR+, CTLA-4+, CD28-), (CD62L+, $CD44^{LO}$, $CD45RB^{HI}$), (CD44-, $CD103^{HI}$), (CD122+, $CD44^{HI}$, Ly49+), (CD122+, PD-1+) or TCR/Qa-1 MHC restriction[120]. Despite their potent capability of suppressing numerous forms of autoimmunity including Experimental Autoimmune Encephalomyelitis (EAE)[121], collagen-induced arthritis[122], and lupus[123,124], few reports have explored the mechanisms by which CD8 Tregs dampen the alloresponse[E]. In the context of islet transplantation, naturally occurring, alloantigen-naive CD122+ CD8 Tregs were found to be more suppressive than their CD25+ CD4 Treg counterparts when transferred to islet allograft bearing immunodeficient $RAG^{-/-}$ recipients[125]. In this setting, CD8 Treg suppression was mediated by IL-10. Injection of IL-15 (which directly

[E] Although many additional reports have documented roles for CD8 Tregs in the rat and xeno-transplant settings, all reports reviewed in this section describe studies performed in the murine setting. Rat and xeno- based transplant platforms have been excluded for the sake of uniformity; all data presented throughout this dissertation have been gleaned from the murine or human setting.

expands CD8 Tregs via their expression of the IL-15 Receptor CD122) further delayed islet allograft rejection in immunocompetent hosts.

Further characterization of CD122+ CD8 Tregs found that the PD-1+ fraction of these cells distinguished them from their non-suppressive CD8 memory T cell counterparts. Specifically, the transfer of 2CTg CD122+PD-1+ CD8 Tregs (derived from transgenic mice in which all CD8 T cells bind the Balb/C derived H-2L^{d} MHC Class I peptide SIYRYYGL loaded in the context of self H-2b) delayed skin allograft rejection in antigen-specific and IL-10 dependent manner[126]. These data fall in line with a previous study demonstrating that antibody mediated blockade of PD-1/PD-L1 broke tolerance that was otherwise maintained by PD-1 expressing OT-I CD8 T cells[127]. Furthermore, blockade of the ICOS/ICOS-L axis promotes the generation of PD-1 expressing CD8 Tregs capable of prolonging cardiac allograft survival[128]. Although not in the context of transplantation, co-stimulation via the CD28-B7.1/2 axis is also required for CD122+ CD8 Treg mediated suppression of the CD4/CD8 IFNγ response[129]. In 2009, Shi and colleagues found that human CXCR3+ CD8 T cells possess similar suppressive functions as murine CD122+ CD8 Tregs as informed by comparative DNA microarray analysis[130]. Whether these CXCR3+ CD8 T cells participate in clinical graft rejection or tolerance has yet to be defined. Looking beyond what markers define a true CD8 Treg or how these cells mechanistically dampen the transplant response, CD8 T cells are absolutely required for long-term allograft tolerance[131].

B Regulatory Cells in Transplantation

B Regulatory Cells (Bregs) are relatively new participants in the transplant response. Even less well phenotypically defined than CD8 Tregs, a population of IL-10 producing

$CD1d^{HI}CD5+$ B cells were first described to suppress inflammatory intestinal disease (2002), inflammatory arthritis (2003), and ear inflammation perpetuated by a delayed type hypersensitivity reaction (2008)[132]. In these models, Breg mediated suppression quelled inflammatory IL-1 production and dampened CD4 TH1 responses. In 2007, Deng and colleagues were first to demonstrate that B cells were absolutely required during anti-CD45RB mediated tolerance induction to cardiac allografts[133]. Building on their findings that B cell deficient recipients of cardiac allografts were resistant to anti-CD45RB mediated tolerance induction, the authors next demonstrated that the adoptive transfer of B lymphocytes from wild-type animals could restore tolerance in otherwise resistant B cell deficient hosts. Expression of CD45, CD40, and B7 was required for Breg mediated tolerance induction. In the context of islet allografting, Tim-1 was later found to be a marker of Bregs that produce IL-10 and IL-4, promote a TH2 response, and possess the ability to directly transfer antigen-specific allograft tolerance[134]. When used in conjunction with anti-CD45RB, a low affinity anti-Tim-1 antibody enhanced islet allograft tolerance induction in a B cell dependent manner[135]. Further characterization of Tim-1+ Bregs has identified their capacity to induce graft-protective CD4 Treg expansion via a TGFβ dependent mechanism[136]. Complementing these observations, in 2009 Walters and colleagues found that BAFF-Tg mice indefinitely accepted islet allografts even in the absence of any tolerance inducing therapy[137]. Establishment of graft tolerance was directly attributable to CD4 Treg mediated activation by BAFF-expanded B cells.

Overall, the role of IL-10 in B cell mediated transplantation tolerance remains in question. In 2010, Zhao and colleagues demonstrated that neutralization of B cell derived IL-10 during anti-CD45RB mediated tolerance induction reduced chronic cardiac allograft vasculopathy and graft-specific alloantibody levels[138]. However, in the context of anti-CD40L

mediated tolerance induction, the absence of B cell derived IL-10 resulted in decreased T Follicular Regulatory (TFHR) cell numbers and enhanced IL-21 production by pathogenic TFH cells[139]. In conjunction with IL-6, enhanced IL-21 led to the generation of TH17 cells that directly precipitated cardiac allograft rejection.

In addition to clinical observations that operationally tolerant (COT) renal allograft recipients possess enhanced B cell numbers (as reviewed above), there now exists evidence that COT patients possess enhanced numbers circulating CD19+CD24^{HI}CD38^{HI} Bregs as compared to patients experiencing chronic allograft rejection[140]. In fact, patients undergoing Alemtuzumab induction therapy possess increased numbers of circulating CD19+CD24^{HI}CD38^{HI} Breg during the first few years after treatment. Circulating B cells in COT patients richly express miR-142-3p, a microRNA that directly regulates TGFβ expression[141]. In the coming decades, further investigation of Breg biology will undoubtedly lead to a deeper understanding of how these cells control the transplant response.

Resistance to Transplantation Tolerance in Autoimmunity

Transplantation represents a potential cure for end organ damage directly attributable to autoimmune disease. Unfortunately, overcoming the transplant response in autoimmunity is a significant hurdle in that grafted tissue is subject to both newly introduced alloimmunity against MHC mismatches within the foreign graft as well as recurrent autoimmunity that initially destroyed a patient's native organ[142]. No better is this clinical scenario modeled than the T1D-prone non-obese diabetic (NOD) mouse, a model in which no treatment has *ever* induced permanent transplant tolerance to grafted islet tissue in the intact NOD immune system. Furthermore, even when the transplanted tissue is not subject to autoimmune attack (i.e. cardiac

or skin allografts), NOD mice remain resistant to any tolerance inducing strategy[143,144]. In comparison, these same agents readily induce permanent transplant tolerance to a variety of organ allografts in nearly all non-autoimmune mouse strains. Overall, an initial failure to tolerate self-antigen likely contributes to an inability to later "learn" to accept foreign tissue as self. At this point, it is important to note that all studies exploring autoimmunity as a barrier to transplant tolerance have so far been conducted in the autoimmune NOD setting. The hypothesis that autoimmunity, in general poses a barrier to transplant tolerance, especially those conditions that necessitate clinical transplantation due to end-organ failure (i.e. SLE), is explored in Chapter V.

Loss of tolerance in NOD mice is polygenic in nature[145]. Thus, there are likely numerous genetic factors that mediate resistance to transplant tolerance in NOD mice. Identification of Insulin Dependent Diabetes (*Idd*) risk loci subsequently led to numerous backcrossing studies in which these regions in NOD mice were replaced with those from a diabetes resistant strain (B6). In nearly all instances, introduction of these loci resulted in either reduced insulitis or protection of diabetes[146]. However, introduction of these loci either alone or in tandem never rendered these congenic NOD mice susceptible to tolerance induction[147]. It can be inferred that no single genetic region renders NOD mice resistant to tolerance induction.

Complementing the initial observation that NOD mice were resistant to anti-CD40L mediated tolerance induction to islet and skin allografts[143], Moore and colleagues similarly demonstrated that NOD mice are resistant to anti-CD45RB mediated tolerance induction to islet and cardiac allografts[144]. Choice of this agent was predicated on its generally non-immunosuppressive effects and ability to induce antigen-specific, graft-protective regulatory cells (as compared to anti-CD40L). The authors first observed that CD4 T cells from NOD mice failed to proliferate to the same extent as CD4 T cells from tolerance-susceptible B6 mice when

exposed to anti-CD45RB. As previous data demonstrated that adequate cell cycle progression is required for the generation of diabetes-protective TH2 cells[148] and furthermore, that CD4 T cell hypoproliferation is characteristic of NOD CD4 T cells[149], the authors speculated that inadequate CD4 T cell proliferation by anti-CD45RB may result in a failure to induce graft-protective cells. Secondly, the authors found that whereas anti-CD45RB upregulated MHC Class II expression on B cells in tolerance susceptible B6 mice, such upregulation was not observed in NOD mice undergoing therapy. As B cells are solely responsible for T cell activation in the NOD system due to dendritic cell and macrophage deficiencies[149], improper B cell alloantigen presentation could result in an improper balance of effector vs. regulatory T cell activation during tolerance induction (T cells are exquisitely sensitive to antigen load). Determining what factors contribute to CD4 T cell hypoproliferation and improper B cell antigen presentation in the NOD system may offer clues as to why this strain resists transplant tolerance.

Enhanced indirect alloreactivity in NOD mice may further contribute to this strain's resistance to transplant tolerance[150,151]. Whereas MHC Class I and II deficient B6 islets are uniformly accepted by non-autoimmune Balb/c recipients in the absence of any tolerance-inducing therapy, these same islets are readily rejected by NOD hosts. Indirect allorejection in the NOD system depends on the presence of B cells as B cell deficient NOD mice uniformly accepted MHC Class I and II deficient islet allografts. As emerging evidence demonstrates that indirect allorecognition represents a more stringent barrier to long-term graft acceptance than direct allorecognition[30], why B lymphocytes enhance indirect alloreactivity in the NOD system remains an outstanding question. Although transient in nature, temporary depletion of B cells prior to CTLA-4Ig mediated tolerance induction in NOD mice delays allograft rejection 4-fold[83].

As B cells are likely regenerated after cessation of treatment, the reemergence of these critical APCs likely reinitiates the aggressive anti-graft T cell response characteristic of this strain.

A number of studies have further explored the roles of BAFF, CD103, ICOS, IL-2, IL-10, IL-21, iNOS, SOCS3, and STAT1 in NOD islet allograft rejection. In general, genetic deletion (NOD.BAFF$^{-/-}$[152] recipients or iNOS$^{-/-}$[153] or STAT1$^{-/-}$[154] donor islet allografts), pharmacologic disruption (depletion of CD103+ cells[155], antibody blockade of ICOS[156], antibody blockade of IL-2[157], or IL-21R-Fc mediated blockade[76]), or enhancement (whole animal overexpression of IL-10[158], or RIP-SOCS3-Tg overexpressing donor islet allografts[159]) of these molecules have at best demonstrated modest prolongation of allograft survival in NOD recipients. Permanent transplant tolerance was never achieved. Although CD4 Tregs can actively establish, maintain, and transfer tolerance in non-autoimmune recipients, there exists only one study demonstrating the role of CD4 Tregs in the NOD allograft setting[160]. In a rather complex set up, the authors found that transfer of polyclonal NOD CD4 Tregs significantly delayed islet-reactive NOD.BDC2.5 mediated rejection of C3H islet allografts (a transgenic model in which all CD4 T cells recognize an islet specific, Chromogranin A derived peptide) as compared to mice receiving polyclonal NOD CD4 Tregs or NOD.BDC2.5 T cells alone. Whether CD4 Tregs in the autoimmune T1D setting are competent of suppressing allograft rejection remains to be answered.

Significance of the Research and Synopsis of the Data

Overall, understanding why the autoreactive immune system resists transplant tolerance demands developing a comprehensive understanding of the cellular and molecular pathways that control immunologic tolerance. In my dissertation, I explore the specific hypothesis that dysregulated T-B cell collaboration in autoimmunity poses a barrier to transplant tolerance.

Ultimately, identifying these key immunologic disruptions may one day improve clinical transplantation outcomes in patients with autoimmunity and reveal new pathways that dictate immunologic tolerance.

In Chapter II, I provide evidence that B lymphocytes are the critical barrier to transplant tolerance in T1D-prone NOD mice and that these cells limit graft-protective CD4 T regulatory cell function. In questioning whether failures to extrinsically regulate deleterious autoimmune B cell function may further perpetuate this strain's generalized resistance to transplant tolerance induction, my findings in Chapter III provide new evidence for a requisite role of CD8 T Regulatory cells in dampening islet immunity and instilling long-term transplant tolerance. Dysregulations at the earliest stage of T and B cell development (the hematopoietic stem cell [HSC] niche) are linked to autoimmune pathogenesis. Thus, in Chapter IV I find that adequate HSC mobilization is required for transplant tolerance induction; moreover, I find that failures to adequately mobilize HSCs in autoimmune NOD mice in part contribute to this strain's generalized resistance to transplant tolerance induction. Finally, I questioned whether other forms of autoimmunity characterized by dysregulated T-B cell collaboration are similarly resistant to transplant tolerance induction. In Chapter V I demonstrate that lupus-prone B6.SLE123 mice are highly resistant to transplant tolerance induction and possess an Effector T Cell population that resists T Regulatory Cell mediated suppression. Although each lupus-derived congenic region was alone sufficient in preventing transplant tolerance induction, in Chapter VI I provide evidence that overcoming a heightened IL-6 response and enhanced T cell glycolysis during transplantation partially restores graft tolerance in these lupus-prone mice. Ultimately, my findings indicate that functional deficiencies in T-B cell collaboration likely

prevent transplant tolerance in autoimmunity while providing rationale approaches for restoring allograft tolerance in these highly immunogenic settings.

CHAPTER II

B LYMPHOCYTES PREVENT TRANSPLANT TOLERANCE IN T1D PRONE NOD MICE BY LIMITING CD4 T REGULATORY CELL FUNCTION

Scientific Goal

Although T1D-prone NOD mice are exquisitely resistant to transplant tolerance induction, the specific immunological barriers driving this phenotype have yet to be elucidated. Stemming from observations that B lymphocytes are the crucial antigen presenting cell in the initiation of T1D in NOD mice, in Chapter II I explore the hypothesis that B lymphocytes similarly represent a key barrier to successful transplant tolerance in this strain. Findings from this Chapter will directly address how B lymphocytes participate in the transplant response in autoimmune T1D. Furthermore, these studies will directly address whether the autoimmune environment possess an intrinsic regulatory potential to later "learn" how to tolerate foreign graft tissue when the deleterious actions of B lymphocytes are absent. Overall, my studies in Chapter II explore whether interrupting deleterious anti-graft T-B cell collaboration via genetic elimination of the entire B cell compartment can directly render the autoimmune environment permissive to transplant tolerance induction.

Introduction

There are presently over 2 million Americans with Type 1 Diabetes (T1D) and within the next year more than 15,000 children will be diagnosed[161]. Autoimmune T1D is characterized by inappropriate T and B cell mediated destruction of the insulin producing beta cells of the

pancreas. In the absence of endogenous insulin, patients are unable to properly regulate glucose homeostasis, which in turn results in an inability to properly store energy and build tissue. A state of metabolic starvation is perceived within the body; the liver undergoes gluconeogenesis, ketone bodies are produced, and fat is broken down. After a period of rapid weight loss, polyphagia (increased hunger), polyuria (increased urination), and polydipsia (increased thirst), patients often present to hospitals in a state of ketoacidosis during which physicians make a diagnosis of T1D. Lifetime insulin therapy is initiated which includes daily blood glucose checks and proper carbohydrate counting. Although improvements in insulin therapy have greatly increased the life expectancy of patients, insulin therapy does not come without risk. Failure to maintain euglycemia and prevent hyperglycemia can result in early blindness, kidney failure, and amputation[162]. On the other hand, too strict adherence to insulin therapy can result in life threatening hypoglycemic episodes that can lead to seizures, coma, and even death[163].

Although insulin therapy is life prolonging, it is not a replacement for a patient's healthy islets. Patients with T1D possessing residual beta cell function have overall reductions in glycosylated hemoglobin A1C levels, reduced incidences of T1D related complications, and fewer episodes of life-threatening hypoglycemic events[164]. Accordingly, researchers and physicians have worked tirelessly to realize the clinical potential of beta cell replacement therapies as a treatment for T1D. One such method, islet transplantation from cadaveric donors, is a promising approach for patients for patients with T1D[165]. Unlike traditional open transplantation surgeries, donor islets are infused into a recipient's portal vein to eventually take residence in the liver as they are too big to pass through the sinusoids. Patients undergoing this relatively non-invasive, outpatient therapy demonstrate improved metabolic regulation and decreased T1D-related complications compared to patients receiving insulin therapy alone[166-168].

Unlike other solid organ allograft survival rates however, only 44% of patients undergoing this procedure remain insulin-free three years after islet infusion[169]. Moreover, islet transplantation has yet to be widely adopted as transplant recipients must undergo chronic immune suppression to prevent foreign graft rejection. As these drug cocktails have significant side effects (including a role for direct graft toxicity[170], e.g. Rapamycin), islet transplantation remains difficult to justify in children with T1D. Overall, realizing the full potential of clinical islet transplantation as a treatment for T1D demands a deeper mechanistic understanding of the immunological barriers that stand in the way of successful organ engraftment in the context of autoimmune diabetes.

The Non-Obese Diabetic (NOD) mouse represents the most widely used model to explore T1D pathogenesis and islet transplantation in the context of autoimmunity[171]. Although the NOD mouse more likely resembles a single case study of T1D in that these mice are completely inbred (within the human population, T1D is known to affect individuals across all backgrounds at an equal frequency), disease pathogenesis in NOD mice aligns closely with clinical T1D. Both NOD mice and patients with T1D share over 60 known Insulin Dependent Diabetes (*Idd*) risk loci, generate anti-insulin autoantibodies prior to disease onset, gradually develop islet infiltrates comprised of beta cell specific T and B cells, and often present with fulminant disease during their juvenile ages. Progression to overt disease in both settings may also require a yet to be identified environmental trigger. Clinically, it has been frequently observed that monozygotic twins often present with T1D during different times in their lives. In some cases, this age disparity can reach 50 years[172]. Similarly, specific environmental cues modulate T1D progression in NOD mice. Whereas germ-free NOD mice develop disease at an increased incidence[173],

introduction of known pathogens can reduce incidence[174] (although the direct mechanism of protection has yet to be identified).

Accordingly, the NOD mouse represents a clinically relevant model to explore the immunologic barriers to islet transplantation in the context of autoimmune T1D. Strikingly however, no strategy to date has ever rendered NOD mice with an intact immune system permanently tolerant to any allografted organ type (as reviewed in the Introduction: *Resistance to Transplantation Tolerance in Autoimmunity*). Although previous studies have demonstrated that individual Insulin Dependent Diabetes (*Idd*) risk loci in part confer some level of resistance to transplant tolerance induction[147], the specific immunologic aberrancies perpetuated by each of these loci have yet to be defined. Along similar lines, although researchers hypothesize that CD4 T cell hypoproliferation, improper B cell antigen presentation, and enhanced indirect alloreactivity characteristic of NOD mice may perpetuate this strain's resistance to transplant tolerance induction[144], there has yet to be any studies in which these immunologic barriers have been diminished in such a way to restore allograft tolerance.

In Chapter II, I explore the hypothesis that autoreactive B lymphocytes represent a key barrier to transplant tolerance in NOD mice. Such reasoning stems from the well-appreciated fact that islet autoantibodies remain the best clinical predictor of T1D[175]. Although the pathogenic role of islet autoantibodies remains controversial, these observations lend credence to the fact that breakdown of B cell islet tolerance is central to the initiation of T1D pathogenesis. The role of B cells as critical APCs in T1D pathogenesis was first defined by studies demonstrating that NOD mice genetically deficient of B cells (NODμMT) are completely protected from disease[176]. Specifically, B cell MHC Class II antigen presentation is required for disease progression[177]. Mechanistically, B cells provide adequate co-stimulation required for anti-islet CD4 T cell

activation[178,179]. Further supporting the role of autoreactive B cells in T1D progression, an elegant body of work generated by Tom Thomas and colleagues has demonstrated that mice possessing transgenic B cells specific for insulin develop diabetes more rapidly[180]. Whether inappropriate and unchecked B cell antigen recognition and presentation also poses a barrier to transplant tolerance induction in the autoimmune NOD environment has yet to be explored.

In the absence of autoimmunity, B cells play a perplexing role in the establishment of allograft tolerance. Whereas they are absolutely required during tolerance induction to cardiac allografts[133], their absence imparts enhanced tolerance induction during islet allografting in non-autoimmune mice[84]. In the context of autoimmunity, recent evidence has demonstrated that the temporary depletion of B cells in autoimmune NOD mice delays islet allograft rejection 4-fold[83]. However, as B cells can rapidly regenerate from the bone marrow after the cessation of treatment, their reemergence is likely to reengage the highly immunogenic anti-graft response characteristic of this strain. This concept mirrors clinical trials in which transient B cell depletion in patients with recent onset T1D demonstrated a modest, but not permanent reprieve from beta cell destruction[181]. Overall, how the B lymphocyte repertoire shapes and later engages the alloreactive T cell repertoire in autoimmune T1D remains unknown. Furthermore, whether autoreactive B lymphocytes participate in the establishment of transplant tolerance has yet to be explored. Defining a specific role for B lymphocytes during the transplant response in autoimmune T1D may reveal new strategies to induce islet graft tolerance and cure T1D. Herein, I provide data demonstrating that the life-long and permanent absence of B cells renders NOD mice fully permissive to transplant tolerance induction. Specifically, I trace this finding to enhancements in graft-protective CD4 Tregs, whose presence and function is absolutely essential in the establishment of permanent transplant tolerance in B cell deficient NOD mice.

B cell deficient NODμMT mice are permissive to transplant tolerance induction

Building on the knowledge that B cell deficient NODμMT mice are protected from diabetes[176], I investigated whether the complete life-long absence of these cells would render NOD mice permissive to anti-CD45RB mediated transplant tolerance induction. In line with numerous reports, B cell sufficient NOD mice completely resisted tolerance induction to C3H islet allografts (Figure 2.1). Strikingly however, a short-seven day course of anti-CD45RB induced robust transplant tolerance to fully MHC-mismatched C3H islet allografts in B lymphocyte deficient NOD mice (MST>150d, Figure 2.1). B cell deficiency alone did not render these mice incapable of mounting an anti-graft response; all untreated NODμMT recipients rejected their islet allografts by 22 days (Figure 2.1). Overall, this observation not only represents the first instance in which permanent allograft tolerance has been achieved in the NOD immune system, but moreover implicates B cells as a critical barrier to transplant tolerance in the setting of T1D.

Transplant tolerance in NODμMT mice is permanent, antigen specific, and highly robust

As the majority of NODμMT mice were permissive to long-term tolerance induction mediated by anti-CD45RB (MST>150d), I next determined whether these mice were truly immunologically tolerant to the transplanted allograft tissue type. To demonstrate that the allograft tissue itself maintained euglycemia rather than endogenous beta cell regeneration, three NODμMT recipients demonstrating >100 days of graft tolerance were nephrectomized of their kidneys containing their islet allografts. Within 2 days, all recipients returned to hyperglycemic states with blood glucose levels >250mg/dL (Figure 2.2A). These same recipients then received

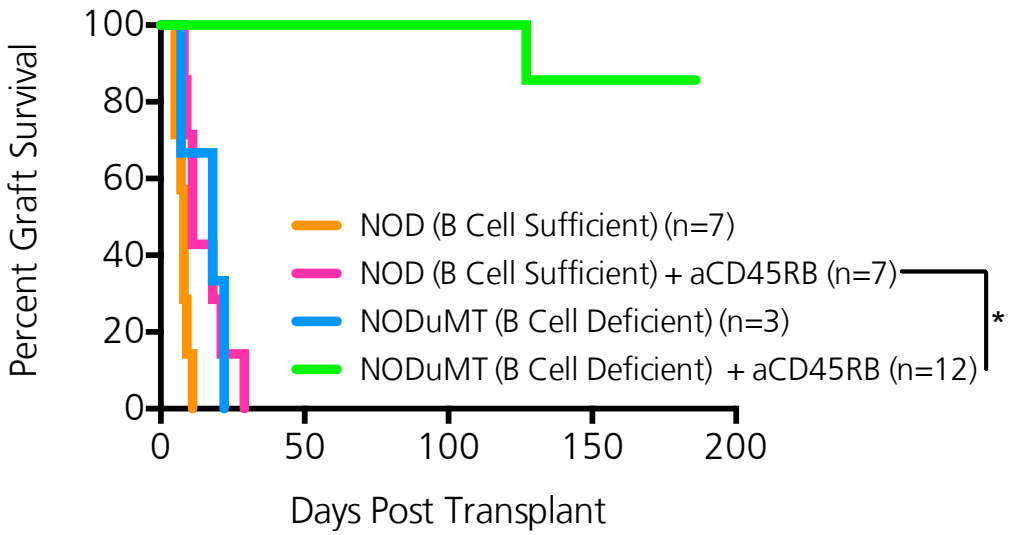


Figure 2.1. B cell deficient NOD mice are permissive to transplant tolerance induction. Briefly, 8-12 week old B cell sufficient NOD and B cell deficient NODμMT mice were made diabetic via a single injection of the beta cell toxin Streptozotocin. Diabetic mice then received fully MHC-mismatched C3H islet allografts under their kidney capsules. Cohorts of mice were then left untreated or received 100μg of the tolerance inducing agent anti-CD45RB on days 0, 1, 3, 5, 7 after transplantation. Blood glucose was monitored and two consecutive readings >250mg/dL denoted rejection. In line with previous reports, B cell sufficient NOD mice were fully resistant to transplant tolerance induction (pink trace). Conversely, nearly 100% of B cell deficient NODμMT demonstrated long-term allograft tolerance when treated with a short course of anti-CD45RB (green trace, *$p<0.001$ by Log-Rank test). Although untreated B cell deficient NODμMT mice demonstrated delayed rejection, the absence of B cells did not affect the ability of these mice to fully reject their islet allografts (blue trace).

a second matched C3H islet allograft in their contralateral kidney in the absence of further treatment. The maintenance of euglycemia was monitored for the duration of the recipients' lifespans. Strikingly, 2 of 3 recipients were fully tolerant to the retransplanted C3H islet allografts (Survival Times reaching 77d and 80d). Although the last recipient did reject its second matched graft 47 days after retransplantation, the time to rejection was 2-fold longer than NODμMT mice receiving islet allografts in the absence of any tolerance inducing strategy (cf Figure 2.1, blue trace). Thus, some level of allograft tolerance was achieved in this recipient albeit not to the same extent as its counterparts. Overall, these data confirm that in the absence of B cells, NOD mice are permissive to anti-CD45RB mediated tolerance induction and that the tolerance established is permanent to the allograft tissue type in question (Figure 2.2A).

I next questioned how robust this state of allograft tolerance was by trying to "break" tolerance using three strategies. Specifically, I hypothesized that ongoing regulatory mechanisms instilled allograft tolerance in these recipients in lieu of immunologic ignorance to the allografted tissue. The first strategy employed a CD4 Treg depleting antibody (anti-CD25, clone PC61) 50 days after tolerance induction in NODμMT recipients[117]. Interestingly, two injections of PC61 on days 50 and 55 after transplantation/tolerance induction failed to break tolerance in any NODμMT islet allograft recipients (Figure 2.2B). Fifty days later all recipients remained euglycemic. To further stress this state of allograft tolerance, I next injected this same cohort of tolerant NODμMT mice with T cells sorted from naïve NODμMT donor mice 100 days after transplantation. This scenario infused a population of graft-reactive effector T cells capable of precipitating rejection as they had never seen therapy. Fifty days later no signs of rejection were noted (Figure 2.2B). I next assessed whether the presence of B cells shapes the alloreactive T

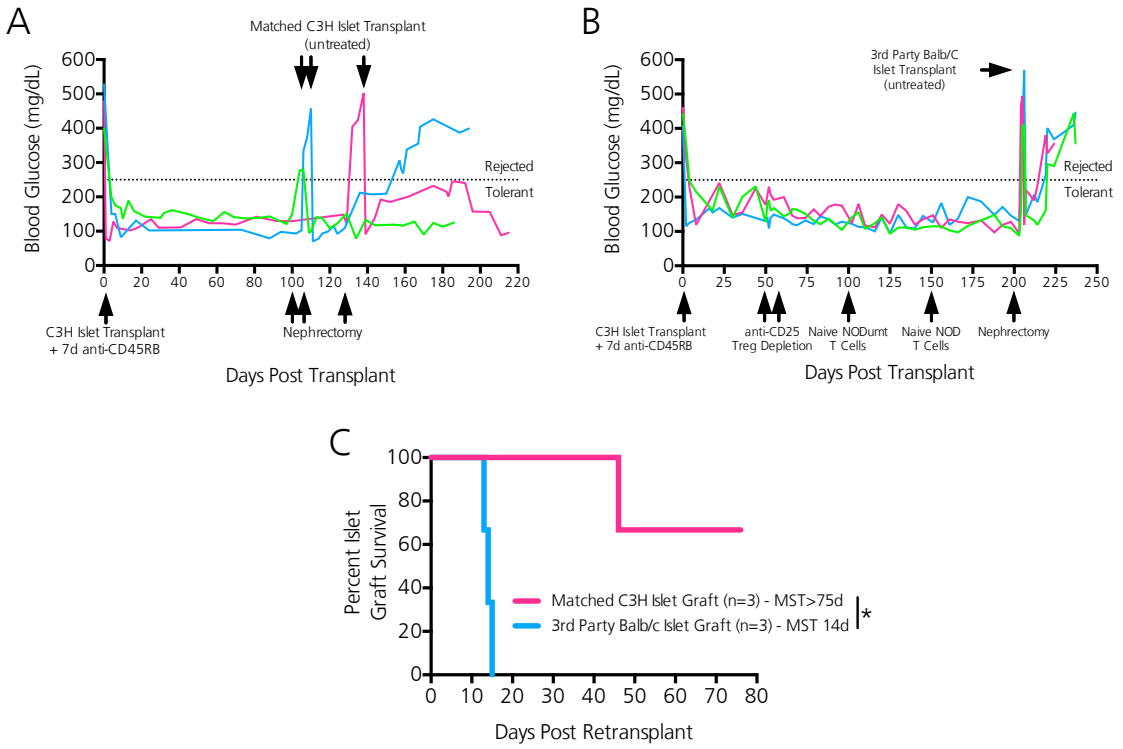


Figure 2.2. Transplant tolerance in NODμMT mice is permanent, antigen specific, and highly robust. A) 100 day tolerant NODμMT mice were nephrectomized of their kidneys containing the islet allograft tissue. All recipients demonstrated a rapid return to hyperglycemia demonstrating reliance on islet allograft tissue rather than endogenous beta cell regeneration in maintaining euglycemia. Placement of a matched C3H islet allograft in the absence of further treatment permanently restored euglycemia in 67% of the recipients demonstrating that these mice were immunologically tolerant to the islet allograft tissue type. B) Allograft tolerance in NODμMT mice is robust as subsequent attempts to "break" tolerance via CD4 Treg depletion (d50) or infusion of naïve T cells from either NODμMT mice (d100) or B cell sufficient NOD mice (d150) did not precipitate rejection. Removal of the islet allograft tissue confirmed that these mice were in fact relying on the graft for maintenance of euglycemia. Furthermore, non-matched 3rd party Balb/c islet allografts were rapidly rejected demonstrating that the tolerant state was not due to a generalized state of immunosuppression. C) Kaplan-Meier curve of retransplanted mice demonstrating that allograft tolerance in NODμMT recipients is antigen specific in that the majority of matched C3H allografts were tolerated in the absence of further treatment whereas non-matched Balb/c islet allografts were rapidly rejected (*$p<0.001$ by Log-Rank test).

cell repertoire in such a way that enhances alloreactivity and decreases susceptibility to regulation (as evidenced by the fact that B cell sufficient NOD mice reject islet allografts with 2-fold faster kinetics than their B cell deficient NODμMT counterparts [cf Figure 2.1]). Thus, this same cohort of tolerant NODμMT mice were injected with T cells sorted from naïve B cell sufficient NOD donor mice 150 days after transplantation. 50 days later no signs of rejection were noted (Figure 2.2B). Finally, I confirmed that this cohort of tolerant NODμMT mice were in fact tolerant to their islet allografts and furthermore, that a general state of immunosuppression did not result in failed rejection. Within 2 days after graft removal via nephrectomy all recipients rapidly returned to hyperglycemia states. Additionally, third-party non-matched islet allografts (Balb/c donor, H-2^d) were rapidly rejected demonstrating intact immune function (Figure 2.2B-C, - MST 14d). Overall, these data confirm that transplant tolerance in NODμMT mice is permanent, antigen specific, and highly robust.

Complementation of NODμMT mice with NOD B cells fails to break tolerance induction

I next hypothesized that complementation of NODμMT mice with B lymphocytes sorted from B cell sufficient NOD mice would render these NODμMT resistant to transplant tolerance induction. Although injection of B cells into immunodeficient SCID mice is a commonly used strategy to prove specific immune phenotypes imparted by B cells, this strategy has so far failed to transfer the B cell deficient μMT setting. In these environments, non-B cell tolerant CD8 T cells rapidly destroy newly introduced B cells in an MHC Class I dependent manner within 14 days after transfer[178]. However, B cell reconstitution in B cell deficient NODμMT mice does provide a brief 2-week window in which these newly introduced B cells survive. Thus, I questioned whether injecting NODμMT mice with B lymphocytes from syngeneic NOD mice on

days -7 and 1 relative to the day of transplantation would render these mice resistant to tolerance induction. Accordingly, this setting would address whether the presence of B cells at the time of transplant imparts a generalized resistance to transplant tolerance. Despite such complementation however, B cell reconstituted NODμMT recipients remained permissive to tolerance induction (Figure 2.3A). Although these recipients did demonstrate a minor retention of injected B cells 2 days before transplantation, the number of peripheral blood resident B cells more closely resembled their non-injected NODμMT counterparts as compared to B cell sufficient NOD controls (Figure 2.3B).

Peripheral CD4 Tregs are expanded in 1' and 2' tolerant NODμMT recipients

Although B cell deficient NOD mice are fully protected from diabetes, their T cell repertoire retains diabetogenic capacity. Transfer of NODμMT T cells to T cell deficient NOD recipients precipitates diabetes directly attributable to prior activation mediated by autoreactive B cells[182]. Additionally, CD4 Treg mediated depletion by PC61 can precipitate diabetes in approximately 80% of NODμMT mice[183,184]. NODμMT are also susceptible to Cyclophosphamide induced diabetes[182]; this agent selectively depletes Foxp3+ CD4 Tregs thereby permitting the unchecked activation of anti-islet effector T cells[185]. Thus in the absence of B cells, CD4 Tregs play a significant role in suppressing the anti-islet response in NOD mice. Thus, I first questioned whether naïve NODμMT mice possessed alterations in their peripheral CD4 Treg compartments that might later permit enhanced allograft regulation. Overall, I observed no striking differences in the frequency of CD25+Foxp3+ CD4 Tregs within the splenic CD4 T cell compartment of naïve NOD and NODμMT mice.

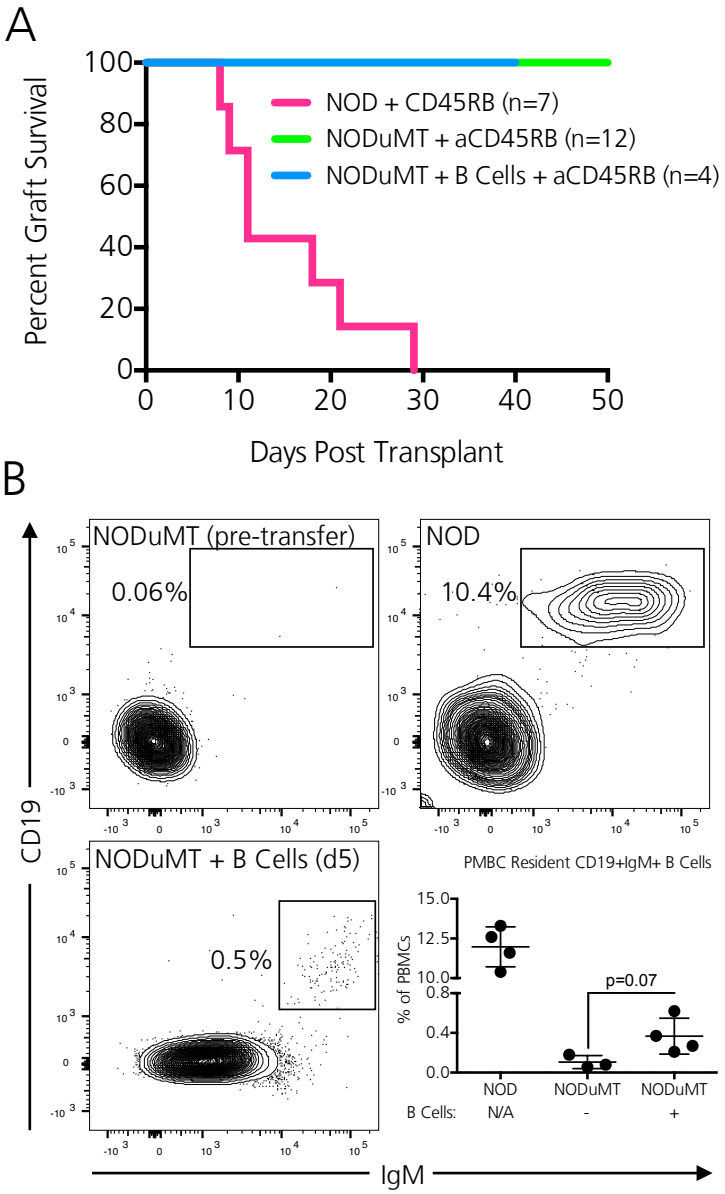


Figure 2.3. Complementation of NODμMT mice with NOD B cells fails to break tolerance induction. A) To determine whether the presence of B cells at the time of transplantation would render NODμMT resistant to transplant tolerance induction, NODμMT mice were injected with 25e6 purified B cells from naïve NOD donors on days -7 and 1 relative to the day transplantation and initiation of anti-CD45RB mediated tolerance induction (d0). Overall, the addition of B cells did not render NODμMT resistant to transplant tolerance induction. B) As B cells are rapidly destroyed by CD8 T lymphocytes upon transfer into the μMT environment, 2 days prior to transplantation NODμMT mice receiving B cells were bled and peripheral B cell frequencies observed. Although a minor retention of B cells was observed prior to transplantation, such complementation did not impart a full recovery of the B cell repertoire (as compared to peripheral B cell frequencies observed in B cell sufficient NOD controls). Significance determined by Student's t-test.

Regardless of the presence of B cells, approximately 8-10% of splenic CD4 T cells were comprised of CD4 Tregs in both settings (Figure 2.4A). However, the overall splenic frequency of CD4 Tregs in NODμMT was nearly twice that of NOD mice (Figure 2.4B). This observation is likely attributable to an overrepresentation of total splenic CD4 T cells due to an absence of B cells.

Enhanced peripheral CD4 Treg frequencies are commonly observed in long-term tolerant non-autoimmune allograft recipients[84]. I next determined whether tolerant NODμMT recipients possessed similar CD4 Treg enhancements. Strikingly, I found CD4 Tregs to be increased in both the splenic and graft draining renal lymph node CD4 T cell compartments of primary (mice tolerant to their 1st transplanted allograft) and secondary (mice tolerant to their 2nd transplanted allograft) tolerant NODμMT recipients as compared to naïve controls (Figure 2.4C). Thus, despite an unchanged CD4 Treg frequency at baseline, the tolerant state observed in NODμMT mice could be directly attributable to permanent peripheral CD4 Tregs enhancements that provide regulation of the anti-graft response.

Islet grafts from tolerant NODμMT mice are encased by graft-protective CD4 Tregs

In the setting of autoimmune T1D, CD4 Tregs are initially activated in the pancreatic draining lymph node in an attempt to control expansion of anti-islet effector cells. However, as peripheral tolerance is broken and effector cells migrate to pancreatic tissue, a high frequency CD4 Tregs follows in an attempt to quell *in situ* damage[186]. In the setting of transplantation this scenario is flipped. CD4 Tregs are initially activated in graft tissue itself after responding to inflammatory cues mediated by surgical intervention[118]. If appropriately activated (i.e. in the

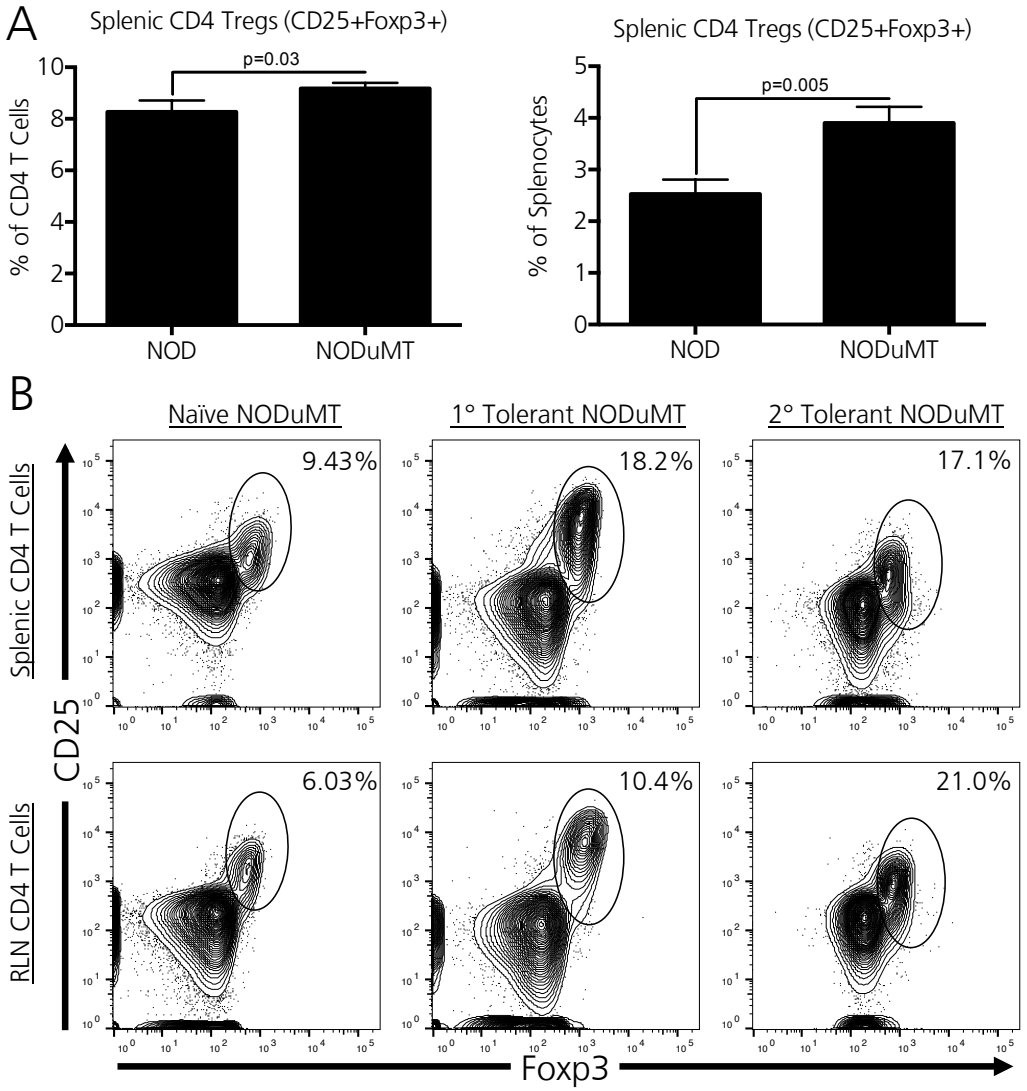


Figure 2.4. Peripheral CD4 Tregs are expanded in primary and secondary tolerant NODμMT recipients. A) Naïve B cell sufficient NOD and B cell deficient NODμMT mice possess similar frequencies of CD25+Foxp3+ CD4 Tregs within their splenic CD4 T cell compartment (left panel). Although NODμMT CD25+Foxp3+ CD4 Tregs make up a higher overall frequency of the entire splenic lymphocyte compartment, this difference is likely due to an overrepresentation of CD4 T cells due to an overall B cell deficiency (right panel). B). In comparison to splenic and draining Renal Lymph Node CD4 Treg frequencies in naïve NODμMT mice, primary and secondary tolerant NODμMT islet allograft recipients demonstrate marked expansions of these populations in their peripheral lymphoid organs. Significance determined by Student's t-test.

context of tolerance inducing strategies) these CD4 Tregs can then migrate to peripheral lymphoid tissues and permanently regulate the anti-graft response.

In further determining how tolerance was maintained in long-term tolerant NODμMT recipients, I entertained two hypotheses. One, that ongoing peripheral regulation restrained the egress of anti-graft effector cells to the allograft tissue itself. Or two that allograft tolerance was further maintained *in situ* by graft-resident CD4 Tregs. Graft tissue from nephrectomized 100d tolerant NODμMT recipients was subjected to frozen immunohistochemistry and stained for insulin and the T cell marker CD3. In line with previous data demonstrating a rapid return to hyperglycemia following nephrectomy[187], strong insulin staining was observed demonstrating intact graft function (Figure 2.5, upper left panel). Strikingly, I also observed a strong CD3+ T cell infiltrate surrounding, but not within the graft tissue. Further analysis revealed that these graft-encasing lymphocytes were comprised of CD4 Tregs cells expressing either CD25 (Figure 2.5, upper right panel) or Foxp3 (Figure 2.5, bottom panels). Overall, these data demonstrates that *in situ* graft regulation may complement a peripheral expansion of protective CD4 Tregs in tolerant NODμMT recipients.

B cells in NOD mice restrain anti-CD45RB mediated CD4 Treg expansion

Anti-CD45RB mediates permanent allograft tolerance via the activation and expansion of graft protective CD4 Tregs[95]. In non-autoimmune settings, anti-CD45RB rapidly induces the expansion of peripheral CD4 Tregs; these cells double in number 7 days of treatment[109]. However, whether the autoimmune T1D environment is permissive to CD4 Treg expansion mediated by anti-CD45RB remains unknown. B6 and NOD mice were administered a standard

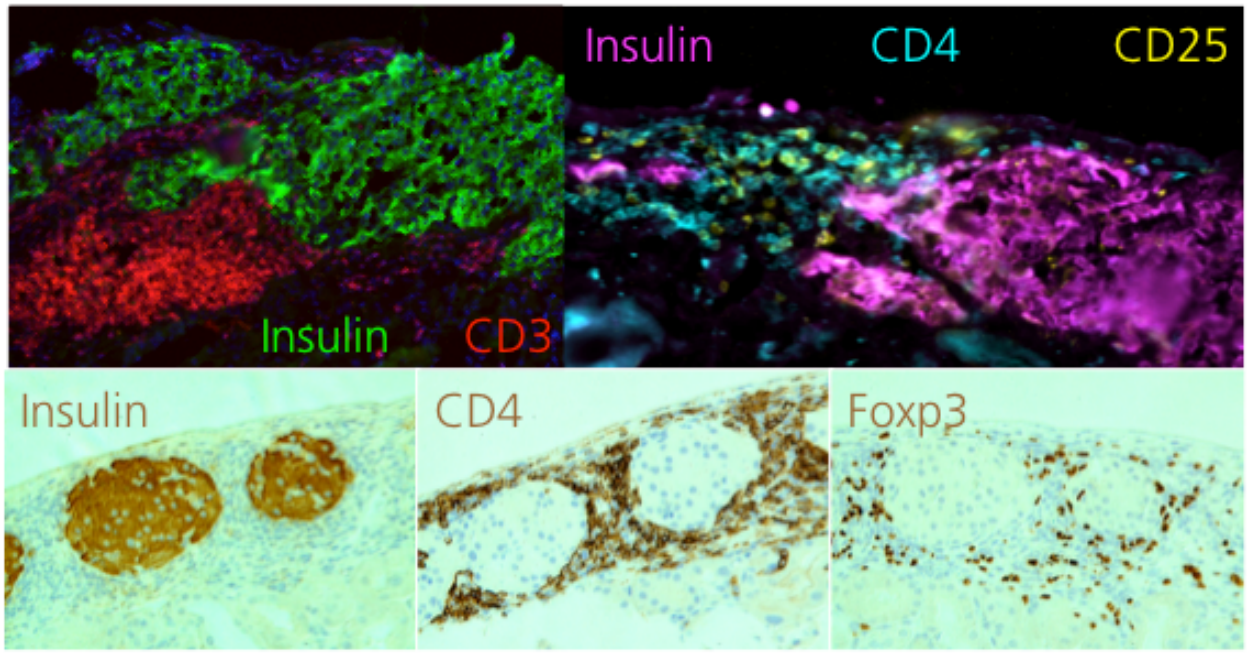


Figure 2.5. Islet grafts from tolerant NODμMT mice are encased by graft-protective CD4 Tregs. Kidney containing allografts were removed from NODμMT recipients demonstrating >100d of tolerance and subjected to frozen and paraffin embedded IHC. Although positive insulin staining (green) confirmed intact islet allograft function, the graft tissue was surrounded by a significant number of CD3+ T cell (red - upper left panel) infiltrate demonstrating that the host immune system was capable of recognizing the islet allograft tissue. However, further frozen section analysis confirmed that a significant portion of this T cell infiltrate was comprised of CD25+ CD4 T cells, lending credence to the possibility of active CD4 Treg mediated graft protection (upper right panel). As no Foxp3 antibody clones have proven reliable for frozen IHC staining, FFPE serial section analysis furthered confirmed a strong presence of Foxp3+ CD4 T cells surrounding the islet allograft tissue (bottom panels). Overall, these data demonstrate that allografts in tolerant NODμMT mice contain a significant number of graft-resident CD25+Foxp3+ CD4 Tregs that may be providing some level of *in situ* graft protection.

7-day course of anti-CD45RB and CD4 Treg expansion was evaluated. In line with previous reports, I noted a relative 2.8-fold increase of CD25+Foxp3+ CD4 T cells in non-autoimmune B6 mice. In comparison, CD4 Treg numbers remained unchanged in NOD mice after treatment (Figure 2.6A,B). From these data, it may be inferred that a generalized resistance to allograft tolerance in the NOD setting may in part result from failures in CD4 Treg activation and expansion.

As enhanced CD4 Treg populations are characteristic of tolerant NODμMT allograft recipients, I next hypothesized that B cells in the NOD setting represented the critical barrier to anti-CD45RB mediated expansion of CD4 Tregs. Naïve NODμMT mice were similarly administered a standard 7-day course of anti-CD45RB and CD4 Treg expansion was evaluated. Overall, the absence of B cells in the NODμMT setting restored anti-CD45RB mediated expansion of CD4 Tregs in their overall splenic frequency and total number (Figure 2.6B). However, it is important to note that unlike treated B6 mice, the frequency of CD4 Tregs within the CD4 T cell compartment of treated NODμMT mice remained relatively unchanged (Figure 2.6A - bottom panel and Figure 2.6B – top graph), suggesting improved CD4 Treg function, diminished effector T cell function, or that some NOD CD4 Tregs were not identified in our staining.

Early CD4 Treg depletion in NODμMT mice impedes transplant tolerance induction

My data implicate that in the absence of B cells, anti-CD45RB mediates a robust 3-fold expansion of CD4 Tregs whose enhanced numbers are characteristic of tolerant NODμMT recipients. Although late-term administration of the CD4 Treg depleting agent PC61 failed to break tolerance in NODμMT allograft recipients (c.f. Figure 2.2B), I hypothesized that CD4

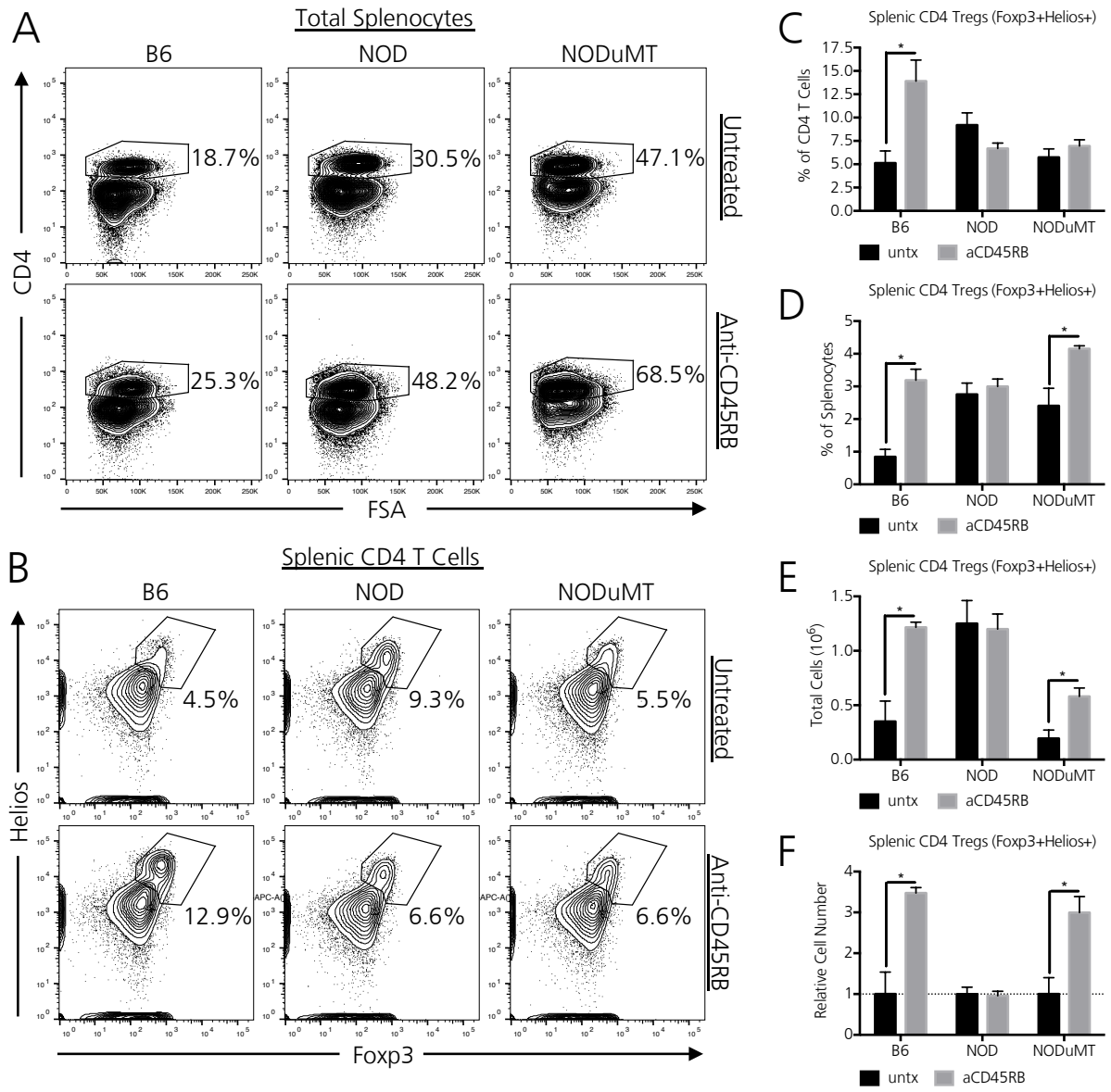


Figure 2.6. B lymphocytes in NOD mice restrain anti-CD45RB mediated CD4 Treg expansion. B6, NOD, and NODμMT mice were either left untreated or administered a standard 7 day course of the tolerance inducing agent anti-CD45RB. A) anti-CD45RB treatment enhanced the overall frequency of splenic CD4 T cells in all recipient strains. B and C) Anti-CD45RB significantly expanded the frequency of B6 CD4 Tregs (Foxp3+Helios+) within this strain's splenic CD4 T cell compartment. D) Although a similar enhancement was not observed in NOD and NODμMT mice, this therapy did increase of the overall splenic frequency of CD4 Tregs in B cell deficient NODμMT mice. E and F) Overall, anti-CD45RB significantly increased the total number of splenic CD4 Tregs in B6 and NODμMT mice, inducing approximately 3.5 and 3-fold expansions over baseline, respectively. Ultimately, the presence of B cells in NOD mice restricts anti-CD45RB mediated expansion of CD4 Tregs. Significance determined by Two-way ANOVA followed by Bonferroni post-test, *$p<0.05$).

Tregs are essential in establishing allograft tolerance at the time of transplantation. Accordingly, diabetic NODμMT recipients were depleted of CD4 Tregs on days -5 and -1 relative to the day of transplantation. On day 0, recipients received a C3H islet allograft followed by a standard 7-day course of anti-CD45RB. Overall, no NODμMT recipients ever achieved long-term allograft tolerance (Figure 2.7A,B); 3 recipients rejected their allografts on days 3, 3, and 11 after transplantation and 2 recipients rejected their allografts on days 62 and 88. Overall, these data confirm that CD4 Tregs are absolutely essential in the establishment of transplant tolerance in the NODμMT setting.

B cells limit Vβ3+ CD4 Tregs in NOD mice

Autoreactive NOD B cells clearly shape the islet-reactive and alloreactive T cell repertoire in NOD mice[188]. Reconstitution of irradiated NODμMT mice with splenic B cells from 4 week-old NOD mice alongside NODμMT bone marrow not only precipitates diabetes, but moreover promotes the development of a rich clonotypic repertoire of pancreatic T cell infiltrate composed of Vβ2, 10, 12, 14, 18, 19 and 20 clones. In the absence of concomitant B cell reconstitution, recipients receiving only NODμMT bone marrow are protected from diabetes and possess an undiversified pancreatic T cell infiltrate repertoire. Accordingly, I questioned whether the absence of B cells in the NOD setting created a skewed T cell clonotypic repertoire that permits not only protection from diabetes, but moreover a susceptibility to transplant tolerance induction. In general, the splenic and thymic CD4 and CD8 T effector cell TCR repertoires did not differ in the absence or presence of B cells (Figure 2.8A,B). However, when examining TCR usage among CD25+Foxp3+ CD4 Tregs, I noted a significant expansion of Vβ3+ clonotypes in both the spleens and thymuses of NODμMT mice (Figure 2.8A,B). Ultimately, prevention of

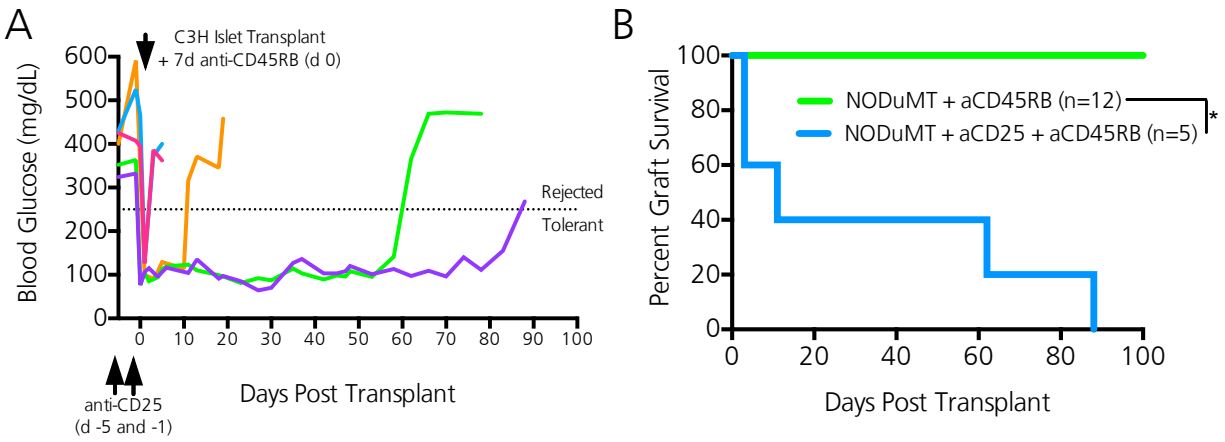


Figure 2.7. Early CD4 Treg depletion in NODμMT mice impedes transplant tolerance induction. A) Diabetic NODμMT mice were administered 500μg of the CD4 Treg depleting antibody PC61 on days -5 and -1 relative to transplant. On day 0, CD4 Treg depleted NODμMT mice were transplanted with C3H islet allografts and administered a standard 7-day course of the tolerance inducing agent anti-CD45RB. Overall, CD4 Treg depletion prior to transplantation and anti-CD45RB therapy rendered all recipients resistant to transplant tolerance induction. Whereas three recipients rejected their grafts with early phase kinetics (days 3, 3, 11), two recipients rejected their grafts with late phase kinetics (days 62 and 88). B) Kaplan-Meier survival curve comparing islet allograft survival between CD4 Treg replete (green trace) and CD4 Treg depleted (blue trace) NODμMT recipients (*$p<0.001$, by Log-Rank Test).

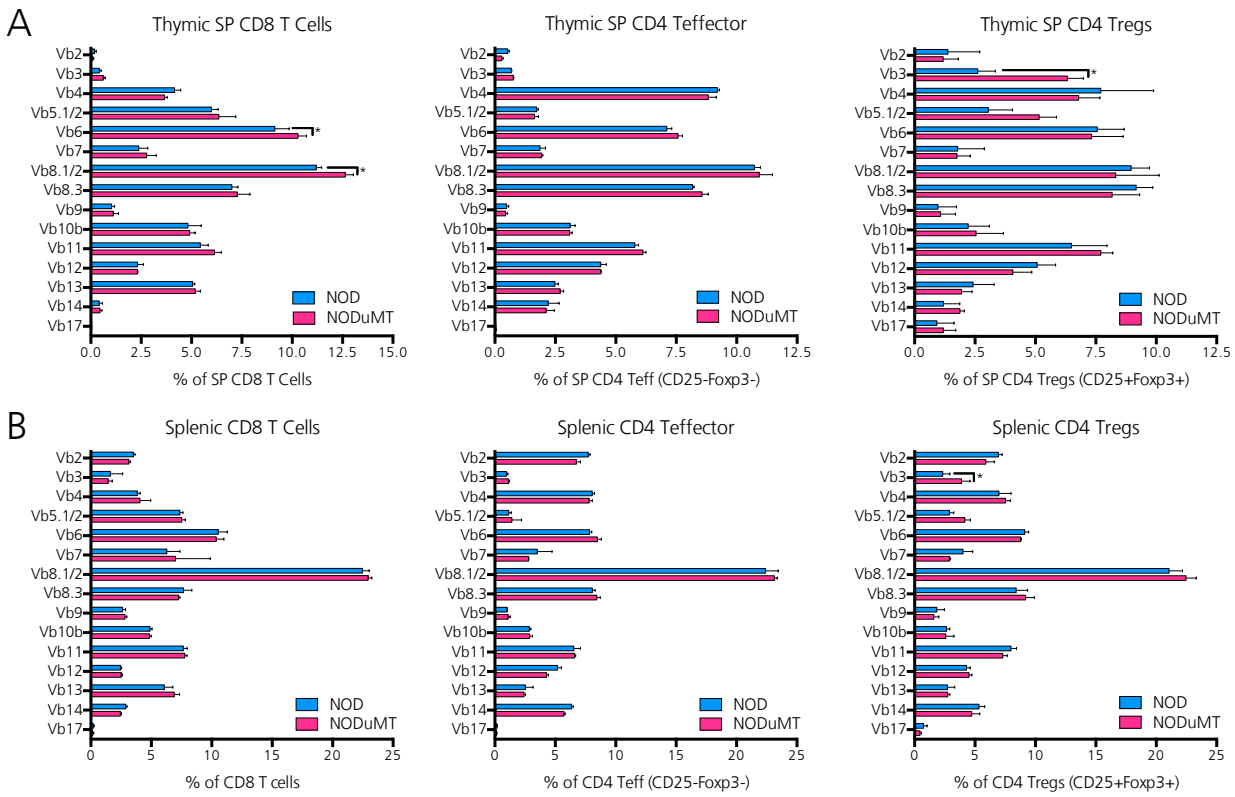


Figure 2.8. B lymphocytes limit Vβ3+ CD4 Tregs in NOD mice. Thymocytes and splenocytes from NOD and NODμMT mice were stained with a screening panel of various anti-Vβ chain antibody clones. A) Overall the presence of B cells had very little effect in shaping the mature Single Positive CD8 and CD4 T effector cell thymic repertoire. However, the absence of B cells in NODμMT mice resulted in a significant over representation of thymic Vβ3+ CD4 Tregs. B) Similarly, the only difference in splenic T cell clonotype usage between NOD and NODμMT mice was an increase in Vβ3+ CD4 Tregs observed in NODμMT mice. (*$p<0.05$ by Two Way ANOVA followed by Bonferroni post test). Data is representative of one experiment confirmed three independent times.

B cell mediated deletion of Vβ3+ CD4 Tregs may confer some level of diabetes protection and tolerance susceptibility to NODμMT mice[189].

Discussion

My findings in Chapter II demonstrate that B lymphocytes are a critical barrier to transplant tolerance in the T1D prone NOD environment. To date, there exists only one additional study exploring the transplant response in B cell deficient NODμMT mice. In 2008, Kupfer and colleagues demonstrated that whereas non-autoimmune Balb/c mice readily accept MHC-I and MHC-II deficient B6 islet allografts in the absence of any tolerance inducing strategy, autoimmune NOD mice readily reject these same islet allografts[150]. They attributed their findings to enhanced B cell mediated indirect alloreactivity in the NOD setting; in comparison to their B cell replete WT NOD counterparts, B cell deficient NODμMT mice accepted MHC-I and MHC-II deficient B6 islet allografts. Thus in the NOD environment, B cells alone initiate the indirectly alloreactive CD4 T cell pool responsible for graft rejection. Although other professional APCs in NODμMT mice remained capable of indirect alloantigen presentation via MHC-II, these cells failed to activate a similar pool of graft-destructive CD4 T cells. In assessing my complementary observation that B cell deficient NODμMT remain capable of rejecting MHC-I/II sufficient allogeneic islets, it therefore can be assumed that the directly alloreactive CD4/CD8 T cell pool in NODμMT mice was adequately selected for during thymic development and that these cells can alone precipitate allograft rejection. Thus, it can be inferred that the slight delay in time to islet allograft rejection in untreated NODμMT mice versus their B cell sufficient counterparts (cf Figure 2.1, blue trace vs. orange trace) could be due to an absence of B cell mediated activation of indirectly alloreactive CD4 T cells. Overall, in the absence of any

tolerance inducing strategy, direct allorecognition in the NOD environment remains a significant barrier to allograft tolerance.

Furthermore, my findings indicate that CD4 Tregs are essential in the establishment of anti-CD45RB mediated transplant tolerance in B cell deficient NODμMT mice. Not only were these cells expanded in the peripheral lymphoid tissues of tolerant NODμMT recipients, but a significant population of CD4 Tregs encased the allograft tissue itself. Although administration of the CD4 Treg depleting antibody PC61 prior to transplantation rendered all NODμMT recipients resistant to tolerance induction, I found that administering this same antibody 50 days after established tolerance failed to "break" the tolerant state. In explaining the latter finding, two possibilities can be considered. One, that CD4 Tregs do not actively maintain established tolerance in NODμMT mice; tolerance could instead result from the immediate inactivation of anti-graft effector cells by short-lived anti-CD45RB activated CD4 Tregs. However, this hypothesis would run contrary to both my findings and previous reports. Specifically, infusion of naive T cells from both B cell deficient and B cell sufficient NOD donors into 100- and 150-day tolerant NODμMT recipients, respectively, failed to precipitate rejection. As these cells were infused in the absence of anti-CD45RB, an on-going, previously established process likely suppressed the anti-graft properties of these naïve T cells. Moreover, two reports published by Trani et al and Deng et al demonstrated that transfer of CD25- CD4 T effector cells from previously tolerant recipients rapidly precipitates allograft rejection in B6 and Balb/c backgrounds[114,190]. In contrast, the transfer of CD25+ CD4 Tregs from previously tolerant recipients imparted tolerance even when transferred alongside naïve splenocytes. These results argue that long-term tolerance is not due to permanent effector cell inactivation, but rather to the induction of long-lived CD4 Tregs capable of actively attenuating the anti-graft response.

Instead, it is more likely that late-term PC61 administration did not fully deplete all of the NODμMT recipients' graft-protective, long-lived CD4 Tregs, although I did not address this directly. Although this antibody is effective in depleting CD25+ cells, a significant number of Foxp3+ CD4 Tregs do not express CD25[186]. Thus, non-depleted CD25- Foxp3+ CD4 Tregs may have in part helped maintained long-term islet allograft tolerance in these NODμMT recipients. Such retention of Foxp3+ CD25- CD4 Tregs post PC61 treatment has been confirmed in NOD and NODμMT mice[183]. Overall, it can be argued that PC61 may not effectively deplete long-lived CD25- CD4 Tregs; however, this same agent may be capable of depleting those CD25+ CD4 Tregs destined to later become graft-protective cells. These data would fall in-line with similar observations seen in the non-autoimmune setting; PC61 breaks tolerance in only 40% of recipients when administered after established tolerance, but is 100% effective in preventing long-term tolerance when administered prior to transplantation/tolerance induction[187].

Whereas B cell sufficient NOD mice resist anti-CD45RB mediated CD4 Treg expansion, the absence of B cells in NODμMT mice permits a nearly 3-fold expansion of splenic CD4 Tregs capable of permanently suppressing the directly alloreactive T cell pool (c.f. Figure 2.6 and 2.7). Although it remains to be defined how anti-CD45RB enhances CD4 Treg number and function in NODμMT mice, a number of hypotheses could be entertained. One, that the absence of B cells in the NODμMT setting represents the "anatomical space" needed for CD4 Treg expansion. Two, that a life-long absence of B cells in the NODμMT setting permits the development of CD4 Treg repertoire capable of responding to anti-CD45RB. Three, that NOD B lymphocytes actively suppress CD4 Treg development in the thymus or periphery (a hypothesis explored in Figure 2.8). Although outside the scope of this project, one could test the first two hypotheses using a basic crisscross platform in which labeled CD4 Tregs harvested from both NOD and NODμMT

mice could be transferred to both NOD and NODμMT recipients undergoing anti-CD45RB treatment. If NOD CD4 Tregs appropriately expand when transferred to NODμMT recipients, one could argue the anatomical space theory. This would be further supported if NODμMT CD4 Tregs failed to expand when transferred to B cell sufficient NOD recipients. Conversely, if NOD CD4 Tregs failed to expand when transferred NODμMT recipients, and moreover that transferred NODμMT CD4 Tregs retained their ability to expand in the presence of B cell sufficient NOD recipients, it could rather be argued that a life-long exposure to B cells renders CD4 Tregs in the NOD setting resistant to the effects of anti-CD45RB. Alternatively, one could further test these hypotheses by determining whether transient B cell depletion in WT NOD mice prior to anti-CD45RB treatment permits CD4 Treg expansion. In fact, it was recently demonstrated that Rituximab mediated B cell depletion of NOD mice engineered to express human CD20 mediates a robust expansion of CD4 Tregs that are capable of suppressing the transfer of diabetes[191].

From a mechanistic standpoint, I am currently investigating whether B cell antigen-specificity, antigen-presentation, or alloantibody production contributes to failed tolerance induction in NOD mice. To address whether graft recognition by alloreactive B cells impedes tolerance induction, I am currently determining whether NOD.HEL mice are permissive to transplant tolerance induction. As these mice possess B cells whose B Cell Receptors are specific to only the exogenous protein Hen Egg Lysozyme [HEL][192] (and thus unable to recognize graft tissue unless the graft tissue expresses the transgenic HEL antigen), I will be able to determine whether B cell antigen-specificity renders NOD mice resistant to transplant tolerance. To determine whether graft antigen presentation by B cells impedes tolerance induction, I recently generated chimeric NOD mice that contain MHC-II deficient B cells (NOD.$B^{H2\text{-}Ab\text{-}/\text{-}}$).

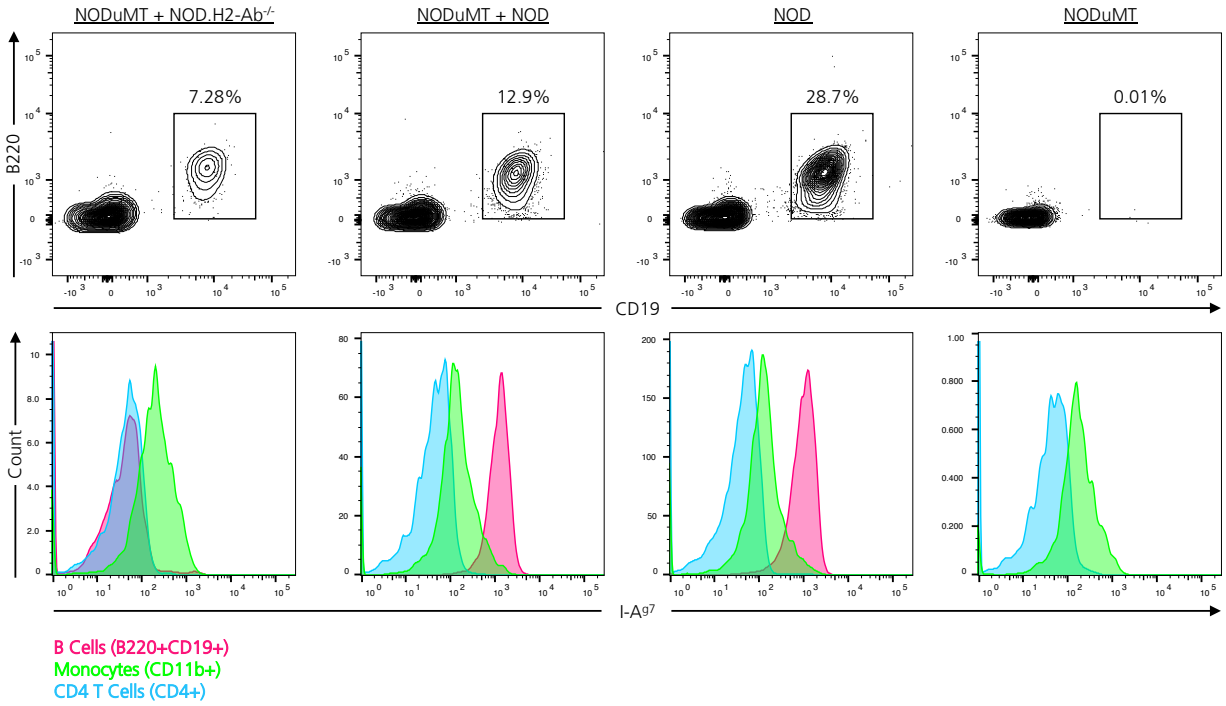


Figure 2.9. Generation of chimeric NOD mice in which only B lymphocytes lack MHC Class II expression. Irradiated immunodeficient NOD.RAG$^{-/-}$ mice were reconstituted with the following combinations of T and B cell depleted bone marrow cells [Column 1: NODμMT (80%) + NOD.H2-Ab$^{-/-}$ (20%); Column 2: NODμMT (80%) + NOD (20%); Column 3: NOD (100%); Column 4: NODμMT (100%)]. Eight weeks after transfer, reconstituted recipients were bled and PBMCs analyzed by flow cytometry. As shown in the upper panel, all chimeric recipients possess circulating B cells (B220+CD19+) except for those reconstituted with NODμMT marrow alone. Whereas the mixed chimeras generated in Column 2 possess B cells (pink histogram) and Monocytes (CD11b+ - green histogram) that both express MHC Class II, the mixed chimeras generated in Column 1 possess MHC Class II null B cells while maintaining MHC Class II positive Monocytes. CD4 T cells (blue histogram), which do not express MHC Class II, are shown as a negative control.

By reconstituting NOD.RAG with mice with 80% NODμMT + 20% NOD.H2-Ab$^{-/-}$ bone marrow, these mice develop and immune cell repertoire in which MHC-II presentation remains intact on other professional APC populations but is deficient on B cells (Figure 2.9)[177]. If I determine that NOD.B$^{H2\text{-}Ab\text{-}/\text{-}}$ mice are susceptible to anti-CD45RB mediated tolerance induction to islet allografts, it could be assumed that B cell mediated graft antigen presentation represents the molecular barrier to tolerance induction in NOD mice. Finally, to address whether B cell derived alloantibody results in failed tolerance induction, I am planning to test tolerance induction in NODμMT mice receiving injections of alloantibody rich serum collected from rejecting NOD islet allograft recipients.

Finally, I questioned how B lymphocytes shape the T cell repertoire in NOD mice. In general, I found effector CD4 and CD8 T cell Vβ receptor usage to be markedly similar between naïve NOD and NODμMT mice (c.f. Figure 2.8). However, I did notice a significant expansion of Vβ3+ CD4 Tregs (CD25+Foxp3+) in both the thymuses and spleens of B cell deficient NODμMT mice. Although Vβ3+ T cells undergo significant deletion in NOD mice due to this strains expression of the mouse mammary tumor virus 3 (MMTV-3) super-antigen, those Vβ3+ clones that do escape deletion represent some of the earliest clonotypes to infiltrate the pancreas[193]. Building on these findings, Krishnamurthy and colleagues recently discovered that genetic deletion of the pro-apoptotic molecule Bim in NOD mice results in protection from diabetes attributable to an expansion of protective Vβ3+ CD4 Tregs[189]. Thus, despite their high propensity for deletion, Vβ3+ T cell clones remain active participants in diabetes progression. Extending these findings, I did notice a mild expansion of Vβ3+ CD4 Tregs in the graft-draining Renal Lymph Node of tolerant NODμMT allograft recipients over their naïve counterparts (data not shown due to insufficient statistics). Whether allograft tolerance can be directly attributed to

Vβ3+ CD4 Tregs specifically remains to be determined. As a Vβ3 activating antibody clone is available (KJ25), future experiments could include transferring expanded Vβ3+ CD4 Tregs sorted from tolerant NODμMT recipients to untreated NODμMT also receiving naïve splenocytes. Overall, defining how B cells restrict CD4 Treg protective function in the NOD environment represents a novel area of investigation (Figure 2.10). Knowledge gained from these studies could one day lead to rational approaches aimed at restoring tolerance to both islet allografts and endogenous islet cells in patients with T1D.

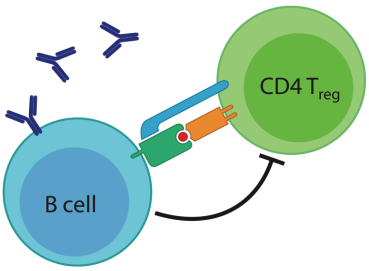
Chapter Findings	Model Figure	Future Work
- B lymphocyte deficient NODμMT mice are permissive to specific, robust, and permanent transplant tolerance induction. - Late CD4 Treg depletion or B cell complementation fails to break transplant tolerance in NODμMT mice. - Peripheral and graft resident CD4 Tregs are expanded in tolerant NODμMT mice. - B lymphocytes restrain anti-CD45RB mediated expansion of CD4 Tregs in NOD mice. - Early CD4 Treg depletion in NODμMT mice impedes transplant tolerance induction. - Thymic and splenic Vβ3+ CD4 Tregs are expanded in NODμMT mice.		- Assess whether NOD B lymphocytes restrict anti-CD45RB mediated CD4 Treg expansion due to "anatomical space" and/or "altered repertoires" theories. - Determine whether pharmacologic B cell depletion in B cell sufficient NOD mice enhances transplant tolerance induction. - Assess whether NOD B cell antigen-specificity, antigen presentation, or antibody production impede transplant tolerance in NOD mice. - Determine the role of Vβ3+ CD4 Tregs in establishing transplant tolerance NODμMT mice.

Figure 2.10. Chapter II – findings and future work.

CHAPTER III

CD8 T REGULATORY CELL CONTROL OF THE TFH-GC B CELL AXIS IS ESSENTIAL IN SUPPRESSING DIABETOGENIC IMMUNITY AND MAINTAINING TRANSPLANT TOLERANCE

Scientific Goal

In Chapter II, I determined that disrupting anti-graft T-B cell collaboration via genetic elimination of the entire B cell compartment in autoimmune NOD mice restores transplant tolerance in this otherwise resistant strain. However, it is also possible that failures in the extrinsic mechanisms that normally regulate the anti-graft B cell response may contribute to the unchecked islet and anti-graft immunity characteristic of this autoimmune strain. In Chapter III I explore the hypothesis that regulation of the immunogenic CD4 TFH - Germinal Center B cell axis by CD8 T Regulatory Cells (CD8 Tregs) is essential to prevent diabetes pathogenesis and to promote transplantation tolerance. To date however, there exists little knowledge of how CD8 Tregs control immunity and promote tolerance in either of these settings. Overall, developing a clearer mechanistic understanding of CD8 Treg function in T1D and transplantation may offer new avenues by which clinicians could halt islet destruction and quell ongoing graft rejection.

Introduction

Circulating islet autoantibodies and graft-reactive alloantibodies remain the best clinical predictors of T1D and Acute Rejection Events in at risk patients[175]. Mechanistically, these clinical observations result from unchecked immunity wherein inappropriately activated T and B

lymphocytes break tolerance. Clinicians have attempted to halt such collaboration by non-selectively targeting the whole B or T cell compartment with anti-CD20 or anti-CD3 depleting therapies, but these approaches have not resulted in permanent islet or graft protection[181,194,195]. Fundamentally, the physiologic regulation of these cellular interactions remains incompletely understood. Identifying pathways that control T-B interactions holds promise to dampen progressive autoimmunity and deleterious graft rejection.

It is now widely appreciated that CD4 T Follicular Helper (TFH) cells actively initiate, maintain, and shape the Germinal Center (GC) B cell response. Upon encountering an immunogenic stimulus, Bcl-6+ TFH cells home to lymphoid germinal centers via their expression of CXCR5 and subsequently activate GC B cells via their expression of the co-stimulatory molecules ICOS, PD-1, and CD40-L and by secretion of IL-4 and IL-21[196]. In the context of T1D, a single study by Kenefeck and colleagues demonstrated that memory T cells from patients with T1D express higher levels of the TFH associated molecules CXCR5, ICOS, PD-1, and Bcl-6 as well as increased frequencies of circulating CXCR5+ CD4 T cells[197]. Although a mechanistic role of TFH cells in diabetes progression remains to be defined, transfer of CXCR5 replete, but not deficient, CD4 T cells can precipitate diabetes in otherwise protected mice. Alternatively in the context of transplantation, a select number of studies have identified TFH/GC B cell clusters in acutely rejected renal allograft biopsies[77]. Although a mechanistic role for TFH cells in transplant rejection has yet to be elucidated, indirectly alloreactive CD4 T cells can give rise to CD4 TFH populations that lead to long-lasting IgG alloantibody responses[75]. Overall, active regulation of aberrant TFH cell function in T1D and transplantation represents a new avenue to quell destructive islet immunity and graft rejection.

Regulation of the TFH-GC B cell axis may be carried out by CD4 T Regulatory Cells (CD4 Tregs)[198,199] and newly identified CD4 T follicular regulatory cells (CD4 TFHR)[200], though the effectiveness of CD4 Tregs against the antibody response may be limited. In addition to these cells, a germinal center selective CD8 T cell also plays an important role in limiting autoantibody production. Despite numerous reports describing CD4 Treg function in T1D and transplantation[201], it is currently unknown whether and how CD8 T Regulatory Cells (CD8 Tregs) protect islets or mediate permanent transplant tolerance. CD8 Tregs have been previously defined by expression of the activation marker CD44 and by expression of the IL-15/IL-2 receptor beta chain CD122[202]. CD8 Tregs can suppress EAE[121,203-205], collagen-induced arthritis[122], lupus[123], and modestly delay skin[126] and islet[125] allograft rejection in non-autoimmune mice. Mechanistically, CD8 Tregs eliminate CD4 T follicular helper cells (TFH) that drive B cell-mediated immunity[123]. Recently, the most potent population of TFH targeting CD8 Tregs was reported to reside within the Ly49 positive fraction of these classically defined CD44+CD122+ CD8 Tregs[124]. These cells regulate the antibody response and quell further B cell-mediated immune activation that would otherwise promote epitope spreading. Therefore, understanding CD8 Treg function in autoimmune T1D and transplantation is a significant new problem in immune regulation that must be part of a comprehensive strategy to adequately control islet destruction and graft rejection.

In Chapter III, I first explore the hypothesis that loss of CD8 Treg function in T1D prone NOD mice facilitates islet immunity. Herein, I discovered that wild-type NOD mice possess a pool of non-functional, classically-defined CD44+CD122+ CD8 Tregs, which may account for my observation that these mice generate exaggerated antibody responses during antigenic challenge. This functional deficiency may result from my observation that NOD mice possess a

profoundly diminished pool of TFH targeting Ly49+ CD8 Tregs within their classically defined CD44+CD122+ CD8 Treg pool. I trace this deficiency to inadequate IL-15 transpresentation by macrophages, a cell known to promote the development, maintenance, and activation of CD8 Tregs[124]. I then demonstrate that these CD8 Tregs can be rescued by an IL-15 superagonist[206,207], thereby restoring their ability to suppress the antigen-specific antibody response and delay diabetes progression. Finally, I extend my findings to explore the role of IL-15 dependent CD8 Tregs in control of the transplant response. Specifically, I demonstrate that in the absence of this cytokine, non-autoimmune mice lack CD8 Tregs, possess enhanced CD4 TFH and GC B cell frequencies, generate increased alloantibody titers, and resist transplant tolerance induction. These studies further define the phenotype and function of CD8-based regulation in T1D and transplant tolerance. Complementing my findings in Chapter II, restoration of CD8 Treg based regulation of the immunogenic B cell response may represent a novel means to restore transplantation tolerance in the setting of autoimmunity.

NOD mice possess nonfunctional, classically defined CD8 Tregs and generate exaggerated antibody responses

In non-autoimmune B6 mice, classically defined CD44+CD122+ CD8 Tregs suppress the antibody response and were recently described to be more potent protectors of islet allografts than their CD4 Treg counterparts[124,125]. To determine whether these classically defined CD8 Tregs were functional in NOD mice, I utilized a well-established *in vivo* CD8 Treg suppression assay (Figure 3.1A). KLH activated CD8 Tregs (CD8+CD44+CD122+) or non-CD8 Treg (CD8+CD44+CD122-) controls were transferred to immunodeficient recipients along with B

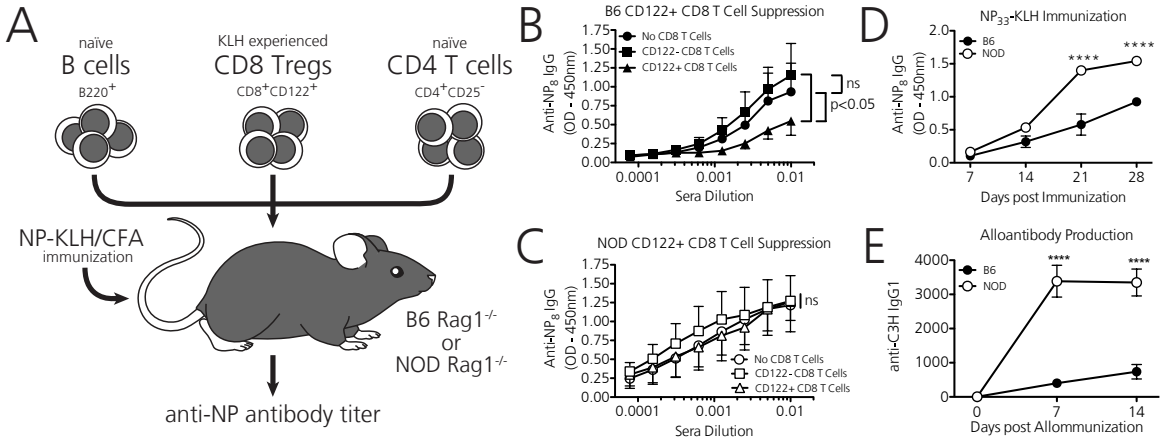


Figure 3.1. NOD mice possess nonfunctional, classically defined CD8 Tregs and generate exaggerated antibody responses. A) *In vivo* CD8 Treg suppression assay in which KLH-activated, classically-defined CD8 Tregs (CD8+CD44+CD122+) and naïve CD4+CD25- T Cells / whole B220+ B Cells are transferred to immunodeficient B6.RAG or NOD.RAG recipients that are immunized and boosted with the test stimulus NP_{33}-KLH. B) Classically defined CD122+ CD8 Tregs (black triangles) from KLH-activated B6 mice readily suppress the anti-NP_8 IgG response as compared to mice receiving CD122- non-CD8 Tregs (black squares) or no CD8 T cells (black circles) as a control. C) In comparison, classically defined CD122+ CD8 Tregs (white triangles) from KLH-activated NOD mice fail to suppress the anti-NP_8 IgG response over mice receiving CD122- non-CD8 Tregs (white squares) or no CD8 T cells (white circles). As compared to CD8 Treg sufficient B6 mice (back circles), CD8 Treg deficient NOD mice (white circles) generate a greater anti-NP_8 IgG response when immunized and boosted with the hapten-carrier NP_{33}-KLH (D) as well as a greater anti-C3H IgG1 alloantibody response when allochallenged with 20e6 MHC-mismatched C3H splenocytes (E). N=3-5 mice per group, ****p<0.0001, ns = non-significant, by semi-logarithmic linear regression analysis followed by y-intercept and slope curve comparison or two-way ANOVA followed by Sidak's multiple comparisons post-test.

lymphocytes and CD4+CD25- T cells from matched, antigen-naïve B6 or NOD strains (CD4 Tregs were not transferred to limit suppression to the transferred CD8 T cell populations). These animals were immunized and boosted with the original test stimulus (Nitrophenyl-Keyhole Limpet Hemocyanin [NP-KLH], a T-cell dependent hapten-carrier) and the relative suppression of the high-affinity anti-NP IgG response was compared between mice receiving CD8 Tregs or non-CD8 Tregs. Whereas classically defined B6 CD8 Tregs suppressed the generation of high-affinity anti-NP_8 IgG antibodies in recipient RAG mice as previously reported (Figure 3.1B), NOD CD8 Tregs failed to suppress this antigen-specific antibody response (Figure 3.1C).

As wild-type NOD mice possess non-functional, classically defined CD8 Tregs, I hypothesized that these mice would generate exaggerated antigen-specific antibody responses when immunized. Compared to CD8 Treg sufficient B6 mice, NOD mice generated a significantly greater high-affinity anti-NP_8 IgG antibody response after immunization and boosting with the hapten-carrier NP-KLH (Figure 3.1D). Alternatively, when immunized intravenously with 20 million MHC-mismatched splenocytes from C3H mice (H-2k), NOD mice (H-2g7) generated a 7-fold (day 7) and 8-fold (day 14) greater anti-C3H IgG1 antibody response than CD8 Treg sufficient B6 mice (H-2b) (Figure 3.1E).

Diabetes-prone NOD mice are profoundly deficient of TFH targeting Ly49+ CD8 Tregs

As the most specific population of TFH-targeting CD8 Tregs has been reported to reside within the Ly49F fraction of classically defined CD8 Tregs[124], I explored whether NOD mice possess an altered pool of Ly49F+ CD8 Tregs as compared to CD8 Treg sufficient B6 mice. Strikingly, NOD mice possess an extremely diminished pool of Ly49E/F+ CD8 Tregs (Figure 3.2A), possessing nearly 11-fold fewer splenic Ly49+ CD8 Tregs at 8 weeks of age as compared

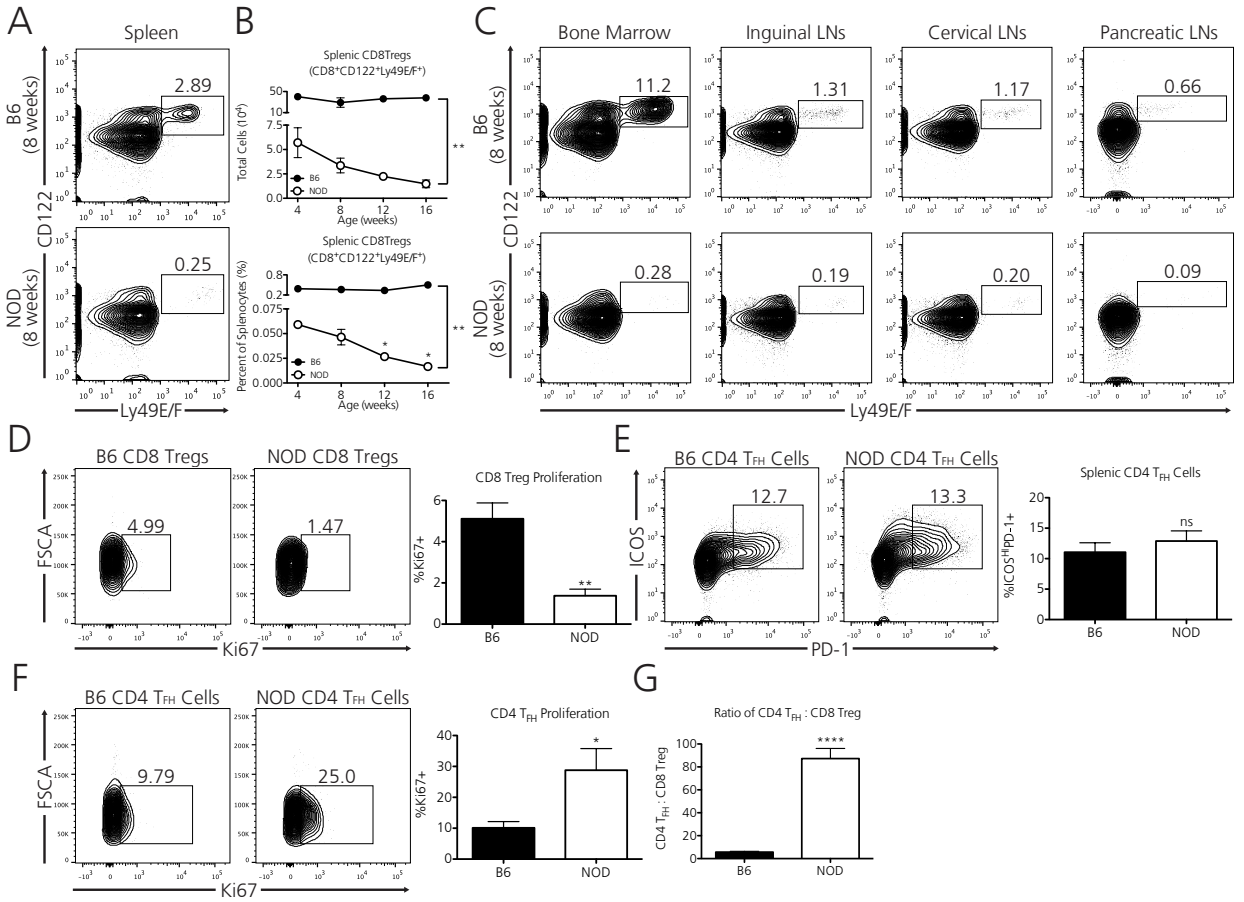


Figure 3.2. Diabetes-prone NOD mice are profoundly deficient of TFH targeting Ly49+ CD8 Tregs. A) Wild-type, 8-week old NOD mice possess 11-fold fewer splenic TFH targeting Ly49+ CD8 Tregs (CD8α+CD122+Ly49E/F+) as compared to non-autoimmune, age-matched B6 mice. B) Whereas healthy B6 mice maintain a relatively robust population of Ly49^{+} CD8 Tregs as they age, NOD mice progressively lose this protective population as they age toward diabetes. C) The observed Ly49+ CD8 Treg deficiency in NOD mice extends to additional lymphoid compartments including the bone marrow, inguinal lymph nodes, cervical lymph nodes, and pancreatic lymph nodes. D) Ly49+ CD8 Tregs in NOD mice divide at a rate three times lower than their B6 counterparts as determined by their Ki67 positivity. Although age-matched NOD mice possess similar percentages of Ly49+ CD8 Treg target TFH cells (E), TFH cells in NOD mice demonstrate a 2.5-fold higher proliferative rate (F), which may account for my observation that CD4 TFH cells vastly outnumber Ly49+ CD8 Tregs in NOD mice approximately 87 to 1 (G). N=3-5 mice per group, *p<0.05, **p<0.01, ****p<0.0001, ns = non-significant, by two-way ANOVA followed by Sidak's multiple comparisons post-test or student's t-test.

to the healthy controls. As allelic differences in the Ly49 locus exist between B6 and NOD strains[208], I tested numerous anti-Ly49F targeting clones in B6 and NOD prior to choosing this specific clone for analysis (Figure 3.3); specifically, the anti-Ly49E/F clone CM4 bound unique populations of CD8 T cells and NK cells in B6 and NOD mice.

Whereas non-autoimmune B6 mice maintain a comparatively robust population of splenic Ly49+ CD8 Tregs throughout their lifetime, NOD mice progressively lose Ly49+ CD8 Tregs as they age as determined by both percentage and total cell number (Figure 3.2B). Moreover, this deficiency extends to additional immune tissue compartments in NOD mice including the bone marrow, inguinal lymph nodes, cervical lymph nodes, and pancreatic lymph nodes (Figure 3.2C). This global Ly49+ CD8 Treg deficiency in NOD mice may in part be due to the lower proliferative capacity of these cells as determined by their baseline Ki67 positivity. Nearly 5% of B6 CD8 Tregs are Ki67+ whereas only 1.5% of NOD Ly49+ CD8 Tregs are positive for this marker of cellular division (Figure 3.2D), suggesting a limitation in available stimulatory factors.

As Ly49+ CD8 Tregs target the action of TFH cells, I explored whether splenic TFH cell populations differed between these two strains. B6 and NOD mice harbored similar percentages of ICOS+PD-1+ splenic TFH cells at 8 weeks of age (Figure 3.2E). However, I observed that TFH cells in NOD mice divide at a 2.5-fold greater rate as determined by their Ki-67 positivity (Figure 3.2F). Accordingly, regulation of these cells may be compromised as TFH cells outnumber Ly49+ CD8 Tregs 87:1. In healthy, non-autoimmune B6 mice the ratio of TFH cells to Ly49+ CD8 Tregs was 6:1 (Figure 3.2G).

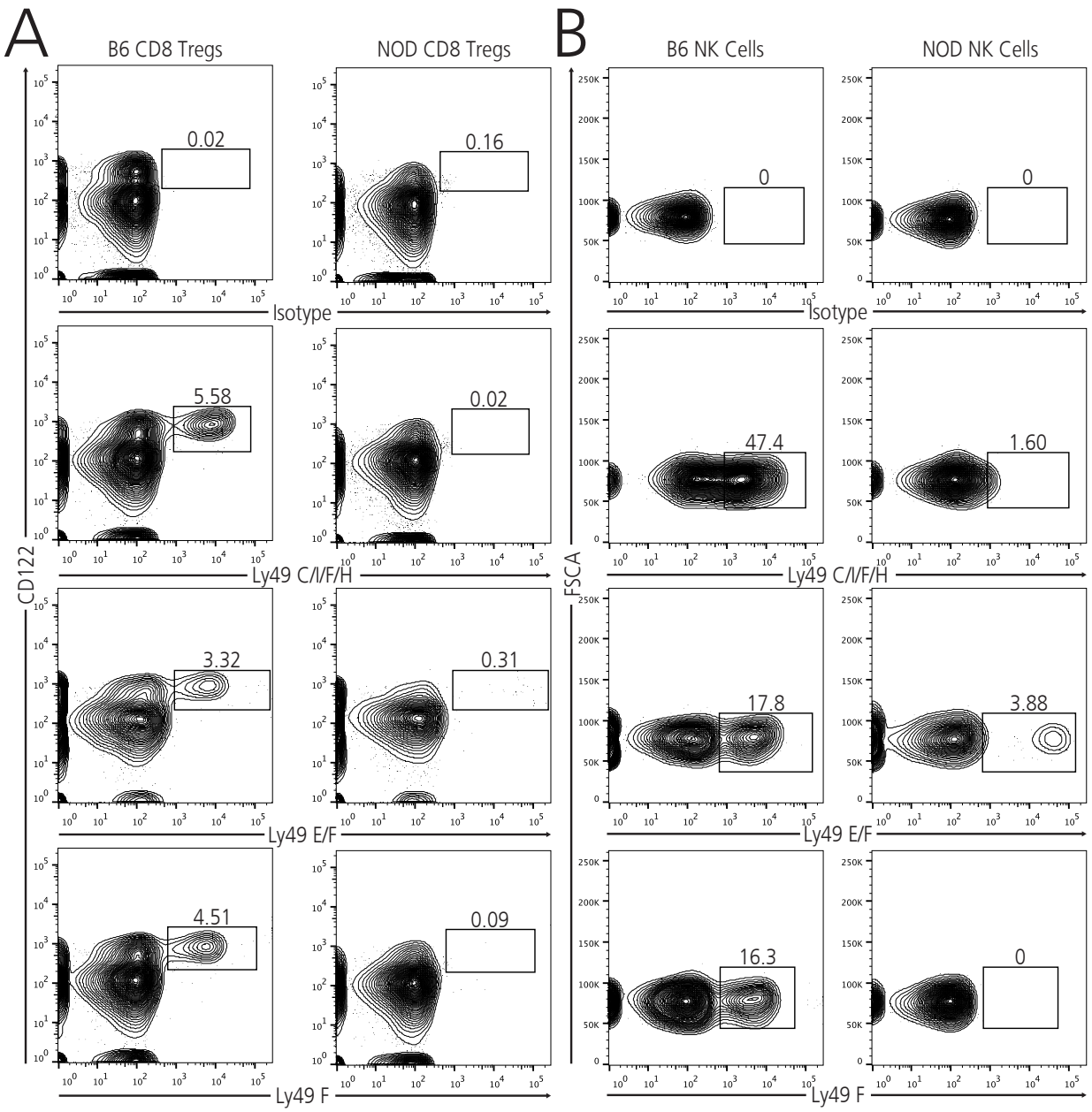


Figure 3.3. The Ly49E/F clone CM4 binds TFH targeting Ly49+ CD8 Tregs in NOD mice. Although NOD mice possess the largest Ly49 haplotype of any known mouse strain, many of the available anti-Ly49 antibody clones fail to detect Ly49 expression in the NOD mouse due to allelic differences. Accordingly, I tested multiple antibody clones that detect Ly49F to enumerate the most potent population of CD4 TFH targeting CD8 Tregs in NOD mice. Specifically, I determined that the anti-Ly49E/F clone CM4 binds a unique population of CD8 T cells and NK cells in NOD mice, whereas the anti-Ly49C/I/F/H clone 14B11 and anti-Ly49F clone HBF-719 fail to detect these populations in this T1D prone strain.

Macrophages from NOD mice inadequately trans-present and upregulate IL-15

The decrease in Ly49+ CD8 Treg proliferation in NOD mice suggested a lack of stimulation or growth supporting factors. I therefore examined whether various populations of Antigen Presenting Cells (APCs) failed to provide adequate survival signals for this regulatory cell population. Notably, work by Kim et. al.[124] revealed that B6 mice deficient in IL-15, the requisite cytokine for CD8 T memory cells and NK cells, lack a splenic population of CD122+Ly49+ CD8 Tregs. However, these animals do retain a small population of CD122+Ly49- CD8 T cells, suggesting that they can sustain a modest population of classical CD8 T memory cells despite the absence of IL-15. Thus, I next questioned whether IL-15 transpresentation by Ly49+ CD8 Treg supporting cells in NOD mice was dysfunctional.

Unlike traditional gamma-chain dependent cytokines such as IL-2, IL-15 is transpresented by its high-affinity receptor (IL-15Ra) to neighboring cells[209]. Thus, as a readout for IL-15 transpresentation, I compared the relative expression of surface IL-15Ra on splenic and bone marrow resident APCs at baseline between B6 and NOD strains. Splenic and bone marrow resident macrophages (CD11b+F4/80+) in NOD mice expressed approximately 2-fold less IL-15Ra at baseline (Figure 3.4A). As CD8 Tregs are activated during episodes of ongoing immunity, I determined whether NOD macrophages increased IL-15 transpresentation during stimulation. Whereas splenic and bone marrow resident B6 macrophages demonstrated approximately 150% and 50% upregulation of surface IL-15Ra after 48 hours of immune stimulation with Poly[IC], respectively, splenic and bone marrow resident NOD macrophages upregulated surface IL-15Ra only 75% and 20% over baseline levels (Figure 3.4B).

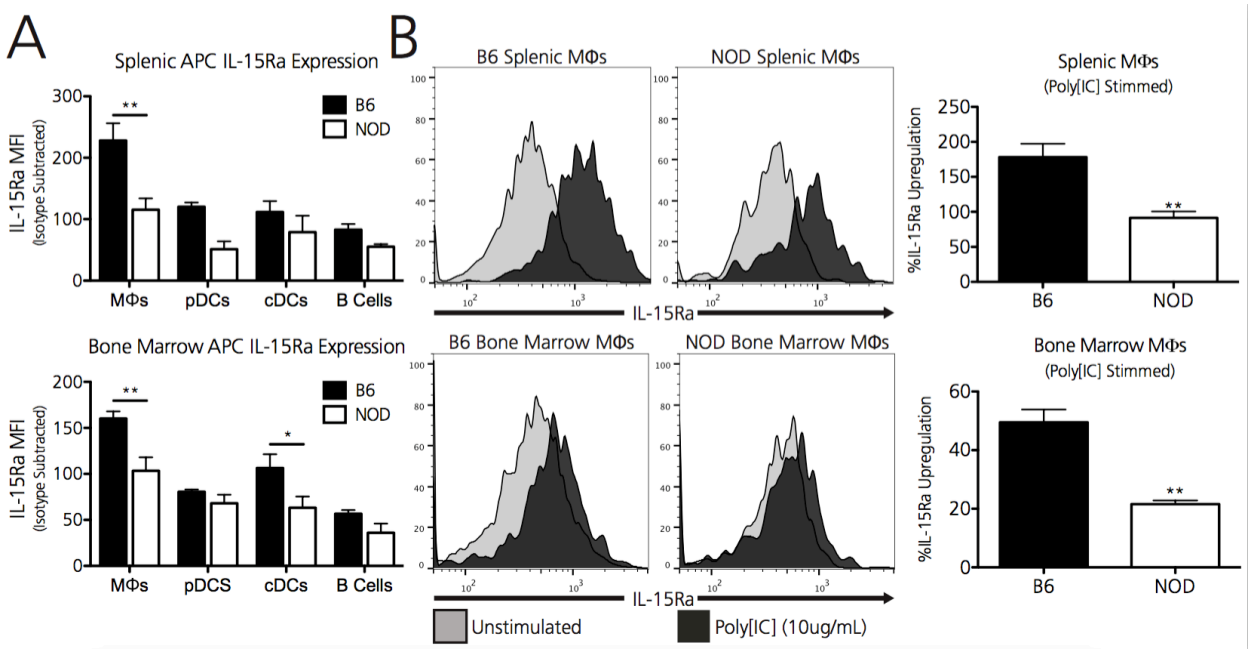


Figure 3.4. Macrophages from NOD mice inadequately transpresent and upregulate IL-15. A) Baseline IL-15 transpresentation, as measured by relative surface IL-15 Receptor alpha expression (IL-15Ra), is significantly reduced on splenic and bone marrow resident NOD Macrophages (MΦs) as well as bone marrow resident NOD Conventional Dendritic Cells (cDCs). B) Splenic and bone marrow resident Macrophages from NOD mice fail to upregulate IL-15Ra expression to the same extent as B6 Macrophages when stimulated with 10μg of the TLR3 agonist Poly[IC]. N=3-5 mice per group, *p<0.05, **p<0.01, by two-way ANOVA followed by Sidak's post-test or Student's t-test.

NOD Ly49+ CD8 Tregs adequately transduce IL-15 mediated survival signals

In addition to the reduced IL-15 availability in the NOD system, Ly49+ CD8 Tregs in this T1D-prone setting could fail to thrive due to inadequate IL-15 signal transduction, which proceeds via the JAK3/STAT5 system[210]. To evaluate IL-15 signaling dynamics in splenic Ly49+ CD8 Tregs, I determined relative STAT5 phosphorylation in B6 and NOD Ly49+ CD8 Tregs in response to IL-15/IL-15Ra superagonist (IL-15C) exposure. Surprisingly, NOD Ly49+ CD8 Tregs phosphorylated STAT5 to the same extent as B6 Ly49+ CD8 Tregs when exposed to increasing IL-15C concentrations in the *ex vivo* setting for 30 minutes (Figure 3.5A). To further define IL-15 mediated signaling dynamics between B6 and NOD Ly49+ CD8 Tregs, I explored STAT5 phosphorylation kinetics over time by exposing B6 and NOD splenocytes to 100, 1000, and 10000 pM concentrations of IL-15C. Similar to my previous finding, IL-15C mediated STAT5 phosphorylation in Ly49+ CD8 Tregs did not differ between strains (Figure 3.5B). Additionally, to determine whether Ly49+ CD8 Tregs signal differently in the whole animal setting, I injected B6 and NOD mice with the maximal dose of IL-15C, which was calculated to achieve a 10000pM concentration at a whole animal blood volume of 2mL. Strikingly, both B6 and NOD Ly49+ CD8 Tregs robustly upregulated STAT5 phosphorylation nearly 15-fold 60 minutes after injection (Figure 3.5C). Despite similar overall pSTAT5 MFI levels between IL-15C stimulated B6 and NOD Ly49+ CD8 Tregs, I did observe a slight bimodality in the pSTAT5 response in stimulated NOD Ly49+ CD8 Tregs. Thus, unlike B6 Ly49+ CD8 Tregs, there may exist a subpopulation of NOD Ly49+ CD8 Tregs that may not respond as robustly to extrinsic IL-15C stimulation.

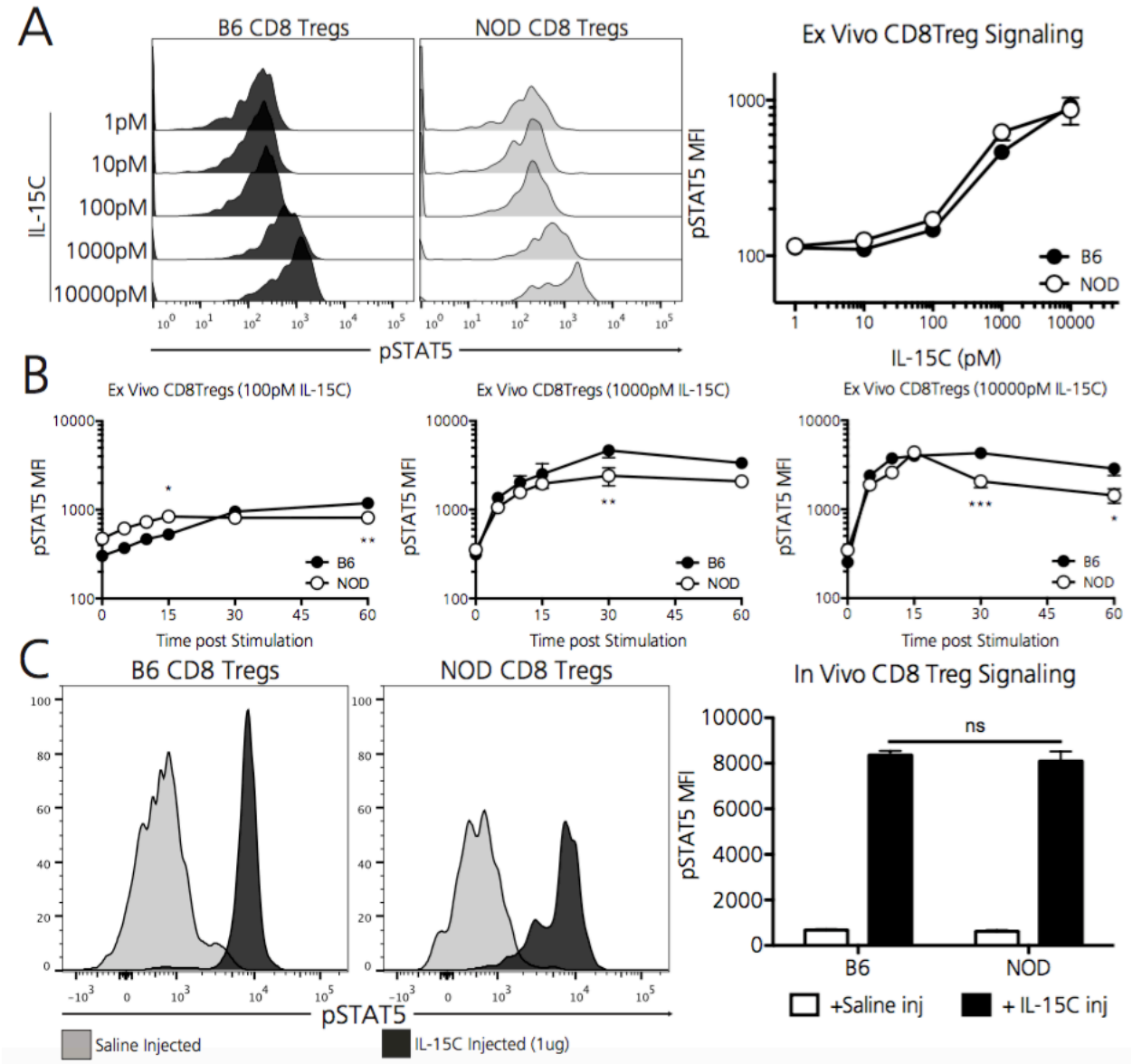


Figure 3.5. NOD Ly49+ CD8 Tregs adequately transduce IL-15 mediated survival signals. A) Within whole plated splenocytes, B6 and NOD Ly49+ CD8 Tregs phosphorylate STAT5 at Y694 to the same extent when exposed *ex vivo* to increasing concentrations of the IL-15C superagonist for 30 minutes. B) B6 and NOD Ly49+ CD8 Tregs phosphorylate STAT5 with similar time kinetics when exposed *ex vivo* to either 100, 1000, and 10000pM of IL-15C. Of note, NOD CD8 Tregs demonstrate statistically lower pSTAT5 levels 30 and 60 minutes after stimulation with the maximal 10000pM concentration of IL-15C (right panel). C) When exposed i.v. to 1µg of *in vivo* stable IL-15C for 60 minutes (calculated to reach 10000pM in a 2mL blood volume), both B6 and NOD Ly49+ CD8 Tregs increase pSTAT5 levels 15-times over animals injected with saline as a control. N=3-5 mice per group, *p<0.05, **p<0.01, ***p<0.005, ns = non-significant by two-way ANOVA followed by Sidak's multiple comparisons post-test.

In vivo administration of IL-15C robustly expands NOD Ly49+ CD8 Tregs and partially rescues their suppressive function

NOD Ly49+ CD8 Tregs sufficiently transduce IL-15 mediated survival signals via STAT5 phosphorylation. Therefore, I explored whether systemically administered IL-15C would expand NOD Ly49+ CD8 Tregs, and moreover, whether these expanded cells would be functionally activated. *In vivo* administration of IL-15C robustly expanded Ly49+ CD8 Tregs, increasing total Ly49+ CD8 Treg numbers 15-fold in B6 mice and 612-fold in NOD mice (Figure 3.6A).

To determine whether these IL-15C expanded NOD Ly49+ CD8 Tregs were functionally rescued, I utilized the CD8 Treg suppression assay described above (Figure 3.1A). Donor NOD mice were immunized with KLH and CD8 Tregs were expanded via concomitant IL-15C administration (1μg/day for 7 days). Recipient immunodeficient NOD.RAG mice then received either IL-15C expanded Ly49+ CD8 Tregs, IL-15C expanded Ly49- non-CD8 Tregs, or no CD8 T cells as a control. These mice then received naïve B cells and CD4+CD25- T cells, as well as the NP-KLH test stimulus. Whereas IL-15C activated Ly49- non-CD8 Tregs failed to suppress the antigen-specific anti-NP_8 IgG antibody response over mice receiving no CD8 T cells, IL-15C activated Ly49+ CD8 Tregs suppressed the antibody response (Figure 3.6B). However, equivalent numbers of transferred IL-15C activated Ly49+ CD8 Tregs in the NOD setting did not suppress the antibody response as completely as non-IL-15C activated Ly49+ CD8 Tregs in the B6 setting (Figure 3.6C), perhaps due to limited IL-15 availability in recipient NOD.RAG mice as compared to B6.RAG mice.

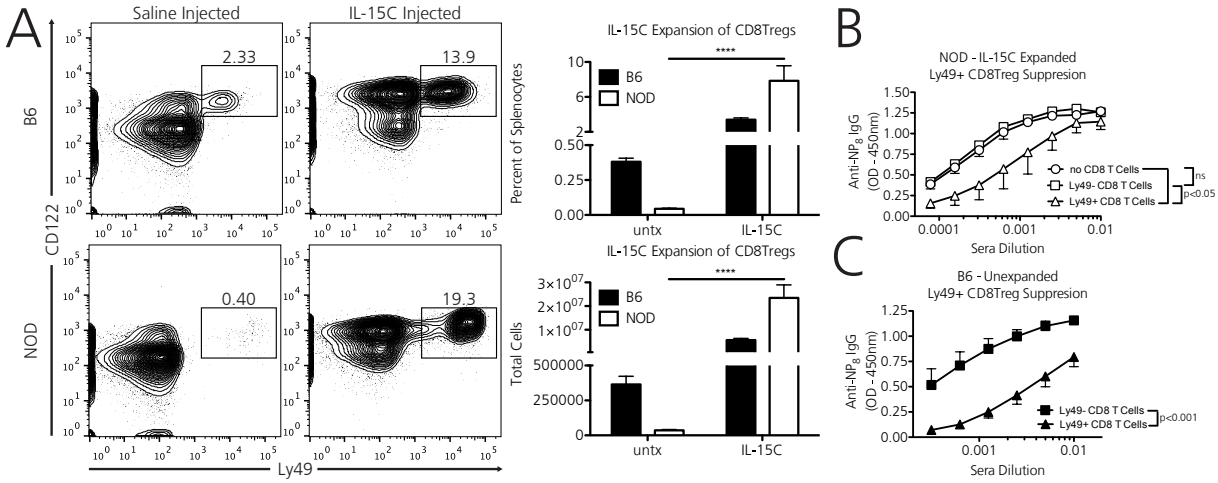


Figure 3.6. In vivo administration of IL-15C robustly expands NOD Ly49+ CD8 Tregs and partially rescues their suppressive function. A) When injected with 2μg of *in vivo* stable IL-15C for 4 consecutive days, B6 and NOD Ly49+ CD8 Tregs expand robustly. B) IL-15C activated Ly49+ CD8 Tregs (white triangles) from NOD mice immunized with KLH partially suppress the anti-NP_8 IgG response as compared to mice receiving Ly49- non-CD8 Tregs from the same donor mouse (white squares) or mice receiving no CD8 T cells as a control (white circles). C) In comparison, non-activated Ly49+ CD8 Tregs from CD8 Treg sufficient B6 mice that did not receive concomitant IL-15C activation (black triangles) robustly suppress the anti-NP_8 IgG response as compared to mice receiving Ly49- non-CD8 Tregs from the same donor mouse (black squares). N=3-5 mice per group, suppression assays shown are representative of three independently repeated experiments. ****p<0.0001, ns = non-significant, by semi-logarithmic linear regression analysis followed by y-intercept and slope curve comparison or two-way ANOVA followed by Sidak's multiple comparisons post-test.

IL-15C activated Ly49+ CD8 Tregs delay diabetes transfer

I next assessed whether IL-15C activated NOD Ly49+ CD8 Tregs could alter diabetes progression. Immunodeficient recipients were injected with either 5e4 CD122- CD8 T cells, 5e4 classically defined CD122+ CD8 Tregs, or 5e4 IL-15C expanded Ly49+ CD8 Tregs from pre-diabetic NOD mice. Two days later, all mice received 5e6 CD8-depleted diabetic splenocytes purified from hyperglycemic NOD mice. Mice receiving IL-15C expanded Ly49+ CD8 Tregs also received a single 1μg injection of IL-15C to maintain CD8 Treg activation. Whereas immunodeficient mice receiving non-activated CD122- CD8 T cells or classically-defined CD122+ CD8 Tregs developed diabetes by 4 weeks on average (MST - 3.9 weeks and 4.1 weeks, respectively, no statistical difference), progression to overt hyperglycemia took on average 10.4 weeks in mice receiving as few as 5e4 IL-15C activated Ly49+ CD8 Tregs (Figure 3.7A). Mice receiving IL-15C activated Ly49+ CD8 Tregs possessed the greatest percentage of splenic Ly49+ CD8 Tregs (CD122+Ly49+, Figure 3.7B) and the smallest percentage of splenic CD4 TFH cells (ICOS+PD-1+, Figure 3.7C).

IL-15 deficient mice lack CD8 Tregs, mount an enhanced alloantibody response, and resist transplant tolerance induction

Hitherto, my data demonstrate that B lymphocytes are a key barrier to transplant tolerance induction in autoimmune NOD mice (Chapter II), and moreover, that failure to regulate aberrant B cell function via CD8 Tregs permits islet immunity in this strain (Chapter III). As complementary data suggest that naturally occurring, IL-15 dependent CD122+ CD8 Tregs can delay skin and islet allograft rejection[125], I next questioned whether intact CD8 Treg control of the CD4 TFH/GC B cell axis was essential to attenuating the alloresponse and establishing

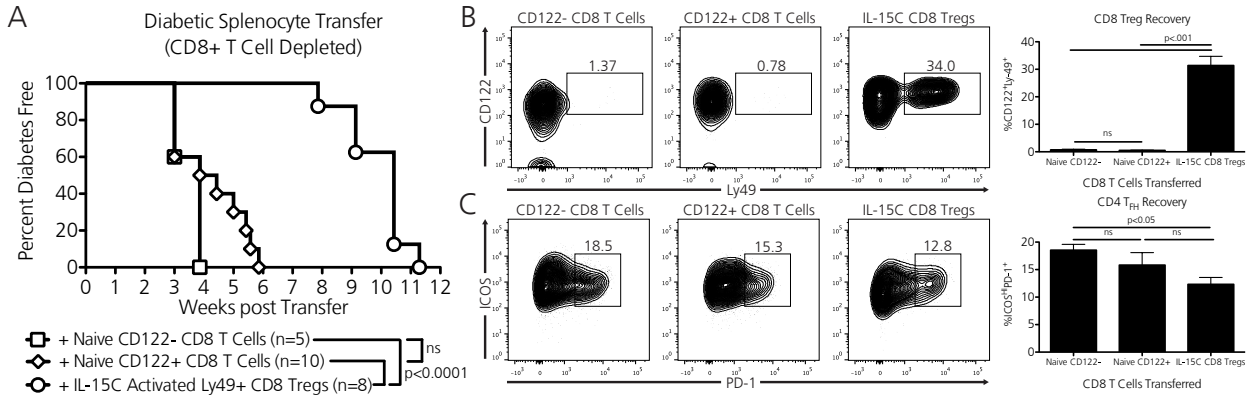


Figure 3.7. IL-15C activated Ly49+ CD8 Tregs delay diabetes transfer. A) A single injection of 5e4 Ly49+ CD8 Tregs FACS purified from pre-diabetic NOD mice receiving 1μg of *in vivo* stable IL-15C for 7 days (white circles) delay diabetes onset 2.6-fold longer than mice receiving either 5e4 CD122+ CD8 T cells (white diamonds) or 5e4 CD122- CD8 T cells (white squares) from untreated, naïve pre-diabetic NOD mice. NOD.RAG mice in all arms received 5e6 CD8-depleted splenocytes from hyperglycemic NOD mice 2 days prior to CD8 T cell infusion. On the day of CD8 T cell transfer, NOD.RAG mice receiving IL-15C activated CD8 Tregs also received a 1μg injection of *in vivo* stable IL-15C to maintain CD8 Treg activation. One week after diabetes onset, mice receiving IL-15C activated Ly49+ CD8 Tregs possessed the largest population of splenic Ly49+ CD8 Tregs (B) and most condensed population of target CD4 TFH cells (C). N=5-10 mice per group, significance determined by Log-Rank test or one-way ANOVA followed by Tukey's post-test, where appropriate.

long-term transplant tolerance. As to avoid any immune aberrancies perpetuated by an autoimmune background, I chose to examine this question in non-autoimmune B6 mice as this strain is susceptible to transplant tolerance induction and possesses an otherwise functional population of CD8 Tregs. Herein, I first explored whether the genetic absence of the CD8 Treg survival cytokine IL-15 altered the CD8 Treg-TFH-GC B cell immune axis in otherwise non-autoimmune, tolerance susceptible B6 mice. As compared to their IL-15 sufficient syngeneic counterparts, IL-15 deficient B6 mice (B6.IL-15$^{-/-}$) lacked splenic CD122+Ly49+ CD8 Tregs and demonstrated concomitant expansions of their splenic CD4 TFH (PD-1+Bcl-6+) and GC B cell (IgM-Fas+) populations (Figure 3.8A). Accordingly, these findings may in part explain why IL-15 deficient B6 mice develop aggravated EAE as compared to their IL-15 sufficient counterparts[211].

Thus, hypothesizing that a loss CD8 Tregs and concomitant expansion of immunogenic CD4 TFH and GC B cell populations would lead to an enhanced transplant response, I subsequently determined whether the absence of IL-15 altered how otherwise non-autoimmune mice respond to alloantigenic challenge. B6 and B6.IL-15$^{-/-}$ mice were immunized with 30e6 MHC-mismatched C3H splenocytes. Although allochallenge increased both the frequency and proliferation of CD4 TFH cells in both strains, allochallenged B6.IL-15$^{-/-}$ mice generated a higher frequency of CD4 TFH cells than their allochallenged IL-15 sufficient counterparts (Figure 3.8B). IL-15 deficiency further resulted in a greater production of anti-C3H IgG1 alloantibody (Figure 3.8B). Overall, these data suggest that in the absence of IL-15, a loss of CD8 Tregs may permit an over exuberant CD4 TFH response that promotes enhanced GC B cell antibody production.

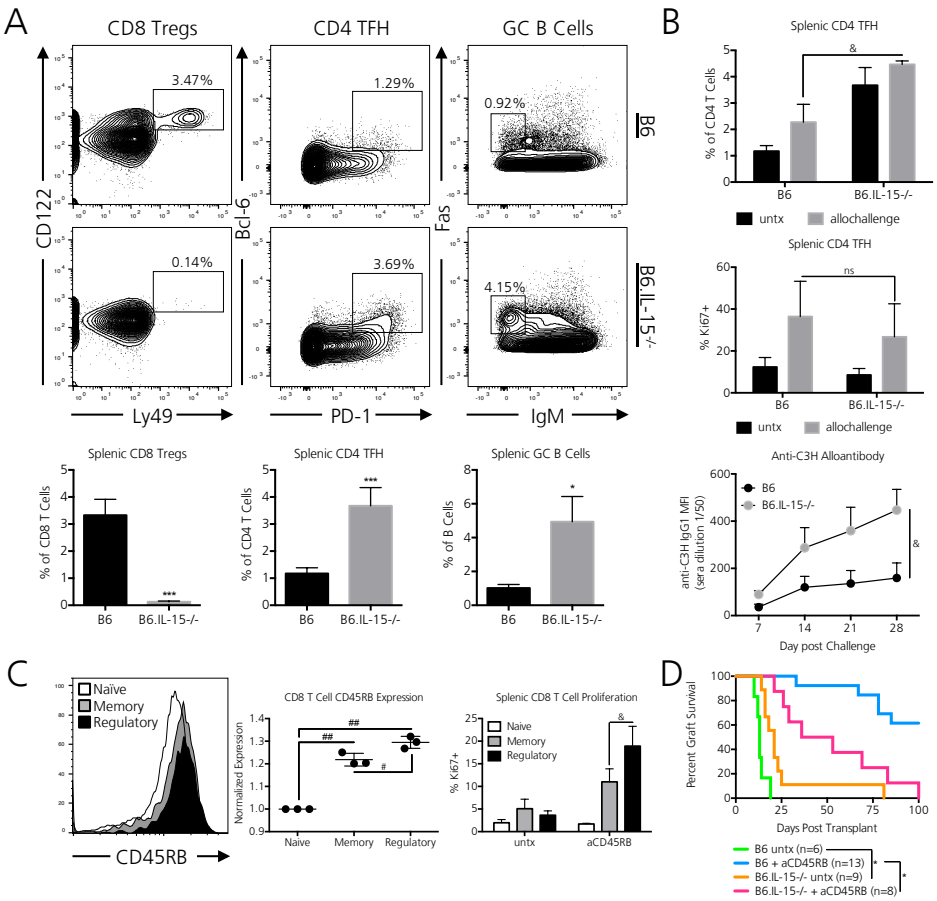


Figure 3.8. IL-15 deficient mice lack CD8 Tregs, mount an enhanced alloantibody response and resist transplant tolerance induction. A) IL-15 deficiency results in a near complete loss of CD8 Tregs and concomitant expansion of target CD4 TFH cells and Germinal Center B cells. (*p<0.05, ***p<0.001 by Student's t-test). B) Although allochallenge increased both the frequency and proliferation of CD4 TFH cells in both strains, allochallenged B6.IL-15$^{-/-}$ mice generated a higher frequency of CD4 TFH cells than their allochallenged IL-15 sufficient counterparts. Moreover, IL-15 deficiency resulted in increased anti-C3H IgG1 alloantibody titers following allochallenge ($^{\&}$p<0.05 by Two-way ANOVA followed by Sidak's multiple comparisons post test). C) To determine whether CD8 Tregs were a potential target of anti-CD45RB therapy, CD45RB isoform levels were compared between naïve (CD122-Ly49-), memory (CD122+Ly49-), and regulatory (CD122+Ly49+) splenic CD8 T cell populations. Overall, CD8 Tregs expressed the highest levels of anti-CD45RB. ($^{\#}$p<0.05, $^{\#\#}$p<0.0001 by One-Way ANOVA followed by Tukey's multiple comparisons post test). Moreover, anti-CD45RB induced CD8 Tregs to proliferate to a greater extent than other CD8 T cell populations. D) To determine whether IL-15 dependent CD8 Tregs were required during anti-CD45RB mediated tolerance induction, chemically diabetic IL-15 sufficient and deficient recipients were transplanted with C3H islet allografts then left untreated or received a standard 7-day course of anti-CD45RB. Although IL-15 deficiency delayed islet allograft rejection in untreated recipients (orange trace vs. green trace), this cytokine was absolutely required for long-term tolerance induction mediated by this therapy (pink trace vs. blue trace, significance determined by Log-rank test).

As anti-CD45RB treatment activates and expands graft-protective CD4 Tregs[109], I next questioned whether CD8 Tregs were also targets of this tolerance inducing therapy. Accordingly, I first examined CD45RB isoform expression on splenic CD8 T cell populations in non-autoimmune, WT B6 mice. In comparison to their naïve (CD122-Ly49-) and memory (CD122+Ly49-) CD8 T cell counterparts, CD8 Tregs (CD122+Ly49+) expressed the highest levels of the CD45RB isoform (Figure 3.8C). To determine whether such increased expression on CD8 Tregs conferred a functional outcome during anti-CD45RB mediated tolerance induction, WT B6 mice received a standard 7-day course of anti-CD45RB. Overall, anti-CD45RB induced CD8 Tregs to proliferate to a greater extent than other CD8 T cell populations as measured by Ki67 positivity (Figure 3.8C). Therefore, I hypothesized that anti-CD45RB mediated CD8 Treg enhancement would be critical to long-term transplant tolerance induction. Accordingly, diabetic IL-15 sufficient B6 and IL-15 deficient B6.IL-15$^{-/-}$ recipients were transplanted with C3H islet allografts and were left untreated or administered a standard 7-day course of anti-CD45RB. Although untreated IL-15 deficient recipients took twice as long to reject their islet allografts, CD8 Treg deficient B6.IL-15$^{-/-}$ mice were completely resistant to anti-CD45RB mediated tolerance induction (Figure 3.8D).

Discussion

CD8 Tregs are a newly defined population of regulatory cells that check the germinal center response, prevent dangerous epitope spreading, and halt autoimmunity[202]. Overall, little is known concerning CD8 Treg mediated control of T1D progression. However, one study by Wu and colleagues demonstrated that vaccination with peptides derived from Myelin Basic Protein (1-9NacMBP or p227) fully protected NOD mice from developing diabetes via activation of

intermediate affinity Qa-1[HSP60 loaded]/TCR restricted CD8 Tregs[212]. Possessing an ability to discriminate self from non-self, these CD8 Tregs inhibited the expansion of Insulin B9:23 peptide loaded cells while permitting the expansion of HEL loaded target cells. Thus, when activated, CD8 Tregs can functionally protect NOD mice from diabetes. However, as activation of CD8 Tregs was essential in promoting the diabetes-protective capacity of these cells, it can be inferred that in an otherwise unperturbed state, NOD CD8 Tregs either lack or lose their diabetes protective function over time. Overall, the mechanisms contributing to CD8 Treg failure in T1D have yet to be defined.

I have determined that at baseline, T1D-prone NOD mice lack a functional population of classically defined CD44+CD122+ CD8 Tregs; this deficiency may account for my observation that NOD mice develop excess antibody responses during antigenic challenge. Recently, the most potent population of TFH-targeting CD8 Tregs was identified within the Ly49F positive fraction of these classically defined CD44+CD122+ CD8 Tregs. Interestingly, the Ly49F isoform of the NK cell family of Ly49 inhibitory receptors is believed to interact with Qa-1[124], which is required for CD8 Treg development and is linked to the diabetes risk locus *Idd24*. This locus has further been connected to the prolonged immune response in NOD mice[213], which is an expected biological consequence of defective CD8 Treg function.

Additional genetic analysis has revealed that NOD mice possess the largest Ly49 haplotype of any known mouse strain[208]. The Ly49 locus in NOD mice contains an overabundance of activating receptors whose function has been linked to diabetes progression. This locus resides in the diabetes susceptibility *Idd6* region on chromosome 6 in NOD mice. NOD mice congenic for the B6 chromosomal region D6 Mit 254 to D6 Mit 14 (NOD.NK1.1 mice) have reduced diabetes incidence[214]. Although the authors suggest improved NK/NKT cell

performance as a mechanism of disease protection, introduction of the Ly49 locus from B6 mice may also restore Ly49+ CD8 Treg function in this NOD congenic strain, although this possibility has not been studied. Thus, although NOD mice possess an extremely polymorphic Ly49 locus, my ability to rescue the antibody suppressive function of these Ly49+ CD8 Tregs and activate them to confer diabetes protection suggests that Ly49 does identify CD8 T cells in NOD with the capacity to regulate islet immunity and antibody production.

In addition to the use of Ly49 as a functional marker for CD8 Tregs, a recent report highlighted the potential role of the Programmed Death Receptor 1 (PD-1) in CD8 Treg mediated suppression of the allograft response. These PD-1+CD44+CD122+ CD8 Tregs from B6 mice delayed rejection of Balb/C skin allografts via an IL-10 dependent mechanism[126]. As TFH cells also express components of the PD-1/PD-1L cellular exhaustion pathway[123], CD8 Treg expression of PD-1 may allow direct TFH cell targeting. In a cytometry-based screening analysis of Ly49+ CD8 Tregs in NOD mice, I detected no expression of PD-1 on CD44+CD122+ CD8 T Cells. This finding is corroborated by a report that wild-type NOD mice lack PD-1+CD122+ CD8 Tregs, which in turn, permitted enhanced islet effector function by their PD-1-CD122+ CD8 T cell counterparts[215]. As my results demonstrate functional rescue of Ly49+ CD8 Tregs by the novel IL-15C superagonist, future studies could investigate whether treatment with IL-15C restores PD-1 expression on and/or IL-10 secretion by CD122+ CD8 T cells in wild-type NOD mice.

As CD8 Tregs require IL-15 for their survival, I next explored whether IL-15 inadequacies in the NOD system contributed to the deficiency of these cells. Herein, I determined that NOD macrophages inadequately trans-present the CD8 Treg-requisite cytokine IL-15. In 2010, Suwanai et al[216] reported that NOD mice possess a defective IL-15 allele, which

underlies this strain's NK cell functional deficiency. Exogenous administration of low doses of IL-15C to the diabetes-protected BDC2.5/NOD mouse preferentially expanded NK cells, which broke islet cell tolerance and rapidly precipitated diabetes. The authors reported no expansion of CD44+CD122+ CD8 T cells, suggesting that in contrast to the high doses of IL-15C used to expand CD8 Tregs in my study, low doses of IL-15C may favor the expansion of diabetes-promoting cells rather than their disease-protective regulatory cell counterparts. In fact, NOD mice demonstrate reduced disease incidence when they genetically lack IL-15[217] or are treated with an anti-IL-15Rb blocking antibody[218]. Thus, although disruption of the IL-15 axis in these systems could interrupt residual Ly49+ CD8 Treg function, I hypothesize that the already profoundly diminished pool of IL-15 Ly49+ CD8 Tregs would be unaffected by any additional loss of IL-15. It was further reported, however, that in the absence of IL-15 dependent NK cells, administration of IL-15 to NOD mice prevented disease[219]. Moreover, in the non-autoimmune B6 setting, co-administration of IL-15 with naturally occurring naïve CD122+ CD8 Tregs prolonged foreign islet allograft survival[125]. Thus, I hypothesize that activating CD8 Tregs with IL-15C specifically, and not their pathogenic IL-15 dependent counterparts, affords disease protection in the NOD setting.

Patients with T1D have been reported to possess non-functional peripheral blood resident CD8 Tregs[220] as well as a unique TFH cell phenotype (described above)[197] that may result from this dysfunction. Specifically, CD8 Tregs from patients with T1D failed to eliminate islet-specific Glutamic Acid Decarboxylase [GAD]-reactive CD4 T cells via TCR-restricted interactions with the non-classical MHC Class Ib molecule HLA-E (the human homologue of murine Qa-1) expressed by target cells. In related clinical studies, patients with recent onset T1D responding positively to anti-CD3 therapy (Teplizumab) demonstrated an expanded pool of

circulating central memory like CD8 T Cells[194]. In fact, CD8 T cells isolated from the peripheral blood of patients treated with Teplizumab possess restored regulatory function as compared to CD8+ T cells isolated from patients treated with a control IgG[221]. These reprogrammed CD8 T Cells upregulated the expression of the regulatory cell identifier GITR, a marker that I also determined to be upregulated on classically defined CD8 Tregs from B6 and NOD mice. Thus, anti-CD3 may reprogram CD8 T cells from an effector to an islet-protective regulatory phenotype. In fact, I have additionally determined that NOD mice treated with a single 50μg injection of anti-CD3 demonstrate a 10-fold expansion of Ly49+ CD8 Tregs seven days later.

In the final portion of Chapter III, I determined that IL-15 deficient mice lack CD8 Tregs, a cellular deficiency which may account for my observation that these mice harbor enhanced frequencies of immunogenic CD4 TFH and GC B cells, mount an enhanced alloantibody response, and resist transplant tolerance induction. It is important to note however that CD122+ NK cells also depend on the cytokine IL-15 for their survival[222]. Thus, from the data presented above it remains to be determined whether the enhanced alloresponse and resistance to transplantation tolerance characteristic of IL-15 deficient mice is due to a functional absence of CD8 Tregs, CD122+ NK cells, or a combination of both cell populations.

This question is highly relevant in that NK cells are active participants in allorecognition[223]. Human NK cells express a unique combination of genetically encoded Killer Immunoglobulin Receptors (KlRs) that scan target cells for the presence of self-MHC-I. In the absence of self-MHC-I expression, NK cells are licensed to lyse target cells via a perforin, a process referred to as the "missing self hypothesis." As KlR/MHC interactions are similarly restricted by MHC haplotype, NK cells can be licensed to target cells expressing foreign MHCs. Evidence for NK cell mediated killing of donor APCs was first demonstrated by Yu and

colleagues in which skin allografts from DBA/2 (H2-d) mice were transplanted onto either T/B cell deplete B6.RAG$^{-/-}$ mice or T/B/NK cell deplete B6.RAG$^{-/-}\gamma c^{-/-}$ mice (both H2-b)[224]. Whereas donor APC recovery 2 weeks after transplantation was nearly undetectable in NK cell replete RAG$^{-/-}$ recipients, donor APCs were readily detectable in NK cell deplete RAG$^{-/-}\gamma c^{-/-}$ recipients. Direct allorecognition and destruction of donor APCs by NK cells is believed to further augment the T cell mediated indirect alloresponse by creating "seed" allopeptides for host APCs[223]. Accordingly, the absence of NK cells in untreated IL-15 deficient islet graft recipients could explain, in part, the 2-fold delay in allograft rejection in that NK cell alloantigen seeding failed to initiate the indirectly alloreactive T cell pool in these mice.

Moreover, an elegant series of experiments performed by Ronald Gill's laboratory demonstrated that NK cells are essential during DST + anti-CD40L or anti-LFA1 mediated tolerance induction[225]. NK cell mediated tolerance induction relied on host MHC Class I expression as well as perforin expression by these innate immune cells. Whether anti-CD45RB similarly requires NK cells to mediate its tolerogenic effects remains to be determined. To directly prove whether failed tolerance induction in IL-15 deficient mice is directly attributable to an absence of CD8 Tregs or NK cells, experiments in which specific antibody mediated depletion of either cell population is performed prior to transplant tolerance induction are currently underway. Alternatively, the Cantor laboratory recently developed a unique mouse strain in which CD8 Tregs cannot ligate and eliminate their target TFH cells[123]; a point mutation in this strain's Qa-1 molecule (D227K) prevents CD8 Treg TCR engagement with Qa-1 and leads to widespread autoimmunity. Using this strain, the requisite role of CD8 Tregs during transplant tolerance could be more directly addressed. Overall, understanding whether and how

CD8 Tregs promote transplant tolerance could one day arm clinicians with an novel means to selectively control the transplant response.

In conclusion, I demonstrate that NOD mice lack a functional population of classically defined CD44+CD122+ CD8 Tregs, a dysfunction that may result from a severe deficiency of the Ly49+ CD8 Tregs that target TFH cells. Despite reduced IL-15 availability in the NOD system, these Ly49+ CD8 Tregs respond adequately to IL-15, can be restored numerically and functionally with a novel IL-15C superagonist, and can prevent diabetes transfer. Moreover, I have determined that IL-15 deficiency imparts a generalized resistance to transplant tolerance induction highlighting a potential role for newly defined CD8 Tregs in control of the transplant response. Overall, IL-15 may activate CD8 Tregs in T1D and transplantation, thereby offering new paradigms for using CD8 Tregs as biomarkers for disease progression or as a novel cell-based therapy (Figure 3.9).

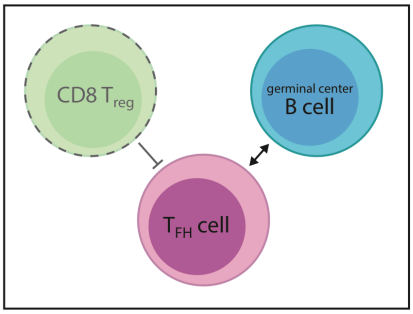
Chapter Findings	Model Figure	Future Work
- T1D prone NOD mice possess non-functional, classically defined CD122+ CD8 Tregs. - NOD mice possess significantly diminished numbers of TFH targeting Ly49+ CD8 Tregs. - NOD Macrophages inadequately transpresent the CD8 Treg survival cytokine IL-15. - NOD CD8 Tregs adequately transduce IL-15 mediated survival signals and expand robustly during exogenous IL-15 administration. - IL-15 expanded NOD CD8 Tregs possess restored suppressive function and delay diabetes transfer. - IL-15 deficient B6 mice lack CD8 Tregs, mount an enhanced alloantibody response, and resist transplant tolerance induction.		- Determine whether diabetes-protected NOD mice possessing the B6-derived, Ly49 locus (NOD.B6Idd6) possess restored CD8 Treg suppressive function. - Assess the role of IL-10/PD-1 in NOD CD8 Tregs. - Determine whether the diabetes protective agent anti-CD3 restores CD8 Treg suppressive function in NOD mice. - Determine the role of Qa-1 restricted CD8 Tregs in the T1D prone NOD environment. - Assess whether failed transplant tolerance induction in B6.IL-15$^{-/-}$ mice is due to loss of CD8 Treg vs. NK cell presence/function. - Define whether NOD CD8 Treg failure contributes to transplant tolerance resistance in this strain.

Figure 3.9. Chapter III – findings and future work.

CHAPTER IV

HEMATOPOIETIC STEM CELL MOBILIZATION IS NECESSARY BUT NOT SUFFICIENT FOR TOLERANCE IN ISLET TRANSPLANTATION

Scientific Goal

In Chapters II and III, I demonstrate that B cell restraint of graft-protective CD4 Treg function, as well as failed CD8 Treg regulation of the immunogenic TFH-GC B cell axis, respectively, represent key barriers to successful transplant tolerance induction. In that the lymphopenic environment characteristic of NOD mice permits the unregulated expansion of immunogenic B lymphocyte clones, I next questioned whether failures at the earliest stage of B lymphocyte development, the Hematopoietic Stem Cell (HSC) niche, further contribute to the generalized resistance to transplant tolerance characteristic of this autoimmune strain. In Chapter IV, I explore the hypothesis that transplant tolerance induction requires sufficient mobilization of the HSC niche. Overall, my findings indicate that the HSC compartment plays an under-recognized role in the establishment and maintenance of immune tolerance and this role is disrupted in diabetes-prone NOD mice. Understanding the stem cell response to immune therapies in ongoing human clinical studies may help identify and maximize the effect of immune interventions for T1D.

Introduction

Long-term success in islet transplantation has been difficult to achieve in recipients with T1D. To date, less than half of patients with T1D undergoing this procedure maintain insulin

independence after three years[165]. The induction of permanent immune tolerance would immensely enhance the outcome of beta cell replacement strategies by protecting the restored islet cell mass from immune destruction. Like the experience in human recipients, animal models of T1D are extraordinarily resistant to transplantation tolerance (as demonstrated in my previous chapters). Even though transplantation tolerance is readily induced in non-autoimmune mice, neither islet nor other organ allografts have shown stable long-term acceptance when transplanted in T1D-prone NOD recipients[143,144,226].

The most successful attempts to induce transplant tolerance in animal models of T1D have included bone marrow transplantation (BMT) as part of the regimen[227]. Extending observations that irradiated NOD mice reconstituted with Hematopoietic Stem Cells (HSCs) from the diabetes-resistant B10.BR and Balb/c strains are protected from diabetes[228], Li and colleagues subsequently found that reconstitution of irradiated, diabetic NOD mice with B10.BR bone marrow at the time of matched B10.BR islet allografting rendered these mice permanently tolerant to these transplanted islet allografts[229]. Similar BMT regimens have also demonstrated some potential in maintaining islet tolerance in patients with T1D. In the context of recent onset T1D, non-myeloablative clinical trials exploring the role of autologous stem HSC infusion have demonstrated some success in restoring insulin independence, increasing C-peptide levels, and decreasing HgA1c levels in recipients[230,231]. Within the context of islet transplantation, however, a single study found that patients receiving islet infusions alongside donor-matched HSC transplantation all succumbed to graft rejection within one year after immunosuppression withdrawal[232]. Specifically, the authors attribute their findings to poor recipient/donor chimerism, which they attribute to the fact that patients did not undergo myeloablation prior to this therapy. Although patients with lympho-hematological malignancies undergoing myeloablation +

allogeneic BMT have later achieved permanent solid organ graft tolerance in the absence of immunosuppression[233], the potential risk of GVHD remains hard to justify over exogenous insulin administration in patients with clinically-manageable T1D.

Overall, it is likely that addition of allogeneic bone marrow creates a state of microchimerism that facilitates the engraftment of matched organs. In addition to the presentation of novel antigens, the introduced bone marrow also contributes to the formation of new cells from hematopoietic stem cell progenitors. The degree to which stem cell progenitor activity contributes to tolerance induction is not well defined.

Recent studies have suggested that bone marrow resident hematopoietic stem cells (HSCs) are not the quiescent cells that have long been envisaged but are instead the master control center of the immune response. In response to a variety of infectious stimuli and cytokine milieus, HSCs are now known to generate a robust effector cell response while maintaining a protective state of immune homeostasis[234,235]. I therefore hypothesized that HSCs play a critical role during the establishment of allograft tolerance by regulating the immune response to the organ transplant.

In Chapter IV, I observe that HSC's are mobilized during tolerance induction with anti-CD45RB in healthy, non-autoimmune B6 mice but not during treatment of T1D-prone NOD mice, which resist transplantation tolerance. Depleting HSCs or inhibiting their mobilization prevented the establishment of tolerance to islet allografts. I observed that the poor mobilization in NOD mice relates to HSC overexpression of CXCR4, which otherwise maintains these pluripotent progenitors in their bone marrow niche. Targeted HSC mobilization improved graft outcomes in NOD mice suggesting that clinical therapies should measure the HSC response and attempt to enhance it in order to maximize immune tolerance in T1D.

The tolerance-inducing agent anti-CD45RB promotes HSC mobilization that is necessary to establish transplant tolerance

Transplant tolerance inducing agents are often partially immune depleting. Thus, I hypothesized that the hematopoietic stem cell (HSC) compartment would respond to this loss of lymphocytes following anti-CD45RB therapy; mobilization in the marrow leads HSCs to leave their quiescent niche and begin to proliferate *in situ*. To my knowledge this hypothesis has not been directly investigated nor has it been studied in the T1D-prone NOD setting. B6 and pre-diabetic NOD mice were left untreated or received a standard 7-day course of anti-CD45RB. On day 8, marrow Lineage Negative, c-Kit+Sca-1+ [LSK] stem cells were identified by flow cytometry; these cells were negative for lineage markers and positive for Sca-1 and c-Kit as illustrated in Figure 4.1A. Anti-CD45RB enhanced B6 stem cell frequency by 30% (Figure 4.1B). In contrast, anti-CD45RB treatment had no effect on HSC frequency in NOD mice (Figure 4.1B).

Because there is no absolute standard for identification of HSCs by cell surface markers, I confirmed the suspected increase in HSC mobilization with a functional assay. To this end, HSC activation was assessed by a colony forming cell (CFC) assay in which bone marrow from anti-CD45RB treated B6 mice developed nearly twice as many colonies per 5000 plated bone marrow cells (BMC) as compared to control (Figure 4.1C). Thus, the tolerogenic agent anti-CD45RB activates bone marrow stem cells in tolerance-susceptible B6 mice.

To determine whether this change in stem cell activity affected tolerance induction, I next depleted HSCs during tolerance induction with ACK2 treatment. Streptozotocin-treated, diabetic B6 mice received a single 500μg dose of ACK2 prior to the day of islet allografting and initiation

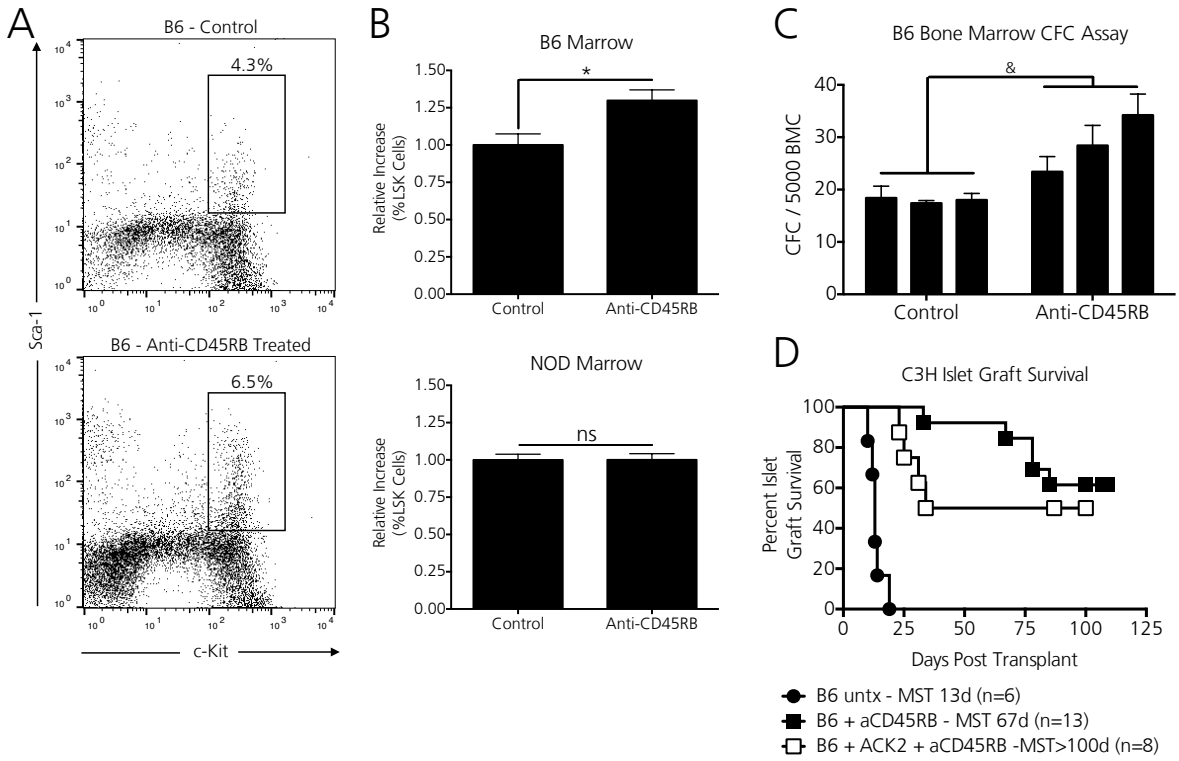


Figure 4.1. Tolerance Induction Requires Stem Cell Mobilization. A and B) Hematopoietic stem cells were identified as LSK cells by staining with a pan-lineage kit to identify lineage negative (Lin^-) cells that were then analyzed for expression of c-Kit and Sca-1 (Lin^- cells shown in plots). Treatment with anti-CD45RB (100μg/day on days 0, 1, 3, 5, and 7) led to an increase in LSK cells in B6 marrow as quantified in panel B relative to untreated mice. The frequency of stem cells in the control group was set to 1 in each experimental repetition (*$p<0.005$ by t-test, 5 independent experiments, n=14 for ctrl and anti-CD45RB). Treatment of NOD mice with the same regimen of anti-CD45RB led to no change in LSK frequency (p=ns by t-test, 3 independent experiments, n=6 for ctrl and anti-CD45RB). C) To verify increased HSC activity in B6 mice, a colony-forming assay was performed. 5000 bone marrow cells from anti-CD45RB treated or untreated B6 mice were plated for 7 days in methylcellulose culture medium and colonies were counted. Marrow from anti-CD45RB treated mice produced more than double the colonies ($^{\&}p<0.0001$ by One-way ANOVA, 3 independent samples per condition in 6 replicates each, repeated twice). D) HSC's were depleted by a single 500μg injection of ACK2 (Tocris Bioscience, Bristol, United Kingdom) one day prior to transplantation. On day 0, chemically diabetic B6 mice were transplanted with allogeneic C3H islets and treated with a standard 7-day course of anti-CD45RB. Depletion of HSC's prior to anti-CD45RB therapy led to more rapid rejection in some recipients.

of anti-CD45RB therapy. Animals depleted of HSCs demonstrated a decrease in tolerance induction with more rapid loss of their islet transplants (Figure 4.1D). Because the ACK2 therapy is short lived, HSCs likely recovered during treatment, which may have limited the effect. I next sought to determine how HSCs were mobilized in order to target the pathway more effectively.

The effect of anti-CD45RB is not directly on HSCs but rather on osteoblasts

I considered that the differences in the response of the two strains could relate to differential expression levels of CD45RB on the cell surface of HSCs. I therefore analyzed CD45RB expression on LSK marrow cells by flow cytometry. Neither B6 nor NOD HSCs demonstrated CD45RB expression (Figure 4.2A). In comparison, a significant portion of lineage positive cells in both strains expressed the CD45RB isoform (Figure 4.2A). Thus, I concluded that tolerogenic agent anti-CD45RB mobilizes HSCs through an indirect mechanism.

The HSC niche is governed in part by osteoblasts, which are derived from the macrophage lineage and would be expected to express CD45RB[236]. As expected, osteoblasts isolated and cultured from B6 mice expressed Rank-L and Osteocalcin (assessed by flow cytometry; these studies were performed in collaboration with Yuantee Zhu, an MD/PhD student in the lab of Florent Elefteriou). Additionally, cultured osteoblasts expressed the CD45RB isoform (Figure 4.2B). To demonstrate that osteoblast expression of CD45RB was functionally relevant, I compared the response of cultured B6 osteoblasts to G-CSF, which is known to mobilize HSCs through an osteoblast-dependent mechanism[237], and anti-CD45RB. In assessing gene expression changes over untreated control cells (Table 2), I found that both agents downregulated osteoblast CXCL12 expression, which should facilitate HSC mobilization.

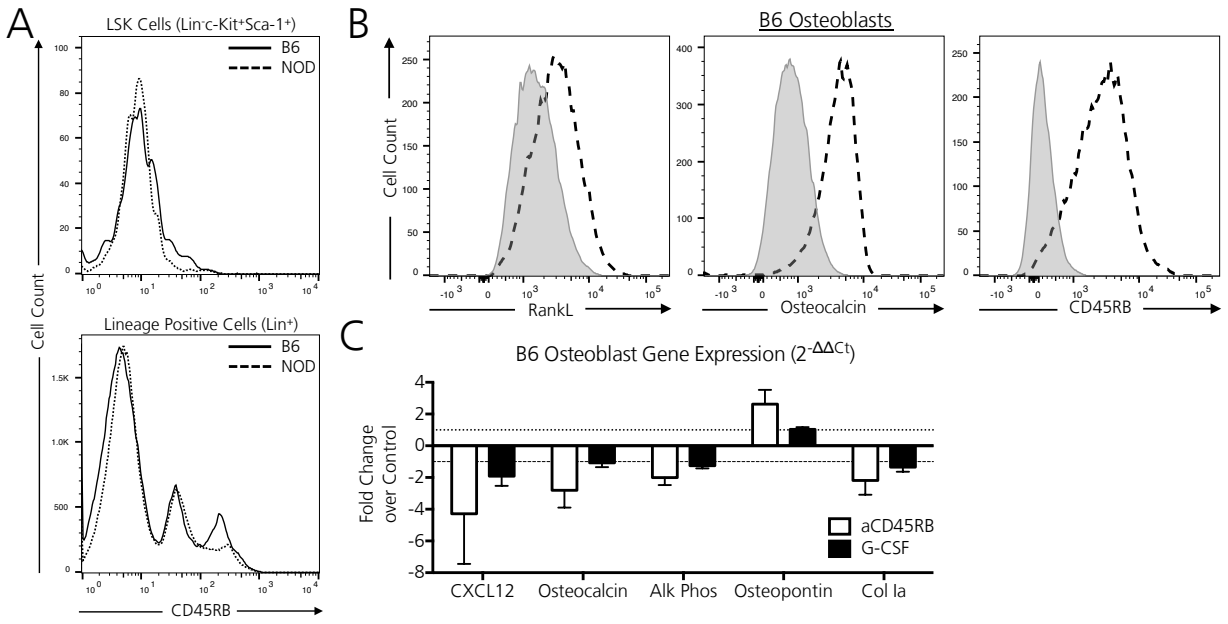


Figure 4.2. Anti-CD45RB targets Osteoblasts. A) Analysis of bone marrow resident HSCs (upper panel) from both B6 and NOD mice showed that neither expressed significant levels of the CD45RB isoform. In comparison, a significant portion of Lin^+ bone marrow cells in both strains expressed CD45RB (lower panel). B) Cytometric analysis of cultured osteoblasts from B6 mice demonstrate expression of Rank-L and osteocalcin as expected. Moreover, these osteoblasts express CD45RB (gray: isotype control). C) Cultured osteoblasts were treated with G-CSF (R&D, Minneapolis, MN), anti-CD45RB, or left untreated (control) to determine whether the expressed CD45RB was functional. As compared to untreated osteoblasts, G-CSF and anti-CD45RB treated osteoblasts downregulated the expression of CXCL12, which normally retains HSCs in their niche. Other osteoblast activation markers were also slightly downregulated, but osteopontin was maintained indicating viable cells.

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
CXCL12	TGCATCAGTGACGGTAAACCA	GTTGTTCTTCAGCCGTGCAA
Osteocalcin	CCGGGAGCAGTGTGAGCTTA	TAGATGCGTTTGTAGGCGGTC
Rank-L	CACCATCAGCTGAAGATAGT	CCAAGATCTCTAACATGACG
Alkaline Phosphatase	ATCTTTGGTCTGGCTCCCATG	TTTCCCGTTCACCGTCCAC
Osteopontin	GATGATGATGACGATGGAGACC	CGACTGTAGGGACGATTGGAG
Collagen 1a	GAGCGGAGAGTACTGGATCG	GTTCGGGCTGATGTACCAGT
GAPDH	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA

Table 2. Primers used to evaluate osteoblast gene expression.

Despite downregulating several other osteoblast specific functional genes, these cells maintained expression of osteopontin indicating cell survival (Figure 4.2C).

Sympathectomy prevents HSC mobilization by anti-CD45RB and abrogates transplantation tolerance

Osteoblast dependent mobilization of HSCs following G-CSF can be prevented by sympathectomy[237], which may also be relevant to nervous system dysfunction in candidate transplant recipients with T1D[238,239]. To determine whether HSC mobilization induced by anti-CD45RB was similarly inhibitable, B6 mice were chemically sympathectomized with 6-hydroxydopamine (6-OHD, 100mg/kg/dose on day -4 and 250mg/kg/dose on day -2) before initiation of a standard 7-day course of anti-CD45RB therapy starting on day 0. Bone marrow resident LSK cells were analyzed on day 8. Consistent with Figure 4.1, anti-CD45RB treatment alone activated HSC mobilization (Figure 4.3A). Pretreatment with 6-OHD ablated anti-CD45RB mediated HSC mobilization (Figure 4.3A).

Because sympathectomy prevented HSC mobilization, I determined whether it also prevented tolerance induction. Following a similar dosing strategy, chemically diabetic B6 mice were sympathectomized with 6-OHD, transplanted with C3H islets, and administered anti-CD45RB for 7 days. Overall, sympathectomy significantly diminished anti-CD45RB mediated tolerance induction with only one recipient demonstrating long-term transplant survival (Figure 4.3B).

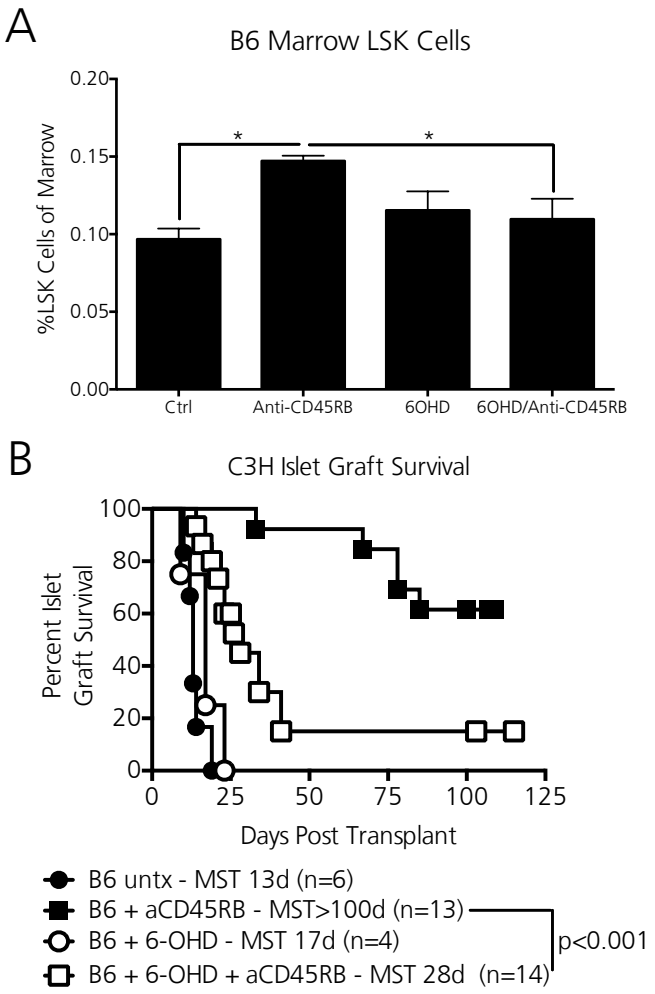


Figure 4.3. Sympathectomy Blocks HSC Mobilization and Tolerance Induction. A) B6 mice were chemically sympathectomized with 6-hydroxydopamine (6-OHD, Sigma Aldrich, St. Louis, MO) on days -4 (100mg/kg) and -2 (250mg/kg) relative to the initiation of a standard 7-day course of anti-CD45RB. The marrow LSK cell response was then measured on day 8. As previously shown, anti-CD45RB induced a significant increase in the percent of LSK cells. Sympathectomy itself had no effect on baseline HSC levels whereas it completely abolished anti-CD45RB mediated LSK response ($p<0.01$ by One-way ANOVA followed by Tukey's multiple comparisons post-test: *$p<0.05$ anti-CD45RB vs. 6-OHD/CD45, *$p<0.05$ anti-CD45RB vs. Control). B) Sympathectomized or control mice received allogeneic C3H islet transplants and were left untreated or treated with anti-CD45RB. Sympathectomy itself had no effect on rejection kinetics. Tolerance induction by anti-CD45RB was abrogated by prior sympathectomy (*$p<0.001$ by Log-rank test).

Blockade of excess CXCR4 enhances NOD HSC mobilization and extends islet transplant survival

I further investigated whether enhanced HSC mobilization would augment islet transplantation in the setting of T1D. Treatment of B6 and NOD mice with G-CSF, which acts with the sympathetic nervous system to modulate osteoblast-mediated HSC retention[237], was highly effective in B6 mice but exhibited only a modest effect on NOD LSK cells (Figure 4.4A). In addition to the role of the sympathetic nervous system (SNS)-marrow axis, HSCs are retained in their quiescent niche by their expression of CXCR4. Flow cytometry analysis demonstrated significantly higher CXCR4 expression on NOD than B6 LSK cells (Figure 4.4B). Targeting CXCR4 with AMD3100 (100μg/day subcutaneous injections for 5 days) resulted in robust HSC mobilization in NOD that exceeded the B6 response (Figure 4.4C). My data complement clinical findings demonstrating that patients with T1D respond poorly to G-CSF mediated HSC mobilization yet appropriately respond to AMD3100 mediated HSC mobilization[240].

I next determined whether HSC mobilization supported improved islet allogeneic transplant survival in NOD mice. Treatment with AMD3100 alone prior to transplantation led to a nearly 7-fold prolongation over anti-CD45RB NOD recipients receiving C3H islet allografts (Figure 4.4D, $p<0.05$). Addition of anti-CD45RB to the AMD3100 pretreatment led to a further, albeit slight increase in allograft survival. However, permanent tolerance induction was not observed suggesting that NOD immune cells resist tolerance induction at multiple steps.

Discussion

My data suggest that proper HSC mobilization and sympathetic innervation are pre-requisites for tolerance induction during islet transplantation. Persons with established T1D are

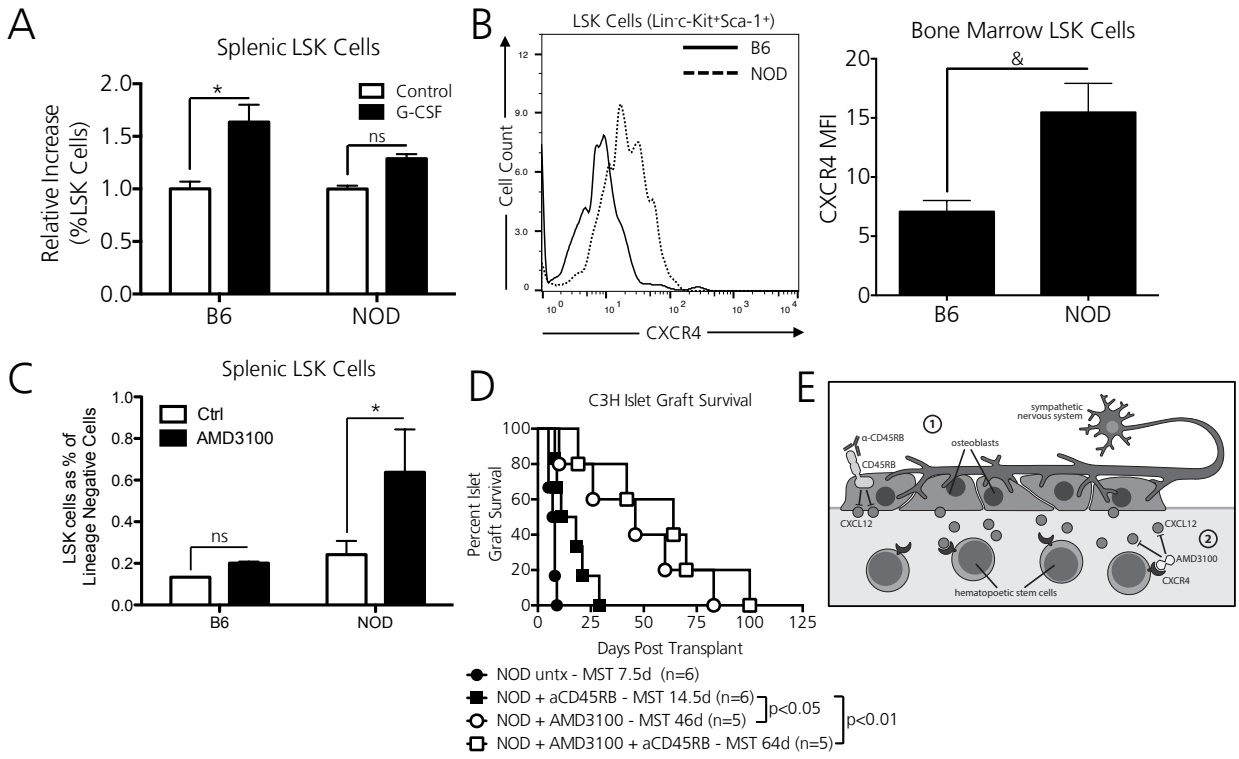


Figure 4.4. Targeting CXCR4 Enhances HSC Mobilization and Extends Islet Allograft Survival in NOD mice. A) B6 and NOD mice were left untreated or treated with G-CSF (250μg/kg/day for 5 days in 8 divided doses). Spleens were harvested 2-3 hours after the last dose. Whereas B6 mice demonstrated a robust increased in LSK cells following G-CSF treatment, little change was seen in NOD mice (*p<0.001 by Two-way ANOVA followed by Sidak's multiple comparisons post-test). B) NOD bone marrow resident LSK cells demonstrated significantly greater CXCR4 expression than B6 LSK cells as measured by flow cytometry (*p<0.05 by t-test). C) Blockade of CXCR4 with AMD3100 (100μg/day for 5 days, subcutaneous injections, Sigma Aldrich, St. Louis, MO) led to significantly increased LSK cell frequencies in the spleens of NOD mice (*p<0.05 by Two-way ANOVA followed by Sidak's multiple comparisons post-test). D) Islet transplantation following pretreatment with AMD3100 (100μg/day on days -4, -3, -2, -1, and 0 relative to the day of transplant) in NOD mice led to a significant prolongation in islet survival, which was further extended by addition of anti-CD45RB although permanent tolerance was not achieved (p values determined by Log-rank test). E) A schematic of the bone marrow HSC niche indicating the presumed action of anti-CD45RB on osteoblasts and the contribution of the sympathetic nervous system (1), as well as the ability of CXCR4-CXCL12 blockade to bypass these steps and promote mobilization in NOD mice (2).

well known to have disturbed sympathetic function[238,239]. These disruptions have been specifically linked to inadequate HSC mobilization in patients with T1D, which others have linked to increased risk for cardiovascular disease[238]. My data suggest that this disruption will impede their responses to immune therapies. Whether persons at risk for T1D have sympathetic dysfunction is less well studied; however, one group determined that patients close to disease onset exhibit some level of autonomic dysfunction suggesting that decrements in the SNS-immune axis may contribute to diabetes progression[143,226]. In the NOD mouse, diabetes has also been correlated with the development of autonomic dysfunction[241]. In addition, multiple nervous system antigens have proven to be the targets of the autoimmune attack[242-244].

My studies in Chapter IV add to these limitations by identifying that T1D prone NOD mice resist SNS-dependent, G-CSF mediated mobilization of HSCs. Furthermore, I determined that excess expression of CXCR4 on NOD HSCs may contribute to failures of bone marrow responsiveness that may in part perpetuate loss of immunologic tolerance; this finding complements previous findings that 1) CXCL12 levels are enhanced in NOD bone marrow, 2) NOD HSCs demonstrate enhanced chemotaxis to CXCL12, and 3) CXCR4/CXCL12 blockade with AMD3100 prevents diabetes in NOD mice[245]. Similar to work published by Fiorina and colleagues, my data confirm that targeting CXCR4 with AMD3100 leads to islet allograft prolongation[246]. In both studies, administration of this agent at the time of transplantation failed to induce permanent allograft tolerance. My work is unique however, in that I connect the HSC mobilization to transplant tolerance induction.

In relating these findings to my observations in Chapter II and III, a number of groups have in part defined how a dysregulated HSC niche in NOD mice may later propagate unchecked anti-islet T and B effector cell function. In 2008, Leng and colleagues found that excess

CXCL12 in NOD bone marrow enhances both naïve T cell and CD4 Treg recruitment to this niche[245]. Although yet to be proven, the authors speculate that CXCL12 driven accumulation of T cells in the bone marrow may perpetuate a state of peripheral lymphopenia that permits an otherwise unchecked homeostatic proliferation of islet reactive T cells. Extending these observations, it may be speculated that this mechanism fosters the unregulated expansion of anti-graft effector T cells in NOD mice that are consequently resistant to anti-CD45RB mediated transplant tolerance induction. Furthermore, it is well documented that NOD HSCs demonstrate enhanced engraftment potential over HSCs from other diabetes-protected strains[247]. Such HSC retention is directly attributable to the *Idd*9 risk locus. Although NOD mice that possess the B10.Idd9.3 locus from diabetes resistant B10 mice (termed NOD.B10.Idd9.3 mice) are protected from diabetes due to enhancements in islet-protective CD137+ CD4 Tregs and reductions in the diabetogenic capacity of CD4 T effector cells, NOD.B10.Idd9.3 mice are no more permissive to anti-CD40L mediated transplant tolerance induction than their WT NOD counterparts[226]. Overall, determining which combinatorial genetic factors prevent HSC mobilization in NOD mice may reveal new targets to restore transplant tolerance in this otherwise resistant strain.

As HSC mobilization may underlie the potential for tolerance induction, measuring the HSC response could represent a new opportunity to identify responder and non-responders to clinical T1D immune therapy. Moreover, it would be anticipated that HSC activity would be a prominent feature of bone marrow transplantation (BMT). In part, HSC enhancement could explain the success of BMT in the NOD model in terms of diabetes prevention and islet allograft potentiation. Furthermore, HSC enhancement may explain the promising preliminary clinical results of autologous transplantation in human T1D. However, the contribution of HSC mobilization has not been examined in detail in any of these settings. My data suggest that

approaches to enhance HSC activity could foster the success of other immune therapies. In addition, my data indicate that tolerance induction is a multi-step process beginning at the level of the HSC. Achieving immune tolerance in T1D will likely necessitate targeting multiple immune pathways with separate therapies.

Overall, my data in Chapter IV suggest that HSC mobilization is a required step in tolerance induction that fails in T1D (Figure 4.5). Improving HSC mobilization improves graft survival but there remain additional obstacles that must also be addressed to obtain durable transplantation tolerance. Nonetheless, further investigation of the hematopoietic progenitor response during T1D prevention and reversal trials may yield additional insight into fundamental aspects of the immune response that lead to beta cell loss.

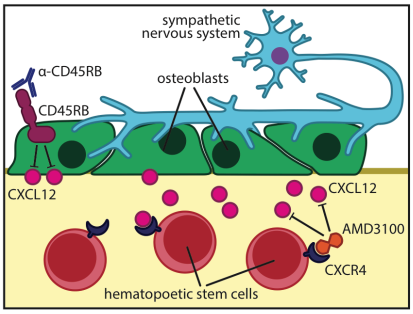
Chapter Findings	Model Figure	Future Work
- The tolerogenic therapy aCD45RB enhances Hematopoietic Stem Cell (HSC) mobilization in tolerance susceptible B6 mice but not in tolerance resistant NOD mice. - Partial HSC depletion impedes transplant tolerance induction in B6 allograft recipients. - aCD45RB targets osteoblasts to downregulate the HSC retaining cytokine CXCL12. - Chemical sympathectomy blocks aCD45RB mediated HSC mobilization and impedes transplant tolerance induction. - Targeting CXCR4 enhances HSC mobilization and extends islet allograft survival in NOD mice.		- Dissect which genetic determinants contribute to enhanced HSC retention in NOD mice. - Characterize the progression of inadequate SNS function in NOD mice over the course of disease development. - Determine whether augmenting HSC mobilization enhances tolerance induction in susceptible strains (e.g. G-CSF administration). - Assess whether the dysregulated IFNγ response in NOD mice contributes to inadequate HSC mobilization required of transplant tolerance.

Figure 4.5. Chapter IV – findings and future work.

CHAPTER V

LUPUS PRONE MICE RESIST IMMUNE REGULATION AND TRANSPLANT TOLERANCE INDUCTION

Scientific Goal

Inadequate regulation of T-B cell collaboration (Chapter II and III), as well as insufficient HSC mobilization (Chapter IV) pose a substantial barrier to transplant tolerance induction in autoimmune T1D. Whether other forms of autoimmunity characterized by similar dysregulations in T-B cell collaboration impede transplant tolerance induction remains to be determined, despite the fact that autoimmune disease represents a significant indication for clinical transplantation. In Chapter V, I explore the hypothesis that resistance to CD4 and CD8 T regulatory cell suppression in Systemic Lupus Erythematosus (SLE) poses a stringent barrier to transplant tolerance induction. Understanding the cellular and molecular aberrancies that contribute to loss of self-tolerance in alternative models of autoimmunity may reveal targetable mechanisms by which the autoimmune environment may be modulated to achieve transplant tolerance.

Introduction

Autoimmune Systemic Lupus Erythematosus (SLE), a disease that affects up to 150 in 100,000 individuals[248], is driven by unchecked collaboration between autoreactive T and B lymphocytes. These deleterious immune interactions manifest in the production of tissue-destructive autoantibodies and generalized inflammation, which can result in permanent damage to multiple organs including the kidney, skin, joints, vessels, lungs, heart, and nervous system.

Although it remains impossible to predict those patients who will progress to clinical SLE, there exists a strong genetic component to this disease. In general, no single genetic polymorphism alone accounts for clinical SLE manifestations. However, mutations in innate immune mediated signaling pathways (C1q, IFNα related signaling molecules IRF5, STAT4, IRAK1, TLR7), adaptive immune mediated signaling pathways (PTPN22, PD-1, LYN, BLK, FcγRII/II), as well as specific HLA haplotypes (HLA-DR2/3) place individuals at higher risk of disease[249]. Furthermore, a number of non-genetic factors (i.e. environmental cues) play a role in disease pathogenesis as there exists only a 14-57% disease concordance rate between monozygotic twins[250]. Associated triggers include UV exposure, sulfa drugs, certain tetracycline/penicillin derived antibiotics, infection, trauma, and pregnancy. There currently exists no cure for SLE. Physicians will prescribe regiments of immunosuppression including glucocorticoids, mycophenolate, cyclophosphamide, azathioprine, or cyclosporine during disease flares with the goal of preventing organ damage. The biologic agents Belimumab (anti-BLyS, which inhibits the B cell survival factor BAFF) and Rituximab (anti-CD20, which depletes B cells) are routinely used clinically. New agents including Tofacitinib (a JAK3/STAT5 signaling inhibitor, which inhibits T cell activation) and Toclizumab (anti-IL-6R, which blocks immunogenic IL-6 signaling) are currently in clinical trials. Despite the promising efficacy of these new agents however, a significant number of patients are now known routinely "escape" the benefits of these drugs[251], suggesting that these agents do not confer true immunologic tolerance.

If inadequately controlled pharmacologically, immune complex deposition and T cell activation can drive glomerular injury to cause Lupus Nephritis, which in 2004, accounted for approximately 1.1% of all cases of End Stage Renal Disease (ESRD) in the United States. Accordingly, of the 17,106 kidney transplants performed in 2014 in the US, 446 cases were due

to lupus-related ESRD based on Organ Procurement and Transplantation Network (OPTN) data as of April 1, 2015. Yet despite intense immunosuppression regimens, patients with SLE remain at risk for Recurrent Lupus Nephritis (RLN) in their kidney grafts, a scenario that places patients at a 4-fold higher risk of organ rejection[252]. In fact, 1 out of every 9 renal transplants in patients with SLE related ESRD were retransplants directly attributable to RLN (OPTN). Complementing these findings, nearly half of patients with T1D receiving islet transplants require a second islet reinfusion just one year after receiving their initial graft[165]. Overall, achieving graft tolerance in the highly immunogenic autoimmune environment requires overcoming both recurrent autoimmunity against the grafted tissue as well as alloimmunity propagated by foreign tissue antigens.

Despite a significant body of work defining tolerance induction in mice and additional evidence that autoimmunity poses a substantial barrier to allograft tolerance[253], tolerance induction in the setting of lupus is poorly understood. It remains unanswered how underlying autoimmunity contributes to graft rejection and to what degree these barriers can be generalized. In contrast to the NOD mouse, there exist many models of murine lupus in which one could explore the transplant response. Characteristic of the outbred NZBxNZW (BWF1), MRL-Fas^{lpr}, BXSB.Yaa, NZM2328, and NZM2410 strains is a propensity to develop fatal lupus nephritis[254]. As these strains age they develop high titers of autoantibodies directed at nucleic acid derivatives. Subsequent renal immune complex deposition ensues leading to glomerulonephritis. Similar to human SLE, murine lupus is a multifactorial disease in which a number of genetic risk alleles act in concert to precipitate loss of T and B cell mediated self-tolerance.

Accordingly, numerous groups have explored whether inadequate CD4 Treg function permits disease pathogenesis in these murine models of SLE[255]. In the context of BWF1 lupus,

neonatal CD4 Treg depletion via the depleting agent PC61 results in rapid development of nephritis with concomitant increases in IL-6 and IFNγ production[256]. Alternatively, transfer of *ex vivo* IL-2/TGFβ expanded CD25+CD62L^{HI} CD4 Tregs attenuates disease when administered after the earliest signs of proteinuria[257]. Thus, from these studies it can be inferred that SLE mice possess a potentially functional pool of protective CD4 Tregs that may later fail to adequately prevent disease pathogenesis.

Building on these observations, Wilhelm and colleagues recently demonstrated that CD4 Tregs from lupus-prone NZM2410 congenic B6.SLE123 mice were able to suppress the proliferation of syngeneic, non-autoimmune B6 effector T cells[258]. Interestingly however, effector T cells from these lupus-prone mice failed to be suppressed by CD4 Tregs from either lupus-prone or non-autoimmune syngeneic mice. In other words, disease progression in these lupus-prone B6.SLE123 mice may be characterized by an evolving effector T cell resistance to CD4 Treg mediated suppression. Complementing these findings, the Mathis laboratory demonstrated that effector T cells from NOD mice also resist Treg mediated suppression[259], thereby suggesting a shared immunologic phenotype across different forms of autoimmunity. From a clinical standpoint, Venigalla and colleagues observed that CD4+CD25- effector T cells from patients with active SLE were less sensitive to autologous CD4 Treg mediated suppression (CD4+CD25+CD127-) as compared to the patients with inactive SLE[260]. Furthermore, patients with SLE undergoing Rituximab mediated B cell depletion demonstrate increases in CD4 Treg frequencies[261], thereby highlighting the deleterious role that other types of effector cells play in restraining CD4 Tregs in this disease (an observation complementing my hypothesis explored in Chapter II). Overall, determining whether SLE is in part perpetuated by effector cell resistance to

CD4 Treg mediated suppression and/or intrinsic CD4 Treg dysfunction remains an active area of investigation.

In addition to the protective role of CD4 Tregs, CD8 Tregs are absolutely essential for preventing lupus-like pathology in murine models. Pioneering work by the Harvey Cantor laboratory demonstrated that genetic elimination of the CD8 Treg restriction element Qa-1 in otherwise non-autoimmune prone B6 mice results in high dsDNA autoantibody titers and renal immune-complex deposition[123]. Mechanistically, these CD8 Tregs eliminate Qa-1 expressing T Follicular Helper (TFH) Cells via perforin. If unchecked, unrestrained CD4 TFH cells can lead to an uncontrolled Germinal Center B cell response characteristic of autoantibody production. Murine CD8 Tregs express CD44, CD122, and the NK-receptor Ly49, depend on the expression of the master transcription factor Helios, and require STAT5 signal transduction to elicit their suppressive function[119,124]. From a clinical standpoint, a number of reports have documented various numerical and functional CD8 Treg deficiencies in patients with SLE[262]. However, as there exists no canonical phenotype of CD8 Tregs in the human setting, the extent to which these cells contribute to clinical SLE remains to be defined.

In Chapter V, I examine immune regulation and tolerance induction in a fully penetrant model of lupus, the B6.SLE123 mouse (a model developed by Edward Wakeland, UTSW, which harbors the lupus-promoting NZM2410 congenic regions SLE1, SLE2, and SLE3 but is otherwise congenic to non-autoimmune B6 mice[263]). As non-autoimmune B6 mice are otherwise susceptible to transplant tolerance induction, choice of this model allowed me to explore the specific contribution of these lupus-promoting loci in the induction of transplant tolerance. Herein, I determined that B6.SLE123 mice, unlike wild-type B6 mice, resist anti-CD45RB mediated tolerance induction to foreign islet allografts, which I have studied to limit the

contribution of recurrent autoimmunity. Mechanistically, effector CD4 T and B cells from this strain resist suppression mediated by both CD4 T Regulatory Cells (CD4 Tregs) and CD8 T Regulatory Cells (CD8 Tregs). Overall, my findings demonstrate that resistance to immune regulation in the autoimmune lupus setting may pose a stringent barrier to transplant tolerance induction and provide a new model for investigation of this clinical challenge.

Lupus prone B6.SLE123 mice possess expanded splenic CD4 TFH and Germinal Center B cells, fluctuating CD4 Treg and CD8 Treg populations, and generate an exaggerated alloantibody response

Lupus-prone mouse strains harbor expanded and activated populations of effector CD4 T and B cells which promote the generation of autoantibodies and the generalized inflammation characteristic of human SLE. T follicular helper (TFH) cells, which promote the activation and expansion of germinal center (GC) B cells, may be prominent in active autoimmunity[196] and are required for SLE progression in Roquin$^{san/san}$ (sanroque) mice[264]. Thus, as expected, 5-month old B6.SLE123 mice harbored 7-times as many splenic PD-1^{HI}Bcl-6^{+} TFH cells and 13-times as many Fas^{+}IgM^{-} Germinal Center B Cells as compared to age-matched B6 controls (Figure 5.1A).

Both CD4 T Regulatory Cells (CD4 Tregs) and CD8 T Regulatory Cells (CD8 Tregs) attenuate the germinal center reaction before it results in tissue-destructive immunity[123,200]. I investigated whether lupus-prone B6.SLE123 possessed altered populations of these regulatory cells. Although B6.SLE123 mice possessed slightly increased percentages of CD4 Tregs and CD8 Tregs at 5-months (Figure 5.1A), immune regulation may be severely compromised in these lupus-prone mice as TFH cells outnumber CD4 Treg and CD8 Treg populations 0.7:1 and 3.3:1, respectively. In comparison, target TFH cells are in an approximately 0.09:1 and 0.3:1

ratios with CD4 Tregs and CD8 Tregs in the background non-autoimmune B6 strain ($p=0.04$ by Mann-Whitney test). I also note that CD4 Tregs in B6.SLE123 mice demonstrate decreased levels of CD25 expression (B6.SLE123: MFI of 249±5vs. B6: MFI of 401±9, $p<0.001$ by student's t test), a phenotype which has been observed in patients with SLE[265]. Finally, although aged B6.SLE123 mice possess significantly increased numbers of $CD25^{+}Foxp3^{+}$ CD4 Tregs as compared to their 7.5 month-old B6 counterparts (B6.SLE123: 9.9±0.73% vs. B6: 5.6±0.29% of CD4 T cells, $p<0.001$ by student's t test), the number of $CD122^{+}Ly49^{+}$ CD8 Tregs is decreased to nearly undetectable levels by 7.5 months of age (B6.SLE123: 0.56±0.24% vs. B6: 4.0±0.04% of CD8 T cells, $p<0.005$ by student's t test), a time at which these mice develop progressive glomerulonephritis (**Figure 5.1B**).

To determine whether the expanded and potentially unregulated TFH and GC B cells in lupus-prone B6.SLE123 mice resulted in enhanced alloimmunity, I immunized both B6 and B6.SLE123 strains with MHC-mismatched C3H splenocytes (H-2k). Fourteen days after immunization, lupus-prone mice generated significantly higher anti-C3H IgG2a, IgG2b, and IgG3 alloantibody titers than non-autoimmune mice (**Figure 5.1C**).

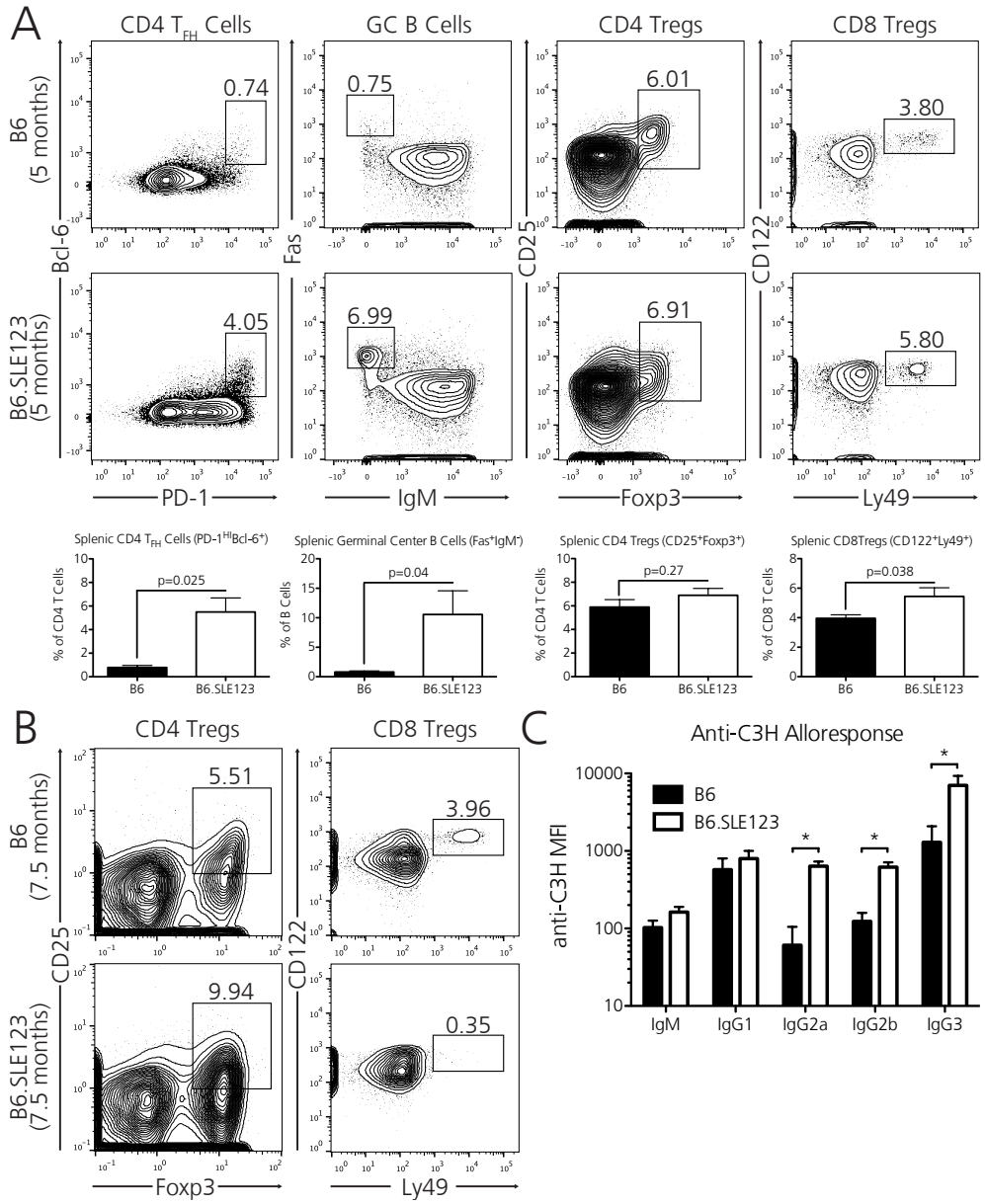


Figure 5.1. Lupus prone B6.SLE123 mice possess expanded splenic CD4 TFH and Germinal Center B cells, fluctuating CD4 Treg and CD8 Treg populations, and generate an exaggerated alloantibody response. A) 5-month old B6.SLE123 mice have expanded splenic populations of CD4 TFH cells (CD4^{+}PD-1^{HI}Bcl-6^{+}), Germinal Center B cells (B220^{+}Fas^{+}IgM^{-}), CD4 Tregs (CD4^{+}CD25^{+}Foxp3^{+}), and CD8 Tregs (CD8^{+}CD122^{+}Ly49^{+}), as observed in the contour plots. B) Compared to 7.5-month old B6 mice (top panels), similarly aged B6.SLE123 mice (bottom panels) possess an expanded population of splenic CD4 Tregs yet completely lack CD8 Tregs, the latter of which may permit unchecked CD4/B cell immunity characteristic of proliferative glomerulonephritis seen in lupus. C) B6.SLE123 mice (H-2b) generate an exaggerated IgG2a, IgG2b, and IgG3 alloantibody response 14 days after immunization with MHC mismatched splenocytes from C3H mice (H-2k). N=5 mice per strain. Significance was determined by student's t-test, *p<0.05.

Effector T Cells from Lupus prone mice resist CD4 Treg suppression of the IFNγ response

CD4 T effector cells from lupus prone B6.SLE123 mice resist CD4 Treg mediated suppression of the proliferative response[258]. As IFNγ produced by activated CD4 T helper cells directly augments IgG2b alloantibody production[266] (cf Figure 5.1C), I questioned whether CD4 T effector cells from this lupus-prone strain would similarly resist CD4 Treg based suppression of the IFNγ response. Naïve CD4 Tregs (CD4+CD25+) were sorted from B6 and B6.SLE123 mice by MACS and plated with MACS purified CD4 T effector cells (CD4+CD25-) from either B6 or B6.SLE123 mice, along with the stimulus anti-CD3 and anti-CD28 in a crossover platform. Although CD4 Tregs from B6.SLE123 mice suppressed IFNγ production by CD4 T effector cells from B6 mice to similar levels as the B6 syngeneic system, CD4 Tregs from B6 mice failed to suppress IFNγ production by CD4 T effector cells from B6.SLE123 mice (Figure 5.2A; data presented herein was generated in collaboration with Amy Major and Ashley Wilhelm, Vanderbilt University), indicating that B6.SLE123 CD4 Tregs were similarly functional despite the slight decrease in CD25 MFI reported above.

To elucidate why CD4 T effector cells from these lupus prone mice resisted CD4 Treg based suppression, I explored the response to TGFβ as this cytokine directly dampens the IFNγ response via a CD4 Treg dependent mechanism[267]. Whole splenocytes from B6.SLE123 mice activated in the presence of exogenous TGFβ showed resistance to suppression of IFNγ production as compared to control (Figure 5.2B). This was supported by reduced pSMAD-2/3 expression (Figure 5.2C) in B6.SLE123 CD4 T cells when exposed to TGFβ. pSMAD-1/5/8 expression in response to BMP-4 (Figure 5.2D) stimulation was intact in B6.SLE123 CD4 T cells indicating that the pSMAD defect was specific to the canonical TGFβ signaling pathway[268].

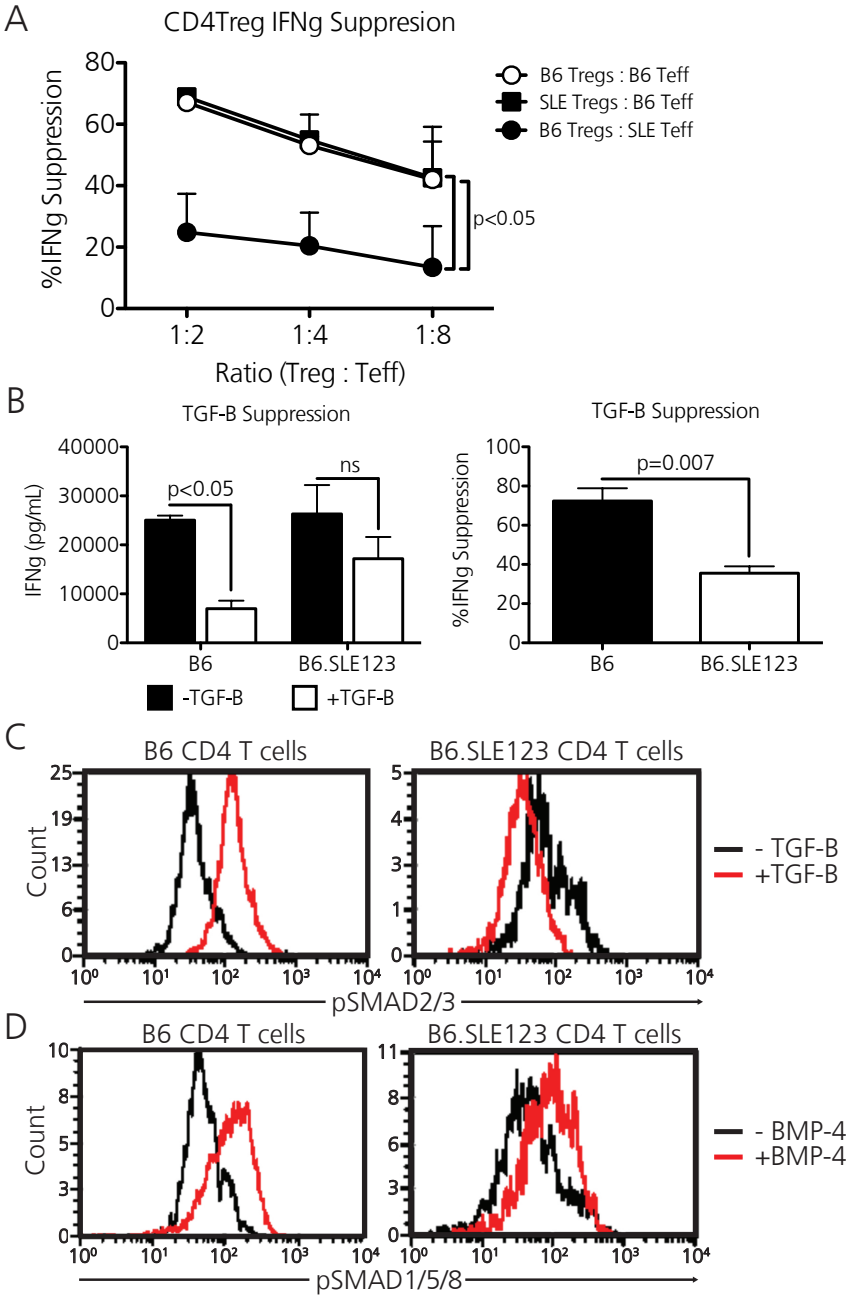


Figure 5.2. Effector CD4 T Cells from Lupus prone mice resist CD4 Treg mediated suppression of the IFNγ response. A) B6.SLE123 effector CD4 T cells resist B6 CD4 Treg mediated suppression of the IFNγ response. B) Plated B6.SLE123 splenocytes are resistant to TGFβ mediated suppression of the IFNγ response. C) B6.SLE123 $CD4^+$ T cells inappropriately decrease SMAD2/3 phosphorylation when exposed to TGFβ; however these cells appropriately phosphorylate SMAD1/5/8 in response to BMP-4 (D). N=3-5 mice per strain/condition. Significance was determined by two-way ANOVA followed by Sidak's multiple comparisons post-test, or by Student's *t*-test, where appropriate. (These data were generated in collaboration with Amy Major and Ashley Wilhelm, Vanderbilt University).

Effector CD4 T and B Cells from Lupus prone mice resist CD8 Treg mediated suppression

Extending my experimental crossover platform, I determined whether effector CD4 T and B cells from B6.SLE123 mice also resisted CD8 Treg mediated suppression[124]. Donor B6 and B6.SLE123 mice were immunized with KLH and CD8 Treg (CD8+CD122+Ly49+) and non-CD8 Treg (CD8+CD122+Ly49-) populations were purified via FACS. Immunodeficient B6.RAG mice were then injected with either of these CD8 Treg or non-CD8 Treg populations, along with MACS purified CD4+CD25- T and B220+ B cells from either antigen-naive B6 or B6.SLE123 mice (Figure 5.3A). Recipient mice were immunized and boosted with the original test stimulus NP-KLH and the high-affinity anti-NP antibody response was compared between groups by ELISA. Whereas CD8 Tregs from both B6 and B6.SLE123 mice suppressed the high-affinity antibody response when targeting B6 CD4 T and B cells, target B6.SLE123 CD4 T and B cells resisted suppression when transferred with either CD8 Tregs from either donor (Figure 5.3B). Thus, although B6.SLE123 CD8 Tregs are able to target effector cells from non-autoimmune mice, target effector cells from autoimmune lupus-prone mice resist CD8 Treg mediated suppression, which could promote an unchecked antibody response.

Lupus-prone mice lack ongoing islet immunity yet resist anti-CD45RB mediated tolerance induction to islet allografts

Anti-CD45RB induces permanent tolerance to foreign allografts via activation of antigen-specific, graft-protective regulatory cells[95,109,114]. After a 7-day course of anti-CD45RB, the absolute number of splenic Foxp3$^+$ CD4 Tregs expanded 1.74-fold in B6 mice and 1.76-fold in B6.SLE123 mice and the percent of proliferating Ki67+ CD8 Tregs increased 2.28-fold in B6 mice and 1.48-fold in B6.SLE123 mice although the absolute number of CD8Tegs were

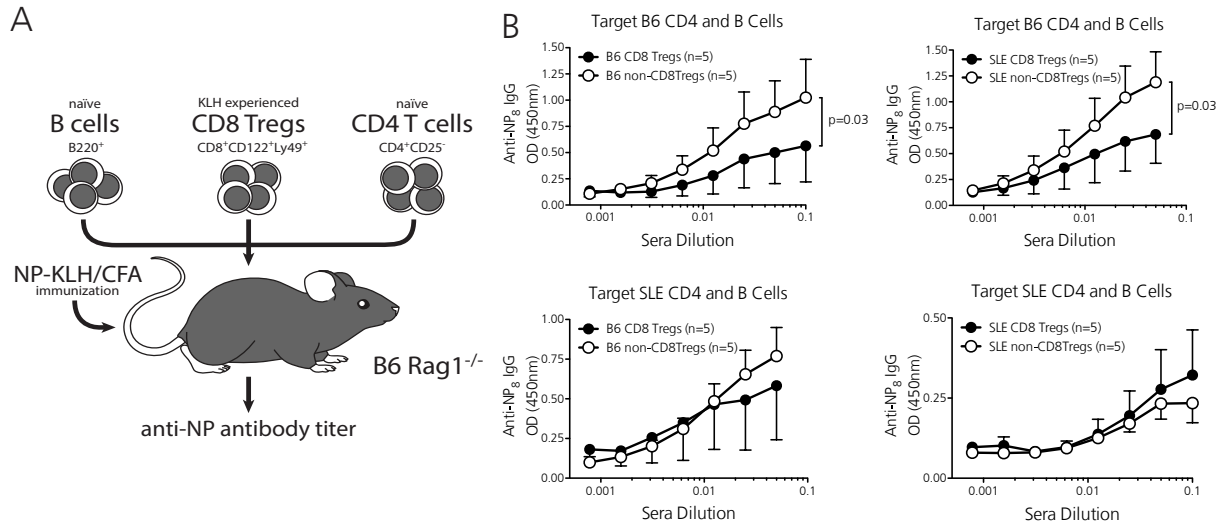


Figure 5.3. Effector CD4 T and B Cells from Lupus prone mice resist CD8 Treg mediated suppression. A) *In vivo* CD8 Treg suppression assay in which KLH-activated CD8 Tregs and naïve $CD4^+CD25^-$ T Cells / whole $B220^+$ B Cells are transferred to immunodeficient B6.RAG recipients that are immunized and boosted with the test stimulus NP-KLH. A similar crossover platform was used as described in Figure 2A. B) CD8 Tregs from B6 and B6.SLE123 mice potently suppress the high-affinity anti-NP_8 IgG response when targeting non-autoimmune CD4/B cells (upper panels). Effector CD4/B cells from lupus-prone B6.SLE123 mice resist suppression when targeted by CD8 Tregs from either B6 or B6.SLE123 mice (lower panels). N=5 mice per experimental group. Significance determined by performing a semi-logarithmic linear regressional analysis followed by y-intercept and slope curve comparison.

unchanged in both. Statistically, there were no significant differences in anti-CD45RB mediated Treg expansion between treated B6 and B6.SLE123 mice. Despite similar Treg expansion in B6.SLE123 mice, I hypothesized that these lupus-prone mice would resist anti-CD45RB mediated tolerance induction to foreign allografts as their regulatory cells would not control anti-graft effector CD4 T and B cells. I performed islet allograft transplantation after determining that B6.SLE123 mice lacked pre-formed anti-insulin IgG that would expose the graft to ongoing, islet-specific immunity (Figure 5.4A). As a positive control, Non-Obese Diabetic (NOD) mice, which resist anti-CD45RB mediated tolerance induction to islet allografts, possessed circulating anti-insulin IgG. A 7-day course of anti-CD45RB induced long-term tolerance to MHC-mismatched C3H islet allografts (H-2k) when transplanted into chemically diabetic non-autoimmune B6 mice (Figure 5.4B, Median Survival Time (MST) >100d vs. MST 13d in untreated controls). However, when lupus-prone B6.SLE123 mice were transplanted with C3H islets and administered anti-CD45RB, all recipients rejected their islet allografts by 40d (MST 21d) (Figure 5.4B). Thus, even in the absence of underlying organ-specific immunity, the autoimmune setting of lupus is highly resistant to transplant tolerance induction.

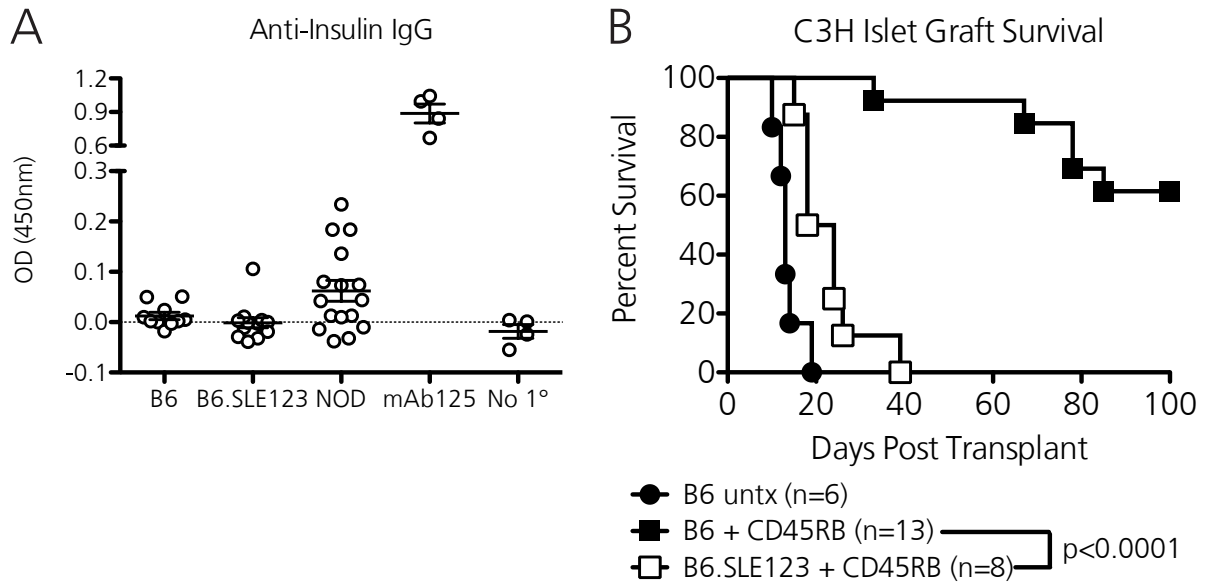


Figure 5.4. Lupus prone mice lack ongoing islet immunity yet resist anti-CD45RB mediated tolerance induction to islet allografts. A) Unlike Type 1 Diabetes (T1D) prone NOD mice (n=14) that develop islet-specific immunity, B6 (n=10) and B6.SLE123 (n=12) mice lack circulating anti-insulin IgG (serum prepared from 8-week old mice). Supernatant from the hybridoma mAb125 (anti-insulin IgG producing) was used a positive control. "No 1°" represents plates coated without serum (negative control). B) Despite lacking islet-specific autoimmunity, lupus-prone B6.SLE123 mice are completely resistant to anti-CD45RB mediated tolerance induction to C3H islet allografts (open squares, n=8, MST 21d). Anti-CD45RB therapy induces long-term tolerance in non-autoimmune B6 mice (closed squares, n=13, MST>100d) whereas untreated B6 mice rapidly reject C3H islet allografts (closed circles, n=6, MST 13d). Significance was determined by the Log-Rank test.

Discussion

SLE is a complex immunologic disease that results in permanent, multi-organ dysfunction. Lupus Nephritis represents a significant cause of ESRD in the US, culminating in approximately 500 renal transplants and 50 re-transplants each year (OPTN). Thus, attenuating autoantibody production and lymphocyte activation before kidney damage results remains a critical barrier to preventing lupus-related ESRD. In Chapter V, I demonstrate that lupus-prone B6.SLE123 mice, despite having no islet-specific immunity, are completely resistant to anti-CD45RB mediated tolerance induction to islet allografts. As this therapy activates graft-protective regulatory cells, I relate this strain's resistance to my observation that effector cells from these mice are highly resistant to both CD4 Treg and CD8 Treg mediated suppression. As future studies move towards linking this resistance to graft rejection directly, it will be vital to understand how Tregs of both the CD4 and CD8 class interact with each other to regulate the immune response to transplant antigens.

In the non-autoimmune setting, the Germinal Center reaction is tightly checked by regulatory T lymphocytes (Tregs)[200]. However, there exists conflicting evidence on the suppressive capacity of CD4 Tregs in the murine setting of lupus. Whereas CD4 Tregs from lupus-prone (NZBxNZW)F1 mice demonstrated similar suppressive capacity as CD4 Tregs from non-autoimmune Balb/c mice, CD4 Tregs from another lupus-prone strain, MRL/MpJ-Fas(lpr/lpr)J mice, demonstrated reduced suppressor function when compared to CD4 Tregs from non-autoimmune CBA/J mice[257,269]. However, these experimental platforms utilized non-congenic strains as controls. To more accurately define the suppressive capacity of lupus-derived CD4 Tregs, I utilized a B6 congenic system, the B6.SLE123 mouse, in which three lupus-promoting genetic regions from the lupus-prone strain NZW2410 have been introduced: SLE1,

which permits the loss of nuclear antigen tolerance; SLE2, which lowers B cell activation threshold; and SLE3, which enhances T cell activation[263]. Using a crossover platform, I demonstrate that CD4 Tregs from B6.SLE123 mice possess similar suppressive capacity as CD4 Tregs from B6 mice when targeting effector CD4 T cells from non-autoimmune B6 mice. I further determined that target CD4 T effector cells from B6.SLE123 mice resist suppression by B6 CD4 Tregs, a finding which complements previous studies demonstrating a similar effector cell resistance to regulation observed in NOD mice.

Importantly, not all models of lupus possess effector cell resistance. Lupus-prone BAFF-Tg mice, which overexpress the cytokine BAFF similar to some SLE patients, possess expanded CD4 Tregs and regulation-sensitive T effector cells. When transplanted with islets, these mice are partially permissive to long-term allograft tolerance[137], further suggesting that effector cell resistance in the B6.SLE123 model contributes to failed tolerance. The opportunity for genetic dissection in the SLE123 system should permit isolation of the cellular mechanism by which effector cells resist regulation in future studies. In particular, recent work by Wong et. al. demonstrates that B6 congenic mice harboring only the SLE1b sublocus possess expanded populations of TFH/GC B cells that generate autoantibodies[270], which may relate to my finding of excess alloantibody production. As chronic alloantibody represents a growing cause of late-stage organ rejection, further dissection of the contribution of each SLE region may provide new opportunities to control this barrier to long-term transplant success[271].

Whereas CD8 Treg control of the germinal center response in lupus is less well defined, CD8 Tregs from the lupus-prone B6.SB-Yaa/J mouse strain did not suppress the high-affinity antibody response when targeting non-autoimmune B6 target CD4/B cells[124]. Although my evidence demonstrates that CD8 Tregs from B6.SLE123 mice maintain suppressive capacity

when targeting non-autoimmune B6 target cells, disease progression in these strains are driven by different genetic factors. Notably, duplication of TLR7 in B6.SB-Yaa/J mice may promote the generation of $TLR7^{HI}$ dendritic cells that favor expansion of non-CD8 Treg memory cells (CD8 Tmem - CD122+Ly49-) thereby overriding the generation of protective CD8 Tregs (CD122+Ly49+)[124]. In comparison, lupus in the SLE123 system is driven by genetic material from the NZW2410 strain that permits over-activation of CD4/B effector cells that resist CD8 Treg mediated suppression. Despite these conflicting observations, defective CD8 Treg suppression due to either CD8 Treg intrinsic dysfunction or extrinsic resistance may permit the unchecked germinal center reaction characteristic of lupus and T1D (cf Figure 3.1C).

In addition to the above barriers, patients with SLE possess circulating autoantibodies directed against the heterodimeric CD94/NKG2 complex[272]. This complex, which is expressed on the surface of NK cells, interacts with the non-classical MHC class Ib molecule HLA-E on target cells. A number of these autoantibodies are known to enhance CD94/NKG2-HLA-E interactions between NK cells and their targets. CD8 Tregs also bind HLA-E, or its murine analog Qa-1, on target TFH cells[123]; therefore, these autoantibodies may favor deleterious NK-HLA-E interactions and thereby impede protective CD8 Treg-HLA-E interactions.

One important caveat of these studies is that islet allografts were placed in the kidneys of lupus-prone B6.SLE123 recipients, an organ that is prone to immune mediated tissue damage due to immune complex deposition and subsequent glomerulonephritis. Thus, it could be speculated that the failed tolerance induction observed in this strain was simply due to heightened, non-specific immunity at the site of transplantation rather than a specific failure of this strain's immune network to be rendered tolerant to allografted tissue. To obviate this potentially confounding factor, all transplant recipients were aged 8-12 weeks, a window that

precedes the earliest signs of renal immune complex deposition and glomerulonephritis by 3 months. To further confirm this strain's generalized resistance to transplant tolerance induction, however, future studies could infuse islet allografts into the recipient's liver via portal vein injection[273]. As the liver is generally not subject to lupus pathology, this site could further confirm my described findings.

In conclusion, lupus-prone B6.SLE123 mice resist CD4 Treg and CD8 Treg based immune regulation, which may account for my observation that these mice have expanded germinal center resident TFH and GC B cells that contribute to an exaggerated alloresponse. Although B6.SLE123 mice lack ongoing islet autoimmunity, these mice are completely resistant to tolerance induction to foreign islet allografts (Figure 5.5). Efforts to promote tolerance to renal allografts in lupus should focus on eliminating or reprogramming anti-graft effector lymphocytes that resist T cell mediated regulation. Introduction of the B6 congenic B6.SLE123 lupus-prone mouse offers a new model in which resistance to immune regulation and transplant tolerance induction can be mapped to specific cell types and/or genetic loci. In sum, I provide a new platform in which researchers can mechanistically dissect the transplant response in SLE.

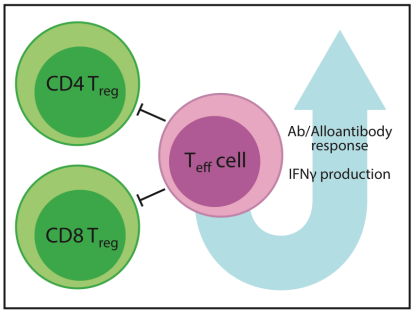
Chapter Findings	Model Figure	Future Work
- Lupus prone B6.SLE123 mice possess expanded CD4 TFH and GC B cells, as well as fluctuating CD4 Treg and CD8 Treg populations. - B6.SLE123 mice mount an exaggerated alloantibody response. - B6.SLE123 CD4 T effector cells resist CD4 Treg mediated suppression of the IFNγ response. - B6.SLE123 B/CD4 T effector cells resist CD8 Treg mediated suppression of the antibody response. - B6.SLE123 mice lack ongoing islet immunity yet resist transplant tolerance induction to islet allografts.		- Assess whether B6.SLE123 mice possess autoantibodies that block Qa-1 mediated CD8 Treg suppressive function and/or enhance Qa-1 mediated NK target cell interactions. - Determine whether heightened, non-specific autoimmunity at the site of allograft placement represents a barrier to transplant tolerance. - Determine whether effector T cell resistance to Treg mediated suppression directly contributes to the observed resistance to transplant tolerance in lupus-prone B6.SLE123 mice

Figure 5.5. Chapter V – findings and future work.

CHAPTER VI

AN ENHANCED IL-6 RESPONSE AND HEIGHTENED IMMUNOMETABOLISM CONTRIBUTE TO TOLERANCE RESISTANCE IN LUPUS-PRONE MICE

Scientific Goal

As demonstrated in Chapter V, effector T cells from lupus-prone B6.SLE123 mice resist CD4 and CD8 Treg mediated immune regulation and transplant tolerance induction to foreign islet allografts despite possessing no underlying islet-specific autoimmunity. Accordingly, determining the degree to which each NZM2410 derived congenic region renders these mice resistant to transplant tolerance may reveal specific genetic regions that control susceptibility to immune tolerance. In fact, many of these genetic regions overlap with known *Idd* risk loci in NOD mice. Thus, findings from these studies may reveal specific aberrancies in T and B collaboration that lead to transplant tolerance resistance across numerous autoimmune conditions. In Chapter VI, I explore the hypothesis that the NZM2410 derived SLE1, SLE2, and SLE3 regions each impart tolerance resistance via unique immunologic disturbances. Via such genetic distillation, I explore the roles of an exuberant IL-6 response and heightened glycolytic function as barriers to transplant tolerance in the setting of murine lupus.

Introduction

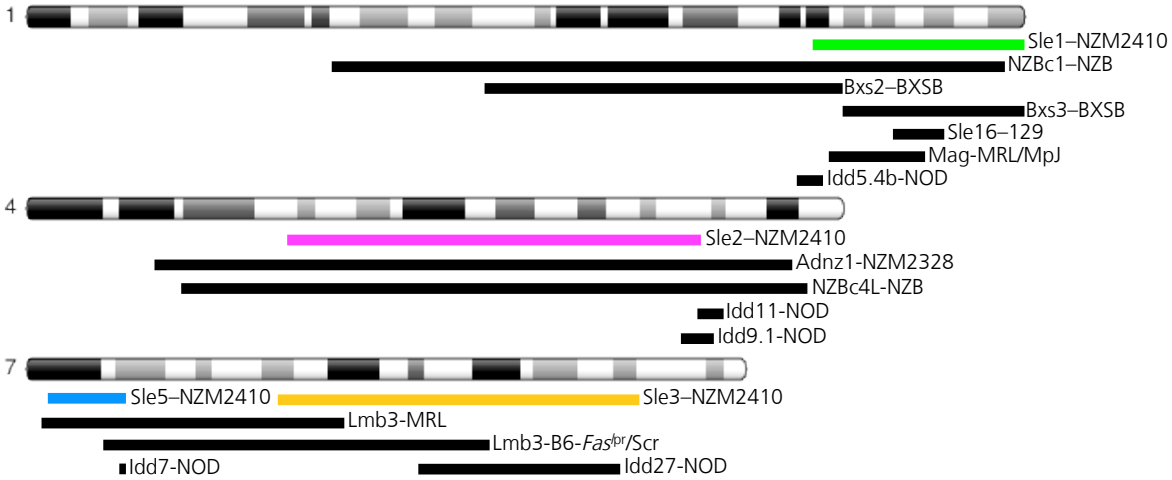
In an attempt to identify the minimal genetic regions required for SLE disease progression, Edward Wakeland demonstrated that just three small genetic regions derived from the lupus-prone NZM2410 strain could transfer fulminant disease to otherwise non-autoimmune

B6 mice[263]. NZM4120 derived regions included the SLE1 risk locus from Chromosome 1 (154-197 Mb), the SLE2 risk locus from Chromosome 4 (50-129 Mb), and the SLE3 risk locus from Chromosome 7 (50.7-124 Mb). One hundred percent of these B6 congenic mice, termed B6.SLE123 mice, succumb to fatal glomerulonephritis. Immunologically, B6.SLE123 mice develop high titers of anti-chromatin and dsDNA autoantibodies whose presence leads to renal immune complex deposition and subsequent renal damage. Interestingly however, single congenic B6.SLE1, B6.SLE2, and B6.SLE3 mice do not develop fulminant disease. Extensive immunophenotyping of these single congenic strains has since revealed that SLE1 mediates loss of nuclear antigen tolerance, SLE2 lowers B cell activation thresholds, and SLE3 lowers T cell activation thresholds. Further dissection of each of these congenic regions by creating subcongenic mice (e.g. B6.SLE1a [Chr1: 170-173 Mb], B6.SLE1b [Chr1: 173-174 Mb], B6.SLE1c [Chr1: 190-197 Mb], and B6.SLE1d [Chr1: 175-188 Mb]) has additionally pinpointed the genes directly leading to specific T and B cell aberrancies. Known immunologic phenotypes conferred by each of these congenic and subcongenic regions are reviewed in Table 3[270,274-294].

Overall, determining how each of the NZM2410 derived congenic regions contributes to the failed transplant tolerance induction observed in B6.SLE123 may reveal the minimal loci that cause this disruptive effect. These studies in the B6.SLE mice will be highly significant in that many genetic loci for SLE are shared with NOD mice, which similarly resist transplant tolerance induction. Moreover, many of these genetic risk regions are syntenic to human disease loci (Figure 6.1) and thus offer potential avenues to improve clinical transplantation outcomes in patients with autoimmunity. Not only will these studies reveal targetable immunologic T and B cell abnormalities that could restore transplant tolerance in B6.SLE123 and NOD mice, but also

Congenic Strain	Subcongenic Strains	Chr	Congenic Interval	Immunologic and Cellular Phenotype	References
B6.SLE1		1	154-197 Mb	anti-H2A/B DNA + anti-Histone IgG CD4Teff resistance to CD4Treg suppression ↑ CD69+/CD40L+ CD4 cell number ↓CD4Treg number with ↓Foxp3 expression ↑DC derived IL-6 ↑CD4 proliferation ↑CD4 IL-2, Il-4, and IFNg production Failed FcgRIIb upregulation in GC B cells ↑Ly-6A/E lymphocyte expression Autoimmune phenotype dependent on Estrogen Receptor alpha	Mohan et al. (1998)[274] Rahman et al. (2005)[275] Kumar et al. (2006)[276] Wan et al. (2007)[277] Yoachim et al. (2015)[278]
	B6.SLE1a.1 B6.SLE1a.2	1	170 Mb 171-173 Mb	Same phenotype as B6.SLE1 mice ↑CD4 ICOS expression from SLE1a.1 locus ↑CD4 expression of PBX-1d splice isoform -> ↓T cell apoptosis	Chen et al. (2005)[279] Cuda et al. (2007)[280] Cuda et al. (2010)[281] Cuda et al. (2012)[282]
	B6.SLE1b	1	173-174 Mb	Restored CD4 Treg and Teff # and function to B6 mice Varied SLAM/CD2 structure and expression in T/B cells Ly108.1 allele impairs B cell anergy, receptor editing, deletion Increased spontaneous GC formation and Tfh cell number	Chen et al. (2005)[279] Wandstrat et al. (2004)[283] Kumar et al. (2006)[276] Wong et al. (2012)[270]
	B6.SLE1c.1 B6.SLE1c.2	1	194-197 Mb 190-193 Mb	Same phenotype as B6.SLE1 mice + Novel CR2 glycosylation-> altered C3d binding altered GC formation and ↓humoral response B6.SLE1c.2: ↓Esrrg expression in T cells -> ↓mitochondrial mass/function and altered metabolic pathway utilization	Chen et al. (2005)[279] Boackle et al. (2001)[284] Chen et al. (2005)[279] Perry et al. (2012)[285]
	B6.SLE1d	1	175-188 Mb	Contribution to lupus pathology currently unknown	Morel et al. (2001)[286]
B6.SLE2		4	50-129 Mb	↓mature B Cells in Bone Marrow early expansion of splenic CD23lo B cells ↑polyclonal/polyreactive IgM ↑B1a cells with ↑CD80/86, CD24, ICAM-1	Mohan et al. (1997)[287] Mohan et al. (1998)[274]
	B6.SLE2a	4	50-80 Mb	strongest contribution to ↑IgM^{lo}CD5+ B1a cell #	Xu et al. (2005)[288]
	B6.SLE2b	4	91-100 Mb	↓IFNa production	Li et al. (2005)[289]
	B6.SLE2c	4	104-117 Mb	strongest contribution to total ↑B1a cell # decreased lymphocyte response to G-CSF	Xu et al. (2005)[288] Lantow et al. (2013)[290]
B6.SLE3.1		7	50.7-124 Mb	↑CD4:CD8 cell ratio ↑activated CD69+CD4 cells ↑I-A2 and CD44 B cell expression ↑T-dependent antibody response ↓CD4 cell proliferation with ↓apoptosis mild penetrant glomerulonephritis When combined with SLE1 -> severe glomerulonephritis IRAK1 dependent dendritic cell hyperactivity Failed renal ↑of Kallikrein during anti-GBM ab ligation	Mohan et al. (1999)[291] Liu et al. (2007)[292] Jacob et al. (2009)[293] Liu et al. (2009)[294]
	B6.SLE3.2	7	53.5-124 Mb	Same as B6.SLE3.1 mice + When combined with SLE1 -> severe glomerulonephritis	Liu et al. (2007)[292]
B6.SLE5		7	4-20 Mb	No observed autoimmune phenotype When combined with SLE1 -> severe glomerulonephritis	Liu et al. (2007)[292]

Table 3. Immunological phenotypes of NZM2410 congenic and subcongenic B6 mice.



Murine Lupus Susceptibility Region	Homologous Human Region Linked to Patients with SLE	Candidate Genes in Regions Shared by Mice and Humans
Sle1	Chr1q21-23	FcgRII, FasL, CD3Z chain, H3 Histone, Serum Amyloid Protein, C-Reactive Protein, CD48
Sle1	Chr1q31-32	Complement-component-4-binding protein, CR2, CD45
Sle1	Chr1q41-42	TGFB2, ADPRT (ADP-ribosyltransferase factor-1)
Sle2	Chr9p22	IFNa
Sle3	Chr11p15	Trim21

Figure 6.1. Genetic overlap of risk alleles shared between tolerance resistant B6.SLE123 and NOD mice. Each of the NZM2410 derived congenic regions overlap with known insulin dependent diabetes risk loci in NOD mice (*Idd* intervals) and also overlap related regions in other autoimmune strains. The mouse gene regions are syntenic to some identified human risk regions supporting future clinical impact of key findings.

provide a new platform from which the regions that control immune tolerance can be genetically distilled.

In Chapter VI, I first determine the extent to which each NZM2410 derived congenic region renders B6.SLE123 mice resistant to transplant tolerance induction. Upon determining that introduction of the SLE1 locus alone renders otherwise susceptible B6 mice fully resistant to transplant tolerance induction, I go on to explore the hypothesis that enhanced IL-6 production and signal transduction in B6.SLE123 mice represents a targetable barrier to transplant tolerance in these NZM2410 congenic lupus-prone strains. Although antibody blockade of the IL-6 Receptor during anti-CD45RB mediated tolerance induction restored some level of tolerance in single congenic B6.SLE1 mice, blockade of this receptor as well as its downstream signaling pathway failed to extend allograft survival in triple congenic B6.SLE123 mice. Thus, extending findings that blocking enhanced CD4 T cell metabolism in B6.SLE123 mice prevents lupus pathology[295], I demonstrate that overcoming dysregulated CD4 T cell metabolism in B6.SLE123 via glycolytic blockade mice significantly extends islet allograft survival in these otherwise tolerance resistant mice. Through use of genetically distilled models of murine lupus, my studies in Chapter VI define specific immunologic barriers that impede transplant tolerance in the setting of autoimmunity and set the stage for future investigation.

Single congenic lupus-prone mice resist transplant tolerance induction to varying degrees

As demonstrated in Chapter V, lupus-prone B6.SLE123 mice are fully resistant to anti-CD45RB mediated transplant tolerance induction to foreign islet allografts despite possessing no underlying islet autoimmunity. However, the extent to which each NZM2410 derived congenic region contributes such transplant tolerance resistance has remained undefined. Accordingly,

single congenic B6.SLE1, B6.SLE2, and B6.SLE3 recipient mice were rendered chemically diabetic, transplanted with C3H islet allografts, and administered a standard 7-day course of the tolerance inducing agent anti-CD45RB (Figure 6.2). B6.SLE1 mice completely resisted transplant tolerance induction and rejected islet allografts with similar kinetics as their triple congenic B6.SLE123 counterparts (green trace). Although B6.SLE3 recipients demonstrated a MST similar to B6.SLE123 and B6.SLE1 mice, 2 of these recipients achieved long-term transplant tolerance (purple trace). The NZM2410 derived SLE2 region conferred the least level of tolerance resistance in that these mice demonstrated a MST of 61d (orange trace). Therefore, the individual perturbations of immunity conferred by each congenic region are sufficient to disrupt transplantation tolerance but to different degrees. Accordingly, I next sought to determine whether a known immunologic aberrancy perpetuated by the SLE1 locus (as shown in Table 3) contributes to the failed tolerance induction observed in B6.SLE123 mice.

Temporary blockade of enhanced IL-6 signaling partially restores transplant tolerance in B6.SLE1 mice but fails to extend allograft survival in B6.SLE123 mice

In elucidating a specific immunologic aberrancy that perpetuates dysregulated T-B cell collaboration in B6.SLE123 mice, work by Wan and colleagues recently demonstrated that heightened levels of IL-6 in B6.SLE1 mice renders this strain's effector T cells resistant to CD4 Treg mediated suppression[277]. Specifically, dendritic cells from B6.SLE1 mice produce higher levels of IL-6 during immunogenic challenge, which when blocked by anti-IL-6 antibodies, restored CD4 Treg suppression of effector T cells. Thus, having determined that introduction of the SLE1 locus alone renders otherwise tolerance susceptible B6 mice fully resistant to

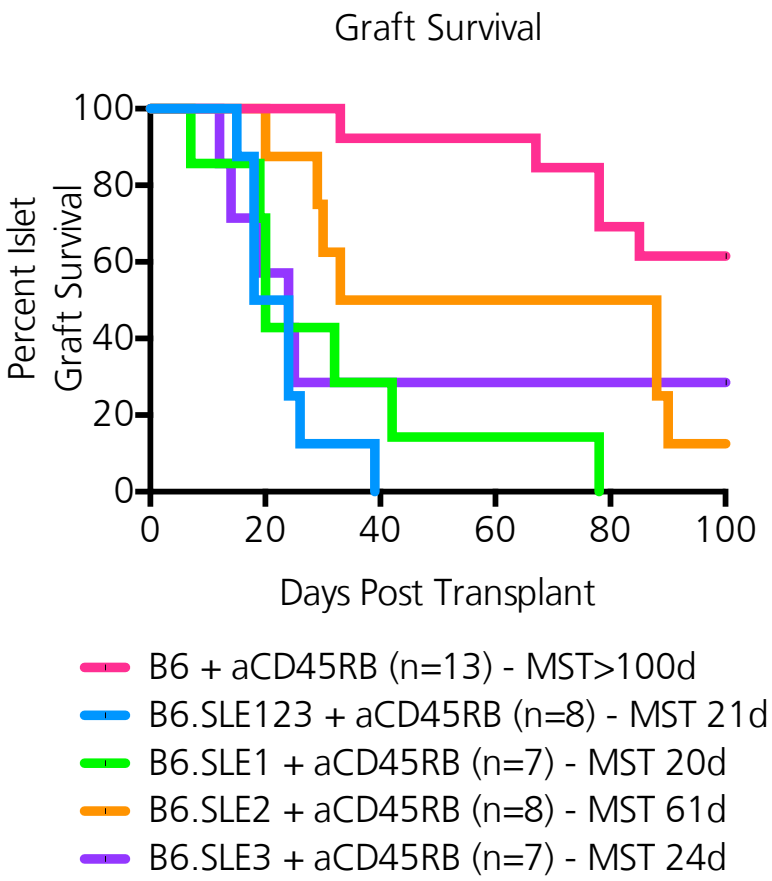


Figure 6.2. Single congenic lupus prone mice resist transplant tolerance induction to varying degrees. To determine to what extent each NZM2410 derived congenic region contributed to the complete transplant tolerance resistance observed in triple congenic B6.SLE123 mice, single congenic B6.SLE1, B6.SLE2, and B6.SLE3 mice were transplanted with C3H islet allografts and administered a standard 7-day course of anti-CD45RB. Overall, B6.SLE1 mice completely resisted transplant tolerance induction and rejected islet allografts with similar kinetics as their triple congenic B6.SLE123 counterparts (green trace). Although B6.SLE3 recipients demonstrated a MST similar to B6.SLE123 and B6.SLE1 mice, 2 of these recipients achieved long-term transplant tolerance (purple trace). Overall, the NZM2410 derived SLE2 region conferred the least level of tolerance resistance in that these mice demonstrated a MST of 61d (orange trace). Reliance on allograft tissue for maintenance of euglycemia in recipients achieving >100d of tolerance was confirmed by unilateral nephrectomy of kidneys containing graft tissue. All nephrectomized mice returned to hyperglycemia a day after surgery confirming allograft tolerance.

transplant tolerance induction, I first explored the hypothesis that an enhanced IL-6 response in part contributes to the failed transplant tolerance induction in B6.SLE123 mice. Splenocytes from B6 and B6.SLE123 mice were mixed with Mitomycin-C treated C3H splenocytes and IL-6 production was measured via ELISA 5 days later. As compared to B6 splenocytes, splenocytes from B6.SLE123 mice generated nearly 3-fold higher levels of IL-6 during the alloresponse - a response that did not differ significantly from B6.SLE123 cells undergoing maximal CD3/CD28 stimulation (Figure 6.3A). Thus, I next determined whether T cells from B6.SLE123 responded differently to IL-6 stimulation, as this cytokine is known to drive pathogenic Th17 and TFH cell differentiation as well as inactivate CD4 Treg suppressive function[110]. Splenocytes from B6 and B6.SLE123 mice were exposed to increasing concentrations of IL-6 for 15 minutes and the relative phosphorylation of STAT3 was compared between CD4 and CD8 T cells between each strain. Overall, both CD4 and CD8 T cells from B6.SLE123 mice demonstrated enhanced phosphorylation of STAT3 during maximal stimulation (Figure 6.3B).

From these observations, I hypothesized that blocking the IL-6 axis during anti-CD45RB mediated tolerance induction could restore tolerance in otherwise resistant B6.SLE1 and B6.SLE123 mice. Specifically, interrupting this axis would attenuate the differentiation of anti-graft CD4 T effector cells while similarly restoring adequate CD4 Treg mediated suppression of these same anti-graft effector T cells. Interestingly, administration of an anti-IL-6 Receptor-α antibody on days -3, -1, 1, and 3 relative to the day of transplantation and initiation of anti-CD45RB therapy significantly extended islet allograft survival in B6.SLE1 mice, in which 2/7 mice achieved long-term tolerance (Figure 6.3C, left panel). However, similar IL-6Rα blockade failed to extend islet allograft survival in triple congenic B6.SLE123 mice undergoing anti-CD45RB therapy (Figure 6.3C, right panel). Accordingly, I next sought to determine whether

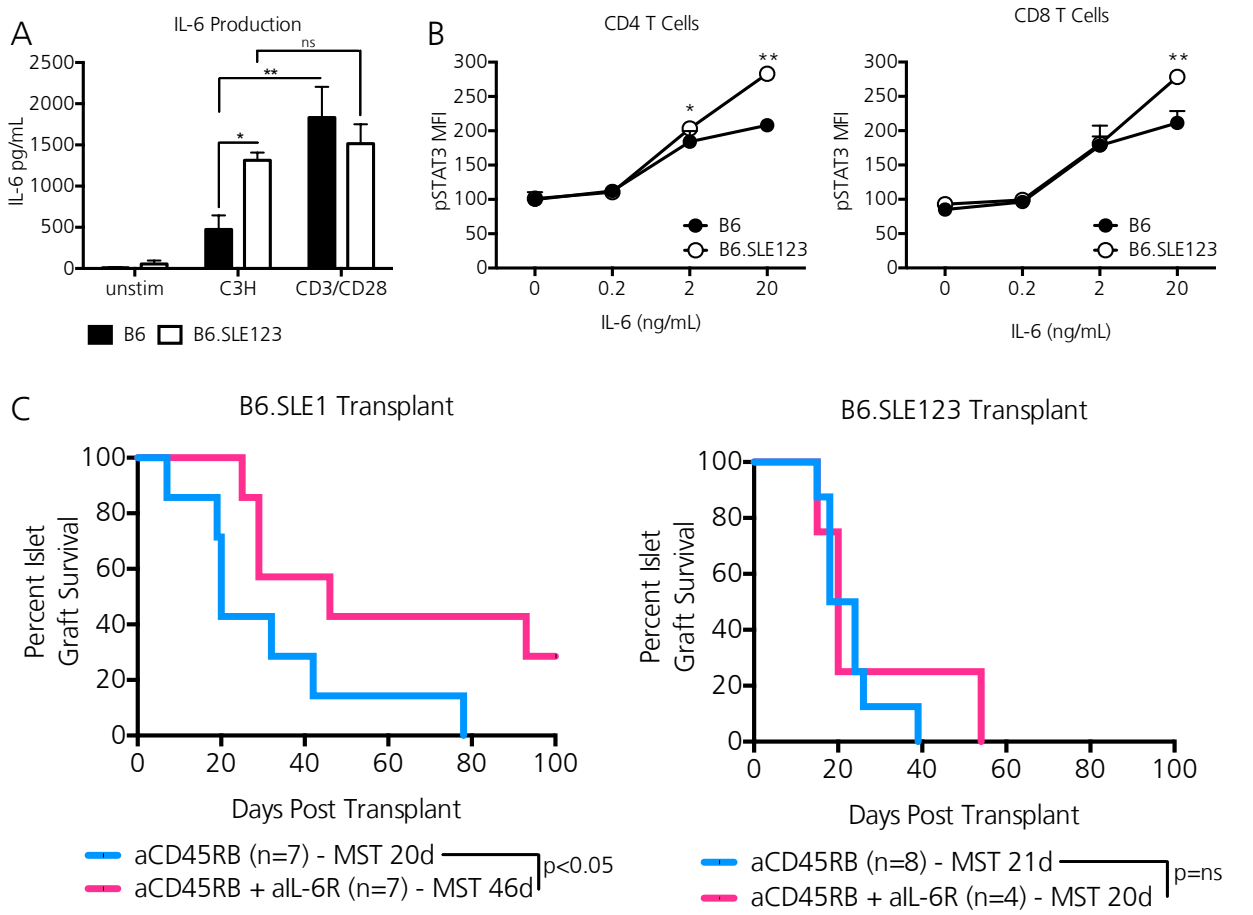


Figure 6.3. Temporary blockade of enhanced IL-6 signaling partially restores transplant tolerance in B6.SLE1 mice but fails to extend allograft survival in B6.SLE123 mice. A) To determine whether the heightened IL-6 response characteristics of B6.SLE1 mice extended to the alloresponse, splenocytes from B6 and B6.SLE123 mice were incubated *ex vivo* with Mitomycin-C treated C3H splenocytes. Overall, B6.SLE123 splenocytes produced nearly 3-fold more IL-6 when exposed to allogeneic target cells. B) To determine whether B6.SLE123 mice responded differently to IL-6, splenocytes from both strains were exposed to increasing concentrations of recombinant IL-6 after which the relative phosphorylation of STAT3 (which is directly down stream of the IL-6 Receptor) was compared between strains. Both CD4 and CD8 T cells from B6.SLE123 mice demonstrated significantly enhanced phosphorylation of STAT3 at the highest concentration of IL-6 (20ng/mL). C) Finally, to determine whether blocking the action of IL-6 during anti-CD45RB therapy could render lupus prone mice susceptible to transplant tolerance induction, B6.SLE1 and B6.SLE123 mice were administered 500µg of an anti-IL-6R blocking antibody on days -3, -1, 1, and 3 relative to the day of transplantation and initiation of anti-CD45RB treatment (day 0). Whereas IL-6R blockade significantly enhanced allograft survival in B6.SLE1 mice with 2/7 recipients achieving permanent tolerance (left panel), addition of this immunologic agent did not alter islet allograft rejection kinetics in B6.SLE123 mice (right panel). (*p<0.05, **p<0.001 by Two-way ANOVA followed by Sidak's multiple comparisons post test; survival differences calculated by Log-rank test.)

IL-6Rα blockade alone was sufficient to block IL-6 mediated signaling in B6.SLE123 CD4 and CD8 T cells.

Enhanced IL-6 signaling blockade in B6.SLE123 mice fails to extend allograft survival

IL-6 is a unique proinflammatory cytokine in that it signals via two distinct mechanisms[296]. Via the classical signaling paradigm, IL-6 first binds the membrane-bound, non-signaling IL-Rα subunit (CD126), which then recruits and heterodimerizes with the gp130 signaling subunit to initiate JAK1/2-STAT3 phosphorylation. In addition to its membrane bound form, a splice-variant of IL-6Rα results in the generation of a secreted and soluble IL-6Rα molecule (sIL-6Rα). When bound to free IL-6, the sIL-6Rα/IL-6 complex can initiative gp130 trans-signaling independent of membrane-bound IL-6Rα, further perpetuating its effects. As CD4 T cells from patients with SLE express increased levels of gp130[297], I hypothesized that enhanced IL-6 trans-signaling in B6.SLE1234 CD4 T cells may represent an additional barrier that may need to be further overcome via downstream signaling blockade. Specifically, the anti-IL-6Rα blocking antibody utilized may be unable to bind and subsequently block soluble sIL-6Rα/IL-6 signal transduction.

Prior to IL-6 stimulation, B6 and B6.SLE123 splenocytes were exposed to the anti-IL-6Rα blocking antibody, the JAK1/3 inhibitor Tofacitinib, the combination of both agents, or left untreated as a control. Cells were then exposed to increasing concentrations of recombinant IL-6 for 15 minutes and relative levels of STAT3 phosphorylation were assessed within CD4 and CD8 T cells. In both stains, each agent individually attenuated STAT3 phosphorylation when cells were exposed to 2ng/mL of IL-6. However at the highest concentration of IL-6 (20ng/mL),

only the combination of both agents was able to significantly attenuate STAT3 phosphorylation in CD4 and CD8 T cells in both strains of mice (Figure 6.4A).

Accordingly, I hypothesized that enhanced IL-6 signaling blockade via the addition of Tofacitinib[298] to the anti-IL-6Rα + anti-CD45RB regiment may render fully resistant B6.SLE123 mice susceptible to transplant tolerance induction. Recipient B6.SLE123 mice received a subcutaneous pump loaded with Tofacitinib (delivery rate of 30mg/kg/day placed during d -4 to 14) and anti-IL6Rα injections (500μg on d -3, -1, 1, 3) relative to the day of transplantation (day 0) and initiation of a standard 7-day course of anti-CD45RB. Overall, addition of both agents failed to extend islet allograft survival over mice receiving anti-CD45RB alone (Figure 6.4B).

Glycolytic blockade via Metformin and 2-DG during anti-CD45RB mediated transplant tolerance induction significantly extends islet allograft survival in B6.SLE123 mice

The above studies demonstrate that intense blockade of proximal IL-6 signaling during the window of transplantation fails to restore transplant tolerance in B6.SLE123 mice. Accordingly, I next sought to determine whether inhibiting a downstream effect of IL-6 could render these mice susceptible to transplant tolerance induction. In addition to the canonical effects of IL-6 mediated STAT3 signal transduction (which includes upregulating genes that control survival [Bcl-XL, Bcl-2], proliferation [cMyc, Cyclin B/D1, Cdc2], cell adhesion [ICAM-1], and cytokine production [IL-6, IL-11, IL-17, IL-17])[296], emerging evidence has demonstrated that STAT3 also increases cellular glycolysis via activation of HIF-1α[299]. Mechanistically, HIF-1α pushes cells to increase glucose uptake (via upregulation of Glut1) and undergo the Warburg effect (aerobic glycolysis) in which cells primarily utilize glycolysis in lieu of oxidative phosphorylation for ATP production. In fact, CD4 T cells from B6.SLE1c mice

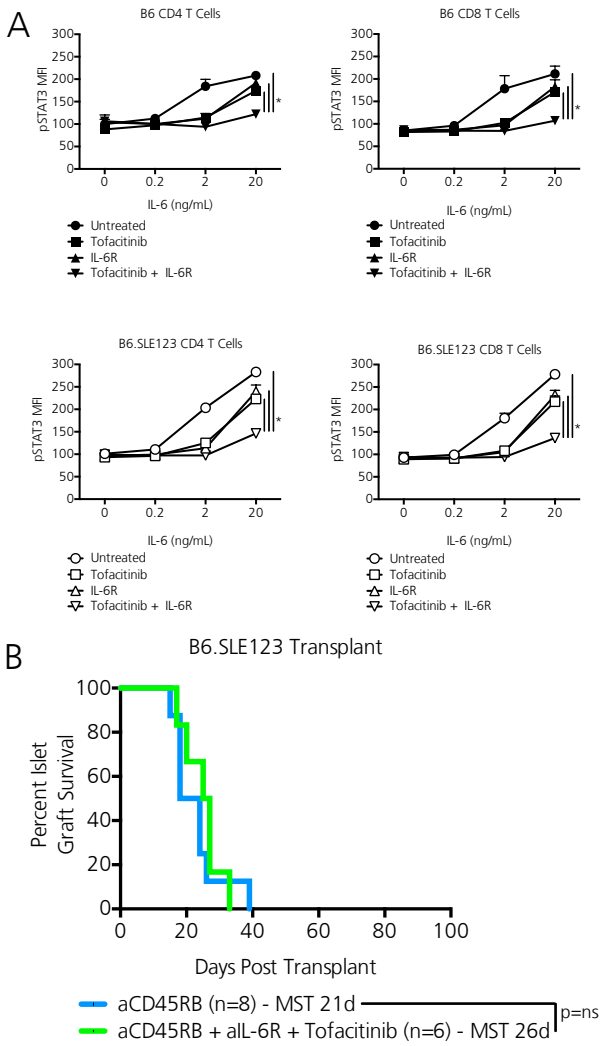


Figure 6.4. Enhanced IL-6 signaling blockade in B6.SLE123 mice fails to extend allograft survival. To determine why IL-6R blockade failed to extend islet allograft survival in triple congenic B6.SLE123 mice, the extent to which anti-IL-6R blocked IL-6 signal transduction, as well as alternative means to block IL-6 signaling were explored. A) Splenocytes from B6 and B6.SLE123 mice were left untreated, or pre-incubated with 10μg/mL of anti-IL6R, 1μM of Tofacitinib, or both agents. Splenocytes were then exposed to increasing concentrations of recombinant IL-6 after which the relative phosphorylation of STAT3 was compared between strains. During stimulation with 2ng/mL of IL-6, anti-IL6R, Tofacitinib, and the combination of both agents successfully inhibited phosphorylation of STAT3. However at the highest dose of IL-6 (20ng/mL), only the combination of both agents significantly attenuated STAT3 phosphorylation. B) To determine whether extended IL-6 blockade could render B6.SLE123 mice susceptible to anti-CD45RB mediated transplant tolerance induction, recipient mice received a subcutaneous pump loaded with Tofacitinib (delivery rate of 30mg/kg/day placed during d -4 to 14) and anti-IL6R injections (500μg on d -3, -1, 1, 3) relative to transplantation and anti-CD45RB (d0). Overall, the addition of both agents failed to extend islet allograft survival over mice receiving anti-CD45RB alone. (*p<0.001, by Two-way ANOVA followed by Sidak's multiple comparisons post test; survival differences calculated by Log-rank test.)

demonstrate increased HIF-1α expression as compared to CD4 T cells WT B6 mice[285]. Furthermore, as work by Yin and colleagues recently demonstrated that CD4 T cells from disease-age B6.SLE123 mice demonstrate enhanced cellular glycolysis, a metabolic pathway which promotes the generation of pathogenic T effector cells[295], I next hypothesized that enhanced CD4 T cell glycolysis in B6.SLE123 mice may in part contribute to this strain's resistance to transplant tolerance. Accordingly, I next explored whether the tolerance inducing agent anti-CD45RB alters CD4 T cell glucose uptake in such a way to attenuate the generation of effector T cells that precipitate graft rejection.

Twelve week old B6 and B6.SLE123 (pre-disease) mice were left untreated or received a standard 7-day course of anti-CD45RB. On day 8, splenocytes were harvested and then exposed to the fluorescent glucose analogue 2-NBDG. In the absence of any treatment, approximately 50% of CD4 T cells from both strains demonstrated significant uptake of 2-NBDG; thus in the pre-disease state, I found that CD4 T cells from B6.SLE123 did not possess any enhancements in glucose uptake that may favor glycolytic pathways. Interestingly however, whereas anti-CD45RB treatment significantly decreased B6 CD4 T cell uptake of 2-NBDG by 60%, CD4 T cells from B6.SLE123 mice resisted the effects of this agent in that glycolytic uptake was only reduced by 20% (Figure 6.5A).

As the anti-metabolic agents 2-Deoxy-D-Glucose (2DG) and Metformin can reverse lupus pathology in B6.SLE123 mice and prevent skin and cardiac allograft rejection in non-autoimmune B6 mice[300], I questioned whether the addition of these drugs during anti-CD45RB therapy could render otherwise resistant B6.SLE123 mice susceptible to transplant tolerance induction. Mechanistically, I hypothesized that the addition of these agents would further enhance anti-CD45RB mediated suppression of the CD4 T cell glycolytic response, thereby

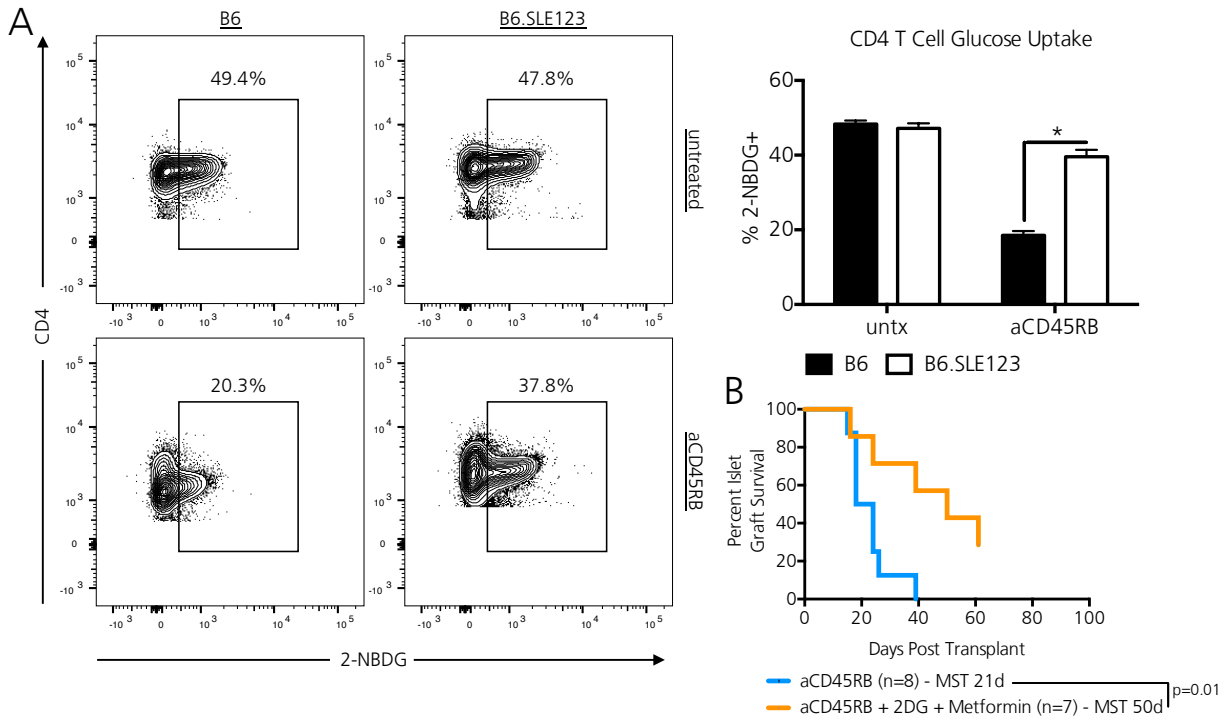


Figure 6.5. Glycolytic blockade via Metformin and 2-Deoxy-D-Glucose during anti-CD45RB transplant tolerance induction significantly extends islet allograft survival in B6.SLE123 mice. As enhanced cellular metabolism is characteristic of activated CD4 T cells in B6.SLE123 mice, I explored whether anti-CD45RB alters CD4 T cell metabolism in such a way that would permit transplant tolerance via attenuation of CD4 T cell activation. A) B6 and B6.SLE123 mice were left untreated or administered a 7-day course of anti-CD45RB. On day 8, splenocytes were then exposed the fluorescent glucose analogue 2-NBDG. Relative glucose uptake in CD4 T cells was analyzed. Whereas anti-CD45RB significantly reduced CD4 T cell glucose uptake in tolerance susceptible B6 mice, this therapy failed to decreased glucose uptake in CD4 T cells from B6.SLE123 mice to the same extent. B) To determine whether glycolytic blockade could restore transplant tolerance susceptibility in otherwise resistant B6.SLE123 mice, recipient mice were administered the anti-glycolytic agents 2-Deoxy-D-Glucose (5mg/mL) and Metformin (3mg/mL) in their drinking water on days -7 to 14 relative to the day of transplant and initiation of anti-CD45RB therapy (day 0). Strikingly, addition of these agents significantly delayed islet allograft rejection 2.5 fold. (*$p<0.001$, by Two-way ANOVA followed by Sidak's multiple comparisons post test; survival differences calculated by Log-rank test.)

preventing the generation of anti-graft effector T cells. Accordingly, 12-week old B6.SLE123 mice were administered 2DG (5mg/mL) and Metformin (3mg/mL) in their drinking water on days -7 to 14 relative to the day of C3H islet transplantation and initiation of anti-CD45RB therapy (day 0). Overall, addition of these agents to the anti-CD45RB treatment regiment extended allograft survival 2.5-fold over B6.SLE123 mice receiving anti-CD45RB alone (Figure 6.B). Although these studies are ongoing, 2/7 recipients have yet to reject their islet allografts 65 days after transplantation.

Discussion

The preliminary data presented in Chapter VI suggest that even a small contribution of autoimmune genes (which alone do not precipitate detectable autoimmune pathology) may be sufficient to prevent transplant tolerance. Using genetically distilled models of murine lupus, I first determined that the NZM2410 derived congenic regions SLE1, SLE2, and SLE3 each preclude anti-CD45RB mediated transplant tolerance induction to varying degrees. Overall, I found that introduction of the SLE1 congenic region alone rendered otherwise susceptible B6 mice fully resistant to transplant tolerance induction. As the SLE1 locus is characterized by an enhanced IL-6 response that renders effector T cells resistant to Treg mediated suppression[277], I subsequently determined transplant tolerance resistant B6.SLE123 mice 1) generate enhanced IL-6 levels during allochallenge and 2) are hyper-reactive to IL-6 mediated signal transduction.

Accordingly, elucidating whether an exuberant IL-6 response renders these lupus-prone mice resistant to transplant tolerance induction is highly warranted in that a number of groups have highlighted the necessary but not sufficient role IL-6 plays in breaking transplant tolerance. Using a model of cardiac transplantation in which exogenous administration of the TLR-9

agonist CpG reliably breaks anti-CD40L mediated allograft tolerance via inactivation of CD4 Tregs, Chen and colleagues determined that both IL-6 and IL-17 were required for CpG's tolerance breaking effects[301]. Extending these findings, Wang and colleagues further demonstrated that injecting IL-6 and IFNβ plasmids into recipient mice receiving cardiac allografts could similarly break tolerance mediated by anti-CD40L[302]. IL-6's allograft tolerance breaking effects are in part attributable to Jagged2 signaling[303], a Notch signaling family member which when activated, increases IL-6 production and thereby renders effector T cells resistant to CD4 Treg mediated suppression. Extending these findings, I hypothesized that a heightened IL-6 response driven by NZM2410 derived congenic region SLE1 may contribute to my observations that B6.SLE123 mice resist transplant tolerance and CD4 Treg mediated suppression. Although IL-6Rα blockade partially restored anti-CD45RB mediated transplant tolerance in single congenic B6.SLE1 mice, this strategy failed to translate to the triple congenic B6.SLE123 setting. It may therefore be speculated that transient IL-6 blockade represents a modifiable barrier in the B6.SLE1 setting; however, additional barriers must be overcome to restore transplant tolerance susceptibility in the fully penetrant B6.SLE123 setting. It is important to note that these studies are limited by the fact that IL-6 blockade was restricted to the window of transplantation; thus, it remains to be addressed whether aberrant IL-6 signaling prior to transplantation establishes an immune milieu that is resistant to transplant tolerance induction. To more directly address this question, future studies could include backcrossing B6.IL-6$^{-/-}$ or B6.IL-6Rα$^{-/-}$ mice with B6.SLE1 and B6.SLE123 mice prior to transplantation.

Exploring how IL-6 impedes tolerance in the setting of autoimmune lupus and transplantation represents a unique opportunity in that the biologic agent Toclizumab (anti-IL-6Rα) is currently undergoing phase I/II clinical trials in patients with SLE and those undergoing

renal transplantation. Overall, this agent has demonstrated modest efficacy in suppressing lupus related anti-dsDNA titers[304] and has shown preliminary success in decreasing Donor-Specific Alloantibody levels[305]. Although the mechanism by which Toclizumab attenuates disease pathology in these clinical settings has yet to be elucidated, numerous groups have previously shown that IL-6 renders effector T cells resistant to Treg mediated suppression[277]. Furthermore, recent evidence indicates that IL-6 alters T cell metabolism in such a way to promote long-term T cell activation. This past year, Yang and colleagues found that IL-6/IL-6R ligation drives downstream phosphorylation of STAT3, which enhances mitochondrial membrane polarity (MMP) in CD4 T cells[306]. Specifically, IL-6 enhances mitochondrial Ca^{2+} levels that ultimately drive long-term IL-4 and IL-21 production in CD4 T cells. As these cytokines are characteristic of CD4 TFH cells that drive the GC B cell response[196], the rapid and sustained disease improvements in RA patients undergoing Toclizumab therapy may in part be explained by attenuation of IL-6 signaling that would otherwise perpetuate the Rheumatoid Factor TFH/GC B cell response. Complementing these findings, IL-6 can directly prevent the generation of induced Foxp3+ CD4 iTregs in the NOD setting[307], further demonstrating the deleterious role of this pathogenic cytokine across different autoimmune conditions. Overall, determining the mechanisms by which IL-6 perpetuates aberrant T-B cell collaboration and resistance to Treg mediated suppression may reveal new means to target and restore immunologic tolerance in SLE, T1D, and transplantation.

In exploring alternative means to restore transplant tolerance susceptibility in the lupus-prone B6.SLE123 setting, I next hypothesized that blocking potential downstream effects of aberrant IL-6 mediated T cell activation may render these mice susceptible to transplant tolerance induction. Specifically, I explored the role of heightened cellular metabolism as a

potential barrier to transplant tolerance induction in that IL-6 is known to enhance cellular glycolysis via activation of HIF-1α[299], a molecule that is significantly upregulated in CD4 T cells from B6.SLE1c mice[285]. As metabolic blockade using the agent 2DG and Metformin can reverse lupus pathology[295] and prevent skin and cardiac allograft rejection[300], I subsequently determined that administration of these metabolic agents during anti-CD45RB mediated tolerance induction extended allograft survival 2.5-fold over B6.SLE123 mice receiving anti-CD45RB alone. At this point however, I have yet to determine how metabolic blockade renders autoimmune lupus-prone mice partially permissive to anti-CD45RB mediated transplant tolerance induction. However, prior to determining that metabolic blockade via 2DG and Metformin significantly extends allograft survival, Lee and colleagues first demonstrated that 2DG and Metformin specifically dampen T cell glycolysis, oxidative phosphorylation, proliferation, and IFNγ production, while concomitantly enhancing CD4 Treg numbers[300]. Accordingly, I plan to assess how T effector and T regulatory cells respond to metabolic intervention in such a way that favors sustained transplant tolerance. Using both *in vivo* and *ex vivo* MLR platforms, I am currently exploring how the metabolic agents 2DG and Metformin alter the alloreactive T cell response and/or pathogenic cytokine production (i.e. IL-6).

Beyond the context of transplantation, researchers have in part defined the metabolic pathways that control T effector cell and Treg function. Work by the Rathmell laboratory recently demonstrated that TH1 and Th17 effector cells rely primarily on glycolytic metabolism whereas their T regulatory cell counterparts rely primarily on oxidative phosphorylation[308]. Extending these observations, a landmark study published by Shrestha and colleagues this past year found that Tregs require the AKT inhibitory protein Phosphatase and Tensin Homolog PTEN to restrain deleterious TH1 and TFH function[309]. Specific ablation of PTEN in Tregs

resulted in enhanced AKT and mTORC2 activity leading to loss of Treg stability and suppressive function. Accordingly, determining the metabolic pathways and signaling networks that control the fate of alloreactive T cells during the transplant response may reveal novel mechanisms that could be targeted to restore transplant tolerance susceptibility in otherwise resistant B6.SLE123 mice. In exploring the role of CD4 T cell glycolytic function in the establishment of transplant tolerance induction, I have recently obtained transgenic mice that selectively overexpress or are functionally deficient of the glucose transporter Glut-1 in CD4 T cells[310] (courtesy of Jeffrey Rathmell, Vanderbilt University). These studies will define a specific role for CD4 T cell glycolytic function in establishing and maintaining transplant tolerance in that these recipients are otherwise free from any underlying autoimmunity. In exploring the role of the AKT-mTORC2 signaling axis in transplant tolerance, our lab recently accrued preliminary data demonstrating that anti-CD45RB downregulates AKT signaling in developing thymic CD4 Tregs of tolerance susceptible B6 mice. Therefore, anti-CD45RB may promote the generation of centrally derived, graft-protective CD4 Tregs via enhancement of the AKT-mTORC2 pathway; future exploration of this signaling pathway is highly warranted in that permanent transplant tolerance instilled by anti-CD45RB requires the generation of these thymically-derived CD4 Tregs[114]. Overall however, it has yet be to defined whether the AKT-mTORC2 axis is 1) required for anti-CD45RB's tolerance inducing effects, and/or 2) dysregulated in such a manner in the B6.SLE123 environment that renders these mice resistant to transplant tolerance induction. However, as CD4 T cells from disease-aged B6.SLE123 mice demonstrated increased phospho-S6 levels[295] (a target directly downstream of mTORC2), disruptions in the CD4 T cell AKT-mTORC2 axis may in part contribute to this strain's resistance to transplant tolerance induction.

In Chapter VI, I introduce a genetically distilled model of transplant tolerance resistance

in that introduction of the NZM2410 derived SLE1, SLE2, and SLE3 congenic loci individually render otherwise susceptible, non-autoimmune B6 mice resistant to transplant tolerance induction. Specifically, I begin to explore how an exuberant IL-6 response and aberrant cellular metabolism perpetuated by these autoimmune derived loci alter the immune network in such a way to resist the induction of transplant tolerance. Overall, identifying the specific genetic components that control immunologic tolerance is of immense importance in that these studies would provide for rationale approaches for the treatment of autoimmune disease and transplantation.

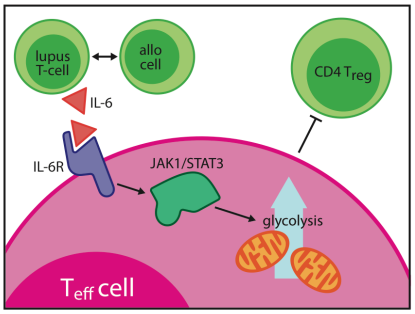
Chapter Findings	Model Figure	Future Work
- Single congenic lupus prone mice (B6.SLE1, B6.SLE2, and B6.SLE3) each resist transplant tolerance to varying degrees. - Temporary blockade of enhanced IL-6 signaling partially restores transplant tolerance in B6.SLE1 mice but fails to extend allograft survival in B6.SLE123 mice. - Glycolytic blockade via Metformin and 2-DG during aCD45RB induction therapy partially restores transplant tolerance in B6.SLE123 mice.		- Determine the requisite role of IL-6 as a barrier to transplant tolerance in lupus prone mice by crossing B6.IL-6$^{-/-}$ or B6.IL-6Ra$^{-/-}$ mice with B6.SLE1 and/or B6.SLE123 mice. - Explore the role of heightened IL-6 signaling in lupus prone mice as it relates to Treg inactivation and conversion to pathogenic effector T cells. - Assess the contribution of altered immune cell signaling in lupus prone mice as it relates to the transplant response and tolerance induction. - Dissect the role of enhanced immunometabolism as a barrier to transplant tolerance in autoimmunity using metabolic blockade and/or transgenic models that disrupt various metabolic pathways.

Figure 6.6. Chapter VI – findings and future work.

CHAPTER VII

DISCUSSION AND FUTURE DIRECTIONS

Overview

Organ transplantation represents a potential life-saving intervention for numerous forms of autoimmunity that result in end organ destruction. However, the autoreactive immune system poses a significant barrier to long-term graft tolerance in that an initial breach in the immunologic mechanisms that maintain self-tolerance may result in an inability to later "learn" how to tolerate foreign organ grafts when treated with tolerance-inducing therapy. To date, this scenario has been best modeled in the autoimmune T1D prone NOD mouse in which no tolerance inducing strategy has ever induced permanent allograft tolerance to any organ type in the intact NOD immune system, including those organs not subject to pre-existing autoimmune attack. In my thesis, I define cellular mechanisms that dysregulate T-B cell collaboration and lead to transplant tolerance resistance in autoimmune backgrounds. In Chapter VII, I discuss the potential implications of my findings, highlight new questions that arise from my data, and outline future directions for my research (Figure 7). Ultimately, identification of specific immunologic aberrancies in T-B cell collaboration will support rationale approaches to improve clinical transplantation outcomes in patients with autoimmunity.

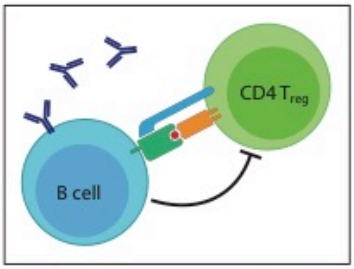
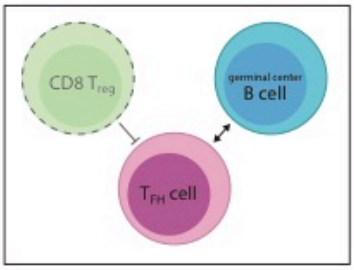
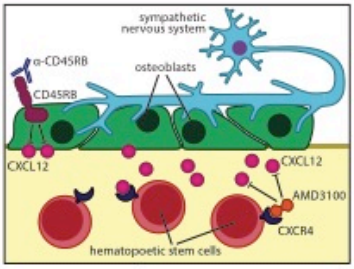
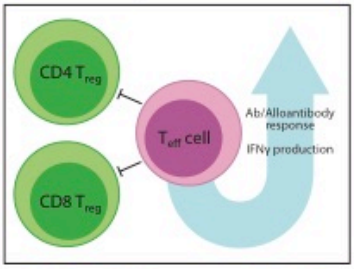
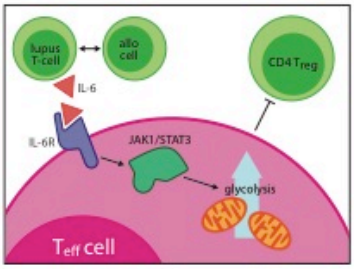
Findings from Thesis	Model Figure	Future Directions
Chapter II -B cell deficiency renders NOD mice permissive to transplant tolerance -NOD B cells restrict the generation of graft-protective CD4 Tregs		Chapter II -Evaluate the role of B cell antigen-specificity, antigen-presentation, and alloantibody production as a barrier to transplant tolerance in NOD mice
Chapter III -NOD mice possess nonfunctional CD8 Tregs that permit islet immunity -IL-15 is required for transplant tolerance		Chapter III -Define whether NOD CD8 Treg failure contributes to transplant tolerance resistance in this strain -Determine whether IL-15 dependent CD8 Tregs or NK cells are required for transplant tolerance
Chapter IV -SNS-dependent HSC mobilization is required for transplant tolerance -Overcoming enhanced CXCR4 mediated HSC retention in NOD mice extends allograft survival		Chapter IV -Determine whether the dysregulated IFNg response in NOD mice contributes to inadequate HSC mobilization required of transplant tolerance
Chapter V -Lupus-prone B6.SLE123 mice resist CD4 and CD8 Treg mediated suppression -Despite possessing no underlying islet autoimmunity, lupus-prone B6.SLE123 mice resist transplant tolerance to islet allografts		Chapter V -Determine whether effector T cell resistance to Treg mediated suppression directly contributes to the observed resistance to transplant tolerance in lupus-prone B6.SLE123 mice
Chapter VI -Each NZM2410-derived lupus-promoting region (SLE1, SLE2, SLE3) is sufficient to resist transplant tolerance -A heightened IL-6 response and enhanced immunometabolism contribute to transplant tolerance resistance in lupus-prone mice		Chapter VI -Further dissect the role of enhanced immunometabolism as a barrier to transplant tolerance in autoimmunity

Figure 7. Summary of findings and future directions.

The Janus-faced roles of B cells in transplant tolerance in autoimmunity

Although T cells represent the end-stage effectors of organ destruction in T1D and transplantation, circulating islet autoantibodies[175] and graft-reactive alloantibodies[311] remain the best clinical predictors of disease progression in their respective settings. These clinical observations likely indicate that loss of B cell tolerance is central to disease progression and may in fact, precede and directly perpetuate loss of T cell tolerance. In examining the role of B cells in T1D progression, it is well appreciated that a life-long absence of B cells in NOD mice protects this strain from developing diabetes[176]. Thus, I hypothesized that a generalized failure to maintain adequate B cell tolerance may contribute to this strain's resistance to transplant tolerance induction. In Chapter II, I demonstrate that the autoimmune B lymphocyte compartment represents a critical barrier to transplant tolerance induction in T1D-prone NOD mice (Figure 7 – Row 1). Specifically, I found that B lymphocyte deficient NODμMT mice are fully permissive to anti-CD45RB mediated, antigen-specific tolerance induction to islet allografts. Mechanistically, the genetic absence of B cells permitted the expansion of graft-protective CD4 Tregs that were essential for imbuing transplant tolerance.

Although my findings complement those made by Lee and colleagues who demonstrated that B cell deficiency in non-autoimmune B6 mice enhances transplant tolerance induction to islet allografts[84], the role of B lymphocytes in resisting or enhancing allograft tolerance is largely dependent on the allograft type in question. In the context of cardiac transplantation, B lymphocytes are instead required for establishing long-term transplant tolerance in non-autoimmune B6 mice[133]. In fact, B cells from these tolerant recipients can transfer cardiac allograft tolerance to otherwise untreated transplant recipients, suggesting the potential role of B Regulatory Cells (Bregs) in regulating anti-graft immunity. Overall, these seemingly

contradictory findings likely indicate that B cells play both deleterious and protective roles in the context of transplantation; thus, achieving durable transplant tolerance will require dampening the graft-destructive functions of these cells while concomitantly enhancing their graft-protective, regulatory functions and determining whether these functions are segregated to separate B lymphocyte subsets.

These data raise a number of questions yet to be explored. One, what role does the autoimmune B cell compartment in NOD mice play in resisting allograft tolerance to organs not subject to recurrent autoimmunity? It is well documented that autoimmune NOD mice resist transplant tolerance to skin and cardiac allografts despite possessing no detectable autoimmunity to these tissue types[143,144]. Two, do B cell sufficient NOD mice resist transplant tolerance due to failures of B cell mediated regulation of the anti-graft response? BCR stimulated, IL-10 producing B cells can prevent diabetes progression in NOD mice[312], suggesting that B cells from this strain may possess masked regulatory potential capable of suppressing anti-graft immunity although this concept has not been extended to transplantation in the NOD model. And three, what is the role of the autoimmune B cell compartment in other autoimmune diseases in resisting allograft tolerance? Although I determined in Chapter V (Figure 7 – Row 4) that lupus-prone B6.SLE123 mice resist anti-CD45RB mediated tolerance induction to islet allografts (despite possessing no underlying islet autoimmunity), it remains to be determined whether autoimmune B cells in this setting mediate their barrier to transplant tolerance.

In addressing the first question, future studies will assess whether B cell deficient NODμMT mice are susceptible to anti-CD45RB mediated tolerance induction to skin, cardiac, or renal allografts. These studies will directly address the role of the autoimmune B lymphocyte compartment in resisting transplant tolerance to allografts not subject to recurrent autoimmunity.

Although these grafts are not subject to recurrent autoimmunity in the NOD setting, these allografts may pose additional barriers to transplant tolerance as they are composed of greater numbers of alloantigen-dense tissue types capable of driving the indirect alloresponse. As NOD mice demonstrate enhanced B cell mediated indirect alloimmunity as compared to their non-autoimmune counterparts[150], these systems will address the role of alloantigen load as a barrier to transplant tolerance in the setting of autoimmunity. From a mechanistic standpoint, the role of autoimmune B cell antigen specificity, antigen presentation, and alloantibody production in the context of skin, cardiac, or renal transplantation could be further addressed using the NOD.HEL transgenic system, chimeric recipients depicted in Figure 2.9, or alloantibody-rich serum transfer system (as described in Chapter II). Addressing how the autoimmune environment resists transplant tolerance to organs subject to only alloimmunity is highly warranted in that a significant number of patients with T1D require renal allografting due to disease-related ESRD (OPTN), the pathogenesis of which is driven by microvascular damage in lieu of an autoimmune process.

Although it remains to be determined whether autoimmune NOD mice possess B cells capable of regulating the transplant response, high-throughput profiling of peripheral blood samples has revealed a transcriptional signature of B lymphocyte gene expression that is strongly associated with immunosuppression-free clinical operational tolerance[97] (however, a direct mechanistic role for Bregs in maintaining clinical operational tolerance has yet to be defined). Thus, it may be speculated that B cell sufficient NOD mice resist transplant tolerance due to inadequate B cell mediated allograft regulation. Although the genetic information that encodes B cell related gene products is spread across numerous chromosomes, future studies could begin to explore this question by creating NOD congenic or chimeric mice that possess B cell related

gene products introduced from tolerance-susceptible B6 mice, whose B cells possess graft-protective regulatory function. In assessing the role of B cell antigen recognition, the B6 congenic regions encoding IgH (Chr12: 113258768-116009954Mb) and Igk (Chr6: 67555636-70726754Mb) could be swapped with those encoded by the NOD genome. In assessing the role of B cell antigen presentation, chimeric mice in which only the B cell compartment expresses B6 derived MHC Class I/II antigen presentation machinery could be generated using commercially available NOD.H2b bone marrow donors[313]. Finally, as numerous reports have associated dysregulated B cell mediated signal transduction with loss of tolerance in NOD mice, the B6 congenic regions encoding the B cell specific signaling components Bruton's Tyrosine Kinase [BTK] (ChrX: 134542334-134583623Mb) or CD19 (Chr7: 126408448-126415140Mb) could be swapped with those encoded by the NOD genome. Interestingly, NOD mice possessing genetic deficiencies of BTK[314] and CD19[315] are significantly protected from diabetes, and CD19 specifically falls within a yet to be assigned *Idd* region.

Furthermore, Tim-1 was recently described to be a marker of Bregs capable of transferring long-term allograft tolerance in non-autoimmune mice[134]. In that an anti-Tim-1 activating antibody enhances transplant tolerance induction in tolerance susceptible B6 mice via a Breg dependent mechanism, future studies could explore the role of Tim-1+ Bregs in establishing transplantation tolerance in NOD mice. Overall, achieving transplantation tolerance in the autoimmune T1D environment in an intact immune system, as remains an important pre-clinical goal, will likely require surmounting deleterious anti-graft function perpetuated by the autoimmune B cell compartment while simultaneously enhancing the graft-protective, regulatory properties of these cells.

Determining whether B lymphocytes represent a generalizable barrier to transplant tolerance across different autoimmune conditions remains to be determined. In addressing this question, future studies will assess transplant tolerance in B cell deficient B6.SLE123, B6.SLE1, B6.SLE2, and B6.SLE3 mice by intercrossing these strains with commercially available B6μMT mice. Not only will these studies reveal whether B lymphocytes represent a key barrier to transplant tolerance in the fully penetrant lupus setting (B6.SLE123 mice), but moreover, will address the role of B cells in contributing to transplant tolerance resistance in the context of genetically-isolated, autoimmune-derived genetic loci (Chapter VI – Figure 7 – Row 5). Via such genetic distillation, use of these single congenic systems may reveal specific, targetable molecular mechanisms by which B lymphocytes resist transplant tolerance in the context of autoimmunity.

My findings in Chapter II provide new data demonstrating that the autoimmune B lymphocyte compartment represents a substantial barrier to achieving transplant tolerance in the setting of autoimmunity. However, the molecular mechanisms by which loss of B cell tolerance in autoimmunity perpetuates a generalized resistance to transplant tolerance remain to be determined. In exploring this concept, emerging data indicate that thymic-resident B cells play an under recognized role in the selection of CD4 T cells. Mechanistically, not only do thymic-resident B cells negatively select potentially autoreactive CD4 T cell clones (by upregulating the expression of AIRE, expressing self-antigen in the context of MHC Class II, and providing CD80 co-stimulation)[316], these thymic resident B cells also positively select for Helios+Foxp3+ thymic CD4 Tregs via similar upregulation of MHC Class II[317]. As thymically-derived CD4 Tregs are required for permanent transplant tolerance induction[114], it remains possible that autoimmune B cells could deleteriously alter the centrally-derived Treg repertoire in such a way

that later restricts the graft-protective function of these cells. Moreover, it could be speculated that autoimmune, thymic-resident B cells inadequately prune the alloreactive CD4 T cell repertoire thereby predisposing the autoimmune environment to heightened allograft immunity.

Because my data demonstrate that B cell deficient NOD mice possess an expanded population of thymic-resident, diabetes-protective Vβ3+ CD4 Tregs[189] (Figure 2.8), future studies will explore the function of these clonotypic cells in establishing transplant tolerance. Although not presented in my thesis, my lab recently discovered that NOD mice possess expanded populations of thymic-resident B cells that possess enhanced ICOS-L expression. Since ICOS plays a significant role in CD4 Treg function in diabetes progression[318], dissecting whether inappropriate ICOS-ICOS-L crosstalk between thymic-resident B cells and CD4 Tregs may offer further clues as to why the autoimmune environment resists transplant tolerance.

Effector T cell resistance to Treg mediated suppression as a barrier to transplant tolerance in autoimmunity

Although immunologic deletion, anergy, and ignorance each represent means by which the immune system selects against potentially autoreactive T and B cell clones, these mechanisms alone do not fully confer self-tolerance. This statement is supported by the fact that patients with no detectable signs of autoimmunity often possess significant numbers of autoreactive lymphocyte clones capable of targeting self-tissue. Overall, adequately regulating these potentially autoreactive clones is essential in suppressing autoimmune pathogenesis. In that a growing body of evidence over the past three decades has demonstrated that failed immune regulation permits autoimmunity, I questioned whether these failures may further pose a barrier to long-term transplant tolerance induction in these highly immunogenic settings.

Complementing well established knowledge that CD4 Tregs actively regulate anti graft-immunity, emerging evidence suggests that a CD122+ subpopulation of CD8 T cells (termed CD8 Tregs) may further aid in graft protection[125]. Thus, I hypothesized that the resistance to transplant tolerance induction observed in NOD mice may in part be due to functional deficiencies in CD8 Treg suppressive function. In Chapter III, I found that CD8 Tregs in autoimmune prone NOD mice inadequately suppress the immunogenic TFH-GC B cell response, a finding that may account for my observation that these mice generate an exaggerated alloantibody response (Figure 7 – Row 2). In determining a putative role of CD8 Tregs in maintaining long-term transplant tolerance, I subsequently determined that the absence of these cells (imbued by a genetic absence of this cell's survival cytokine IL-15) rendered otherwise tolerance-susceptible B6 mice resistant to transplant tolerance induction. In further exploring the role of failed immune regulation as a barrier to transplant tolerance induction, in Chapter V I provide evidence that effector T cells from lupus-prone B6.SLE123 mice resist CD4 and CD8 Treg based suppression, a finding which may account for my observation that these mice resist anti-CD45RB mediated transplant tolerance induction to foreign islet allografts (Figure 7 – Row 4). Overall however, it remains to be determined whether such resistance to Treg based suppression directly perpetuates resistance to transplant tolerance in autoimmunity.

To explore this question in the T1D-prone NOD setting, future experiments will include testing anti-CD45RB mediated transplant tolerance in NOD.RAG mice reconstituted with effector CD4 T cells and CD4 Tregs sorted from either NOD (suppression resistant) or B6.H-2g7 (suppression susceptible[259]) donors. To explore this question in the lupus-prone B6.SLE123 setting, future experiments will include testing anti-CD45RB mediated transplant tolerance in B6.RAG mice reconstituted with effector CD4 T cells and CD4 Tregs sorted from either B6

(suppression susceptible) or B6.SLE123 (suppression resistant) donors. In either setting, a similar crisscross platform will be used as described in Figure 5.2/3. It is important to note a limitation of this adoptive cell transfer system in that immunodeficient recipients reconstituted with mature lymphocytes resist pharmacologic induction of transplant tolerance due to the unchecked homeostatic proliferation of transferred cells[319]. However, since these reconstituted/treated recipients still demonstrate a prolonged time to allograft rejection as compared to reconstituted/untreated allograft recipients, deviations from the baseline "tolerance-susceptible" condition (recipients receiving both CD4 T effector cells and CD4 Tregs from either B6.g7 or B6 donors alone) could be used to assess the direct role of effector cell resistance to Treg based suppression as a driver of transplant tolerance resistance.

Determining the molecular mechanisms by which effector T cells resist Treg based suppression remains highly warranted in that such knowledge may not only help restore transplant tolerance in autoimmunity, but moreover, may provide novel insight into the mechanisms that permit loss of tolerance in autoimmunity. In exploring this concept within the context of autoimmune T1D, it appears that the origin of effector cell resistance is not due to ongoing autoimmunity as both T effector cells from 3 week old NOD mice as well as mature CD4+CD8-CD24^{LO} thymocytes resist Treg suppression[259]. It is speculated that dysregulated signaling in NOD effector T cells may imbue these cells with a generalized resistance to Treg suppression, although this hypothesis has yet to be confirmed. Accordingly, future dissection of TCR signaling pathways during anti-graft immunity may reveal new mechanisms by which the autoimmune environment resists transplant tolerance.

Within the context of SLE, it similarly remains to be defined why effector T cells in this strain resist Treg mediated suppression. From a molecular standpoint however, it has been

observed that effector T cells from lupus-prone mice increase Treg production of IL-17[258]. Interestingly, it has been recently demonstrated that enhanced levels of IL-6 in the autoimmune environment can directly lead to the loss of Foxp3 expression in $CD25^{LO}$Foxp3+ Tregs (termed exTregs)[110]. These exTregs subsequently transdifferentiate into pathogenic TH17 cells - a cell population which directly enhances anti-graft immunity. Thus, it could be hypothesized that effector T cells in the autoimmune SLE environment promote Treg inactivation, converting otherwise graft-protective Tregs into graft-destructive TH17 cells. I found that B6.SLE123 mice possess an overall reduction of CD25 expression in their CD4 Treg population (Chapter V), that both CD4 and CD8 T cells from these mice are hyperresponsive to IL-6, and moreover that these mice generate enhanced IL-6 levels during allochallenge (Chapter VI). Thus, it could be speculated that the SLE environment (and by extension, other autoimmune conditions) resists transplant tolerance due to CD4 Treg inactivation. Overall, determining the role of Treg inactivation and conversion to pathogenic effector cell types could offer new knowledge concerning the barriers to transplant tolerance in autoimmunity. As *in vivo* CD4 Treg fate mapping models are available on both the B6[110] and B6.SLE123 (courtesy of Amy Major, Vanderbilt University) backgrounds, the role of CD4 Treg inactivation during transplant tolerance can be directly addressed in both the tolerance susceptible and resistant environments.

In addition to the role of CD4 and CD8 Tregs, emerging evidence suggests that newly defined CD4 TFH Regulatory (TFHR) Cells (Foxp3+Bcl-6+) aid in the suppression of the immunogenic TFH-GC B cell response[200]. In determining whether these cells directly suppress autoimmune phenotypes, Wu and colleagues recently found that TFHR deficient B6 mice (Foxp3-Cre x flox/Bcl-6/flox) generate higher anti-dsDNA IgA titers during pristane-induced lupus[320]. Although I did not directly explore the role of these cells in my dissertation, future work

will include determining whether and how TFHR cells promote either self and/or graft tolerance in the context of autoimmunity.

Targeting a single component of the immune system vs. the network as a whole

Within the context of autoimmunity, a number of groups have explored whether targeting multiple components of the immune system may enhance allograft tolerance in NOD recipients. In fact, the most potent tolerance inducing strategies (outside of conventional immunosuppression regimens that are dosed continuously until rejection or those utilizing graft matched BMT protocols that lead ultimately to GVHD) have included those that simultaneously inhibit effector cell co-stimulation (via anti-CD40L) and enhance regulatory cell suppression (via anti-CD45RB). Specifically, administration of the combination of these agents extends islet allograft survival in NOD mice 2.5-fold over recipients receiving either therapy individually[321]. However, it is important to note that permanent tolerance was never achieved in any of these recipients. Thus, there still exists an immense need to discover and overcome a number of yet to be determined barriers to transplant tolerance in the setting of autoimmunity.

This need formed the basis of my investigations in Chapter II in which I explored whether a specific cell type mediated the generalized resistance to transplant tolerance observed in NOD mice. My finding that B lymphocytes restrict the generation of graft-protective CD4 Tregs in NOD mice would support the hypothesis that attenuating autoreactive B cell function while simultaneously enhancing CD4 Treg function may enhance transplant outcomes in autoimmune NOD mice. In fact, transient B cell depletion prior to anti-CD40L mediated co-stimulation blockade has been found to extend islet allograft survival 4-fold in NOD mice vs. those recipients receiving anti-CD40L alone[83]. However, an important caveat to these studies

was that the anti-CD22/calicheamicin agent used in these studies did not fully deplete the B lymphocyte compartment in recipient WT NOD mice. Thus, assessing whether therapies that more effectively augment CD4 Treg function (i.e. anti-CD45RB) during enhanced B cell depletion protocols could further render NOD mice susceptible to transplant tolerance induction remains to be determined.

In further exploring how transplant tolerance is immunologically achieved, in Chapter IV (Figure 7 – Row 3), I determined that HSC mobilization dependent on sympathetic nervous system activity is a necessary component to anti-CD45RB mediated transplant tolerance induction. In that anti-CD45RB alone failed to mobilize HSCs in tolerance resistant NOD mice, I found that the CXCR4 antagonist AMD3100 could overcome the enhanced HSC retention characteristic of this strain. When this agent was administered either alone or in combination with anti-CD45RB, NOD recipients receiving islet allograft demonstrated 6- and 7-fold extensions in islet allograft survival. Overall, these data suggest that HSC mobilization during transplantation is a critical step in achieving long-term transplant tolerance. Because failures to properly mobilize HSCs may contribute to transplant tolerance resistance in autoimmunity, these findings lend credence to exploring the efficacy of additional agents that enhance HSC mobilization during clinical transplantation. Along these lines, a recent clinical trial found that G-CSF mediated HSC mobilization reduced the incidence of acute rejection episodes and decreased allograft vasculopathy in patients receiving cardiac allografts[322]. Furthermore, as ATG/G-CSF treatment protocols have recently demonstrated modest success in maintaining islet survival in patients with recent onset T1D[323], these clinical findings and my data presented in Chapter IV lend credence to the fact that effective HSC mobilization may be a critical component in achieving transplant tolerance in autoimmunity.

Overall, HSC mobilization and activity is necessary to generate a robust and proper effector response while maintaining immune homeostasis[235]. Extending these findings, my data suggest that during the transplant response the HSC niche plays a key role in determining the tolerant outcome. In infectious literature, IFNγ plays an important role in modulating the HSC compartment[234]. Although somewhat paradoxical, IFNγ is also required for anti-CD40L/CD28 mediated transplant tolerance to skin and cardiac allografts in non-autoimmune mice[324]. Although IFNγ generally plays a harmful role in autoimmune T1D[325], inadequate IFNγ function may further relate to a failure to act on the HSC compartment, thereby contributing to the generalized resistance to transplant tolerance observed in NOD mice. Future studies will explore whether dysregulated IFNγ mediated mobilization of the HSC niche contributes to transplant tolerance resistance in autoimmune recipients.

In exploring new avenues to achieve transplant tolerance in the absence of conventional immunosuppression, recent evidence has demonstrated that blocking T cell metabolism may offer a novel means by which allograft rejection can be prevented[300]. Although these recent studies utilized a model in which continuous administration of the anti-metabolic agents Metformin, 2-Deoxy-D-glucose, and 6-Diazo-5-Oxo-L-Norleucine prevented cardiac allograft rejection, the authors did note that these agents can effectively attenuate the expansion of IFNγ producing T effector cells while concomitantly increasing CD4 Treg numbers. As CD4 T cells from B6.SLE123 mice possess enhanced metabolic function including roles for heightened cellular glycolysis and mitochondrial oxidative metabolism[295], in Chapter VI I explored whether temporary metabolic modulation of CD4 T cell function during anti-CD45RB therapy could render B6.SLE123 mice susceptible to transplant tolerance induction. I hypothesized that metabolic conditioning during anti-CD45RB mediated tolerance induction would enhance the

inactivation of anti-graft effector T cells while augmenting the generation of graft-protective CD4 Tregs. In fact, I found that the addition of the anti-metabolic agents Metformin and 2-Deoxy-D-Glucose significantly extended allograft survival in otherwise tolerance resistant B6.SLE123 mice. Although the exact mechanism of graft protection in this setting remains to be determined, future studies will include assessing how the tolerance inducing agent anti-CD45RB alters specific metabolic parameters in both effector and regulatory T cell populations in both transplant susceptible (B6) and resistant strains (B6.SL123, B6.SLE1, B6.SLE2, B6.SLE3 and NOD). While outside the context of solid-organ transplantation, recent work by Nguyen and colleagues demonstrated that alloreactive T cells in GVHD rely primarily on aerobic glycolysis[326]. Glycolytic blockade via inhibition of mTORC1 or PFKFB3 ameliorated alloreactive T cell mediated GVHD morbidity and mortality. As these molecules are directly downstream of PTEN, a molecule required for CD4 Treg suppressive function, beneficially targeting the PTEN-PI3K-AKT-mTOR pathway in alloreactive T cells that favors Treg function may offer a novel means by which enhancing transplant tolerance in autoimmunity could be achieved as part of a multi-agent, tolerance-inducing strategy.

Heterologous immunity as barrier to transplant tolerance in autoimmunity

Non-specific immunologic insult represents a significant barrier to transplant tolerance in the clinical setting; acute rejection events are often associated with unrelated pathogenic infections[327]. Although the molecular mechanisms driving such "heterologous immunity" during transplantation have yet to be determined, Adams and colleagues found that the transfer of either CD8 effector or central memory T cells from mice previously infected with LCMV could rapidly precipitate skin allograft rejection in otherwise tolerance-susceptible syngeneic recipients

(mediated by DST + MR1 mediated co-stimulation blockade)[328]. Specifically, the exposure to LCMV generated a cross-reactive, alloreactive memory CD8 T cell pool capable of driving allograft rejection. Whether such exposure led to the activation of alloreactive cells via molecular mimicry (in that a viral bound self MHC resembled self-peptide bound to foreign MHC) or by bystander activation remains to be determined.

It can be speculated that heterologous immunity, driven instead by autoreactive T and B lymphocytes in lieu of a pathogenic agent, could represent a barrier to transplant tolerance in autoimmunity. In these autoimmune milieus, inappropriately activated autoreactive T and B lymphocytes could in theory promiscuously activate bystander alloreactive T cells (which constitute 5-10% of T cell repertoire[30]) that would later resist transplant tolerance induction. Alternatively, these same autoreactive T and B lymphocytes that target self-antigen may generate a memory T cell pool capable of cross-reacting with allogeneic graft tissue. Regardless of whether these alloreactive T cells are generated by bystander activation or molecular mimicry, my data in Chapter III and V demonstrate that autoimmune NOD and B6.SLE123 are unable to adequately control the immunogenic CD4 TFH-GC B cell axis, an axis which when inappropriately regulated, results in the outgrowth of antigen non-specific GC B cells as determined by Linterman and colleagues[200]. Although inappropriately activated GC B cells in NOD and B6.SLE123 mice generate autoantibodies directed at islet and nuclear proteins, respectively, it remains to be determined whether these autoimmune mice also possess antibodies capable of cross-reacting with allogeneic tissue prior to transplantation although this was not observed in my baseline alloantibody measures prior to stimulation. However, as my data demonstrate that both NOD and B6.SLE123 mice generate enhanced antibody and specifically alloantibody titers during immunization, it can be inferred that these autoimmune environments

are genetically predisposed to generating heightened GC B cell responses which are potentially capable of generating highly immunogenic, graft-reactive alloantibodies. Genetic sequencing and comparative analysis of the TCRs and BCRs emerging from the alloresponse may offer insight into whether and how the autoimmune environment is skewed toward generating a heightened alloresponse.

Transplant tolerance as a dynamic, ongoing, and highly regulated process

In Chapter II, I determined that allograft tolerance in B cell deficient NODμMT mice is an ongoing, antigen-specific, and highly regulated state mediated by graft-protective CD4 Tregs. Although I found that infusion of graft-inexperienced, naive T cells from NOD mice failed to precipitate rejection in tolerant NODμMT recipients (thereby suggesting that graft-protective CD4 Tregs actively prevent the anti-graft function of naïve T cells), it remains to be determined whether these same graft-protective CD4 Tregs could overcome the aggressive memory T cell response characteristic of this strain. Accordingly, future studies could include infusing memory T cells from rejecting NODμMT or NOD mice into tolerant NODμMT allograft recipients. In the event that these anti-graft memory cells precipitated rejection, these findings may reveal a new barrier to transplant tolerance in the autoimmune setting - specifically a failure to regulate the allograft memory response.

In exploring the role of graft memory in the context of transplant tolerance, Miller and colleagues recently determined that in non-autoimmune settings, the memory of graft regulation dominates over the memory of graft rejection. Using a model in which anti-CD40L mediated cardiac allograft tolerance was broken by *Listeria* infection, the authors determined that placement of a second matched graft after pathogen clearance was accepted indefinitely without

further treatment[329]. The restoration of graft tolerance was directly attributable to CD4 Tregs in that anti-CD25 mediated depletion of this cell population rapidly precipitated rejection of second matched cardiac allografts.

These findings implicate that the memory of graft regulation can override the memory of graft rejection; although graft tolerance can be transiently overridden during inflammation, this state can re-emerge after inflammation has ceased. Extending these findings, it is well documented that breakthrough lupus nephritis in renal allografts can directly lead to acute rejection events and subsequent graft failure[252]. From these studies, it could be argued that immunosuppression at the earliest signs of acute rejection should entail not only those agents capable of halting effector T cell function, but those that concomitantly enhance graft-protective regulatory cell function. In that graft tolerance is an ongoing and highly regulated immunologic process, determining how to specifically enhance graft regulation during episodes of unrelated immunologic insult may offer new insights into achieving clinical operational tolerance.

Innate immune cells: a potential barrier to transplant tolerance in autoimmunity?

Within the B6.SLE123 setting, the SLE1 locus directly perpetuates an enhanced DC derived IL-6 response, a cytokine whose effects directly render T effector cells resistant to Treg mediated suppression[277]. Although blockade of the IL-6 signaling axis partially restored transplant tolerance in B6.SLE1 mice (Chapter VI), it remains to be determined whether additional aberrancies in DC function contribute to my observation that B6.SLE123 mice resist transplant tolerance induction (Chapter V). Interestingly, Wan and colleagues documented that B6.SLE123 DCs undergo reduced apoptosis and induce greater CD4 T cell proliferation despite

possessing reduced levels of MHC Class II and the co-stimulatory molecules CD40, CD80, and CD86[277].

Accordingly, further dissection of the role of DCs in establishing transplant tolerance in the setting of autoimmune lupus is highly warranted in that a number of reports have recently documented that infusion of tolerogenic DCs can attenuate lupus pathology via enhancements in CD4 Treg function[330]. Similar to exploring whether the genes responsible for resistance to transplant tolerance in B6.SLE123 mice are perpetuated by dominant or recessive genetic polymorphisms (as extended from the transplant studies involving F1 B6 x NOD mice – see below), future studies will include testing transplant tolerance in F1 B6 x B6.SLE1, B6 x B6.SLE2, and B6 x B6.SLE3 recipients. Overall, determining why the autoimmune environment remains resistant to transplantation tolerance demands exploring the entirety of the immune network, including a role for those cells of the innate immune system.

Although it is well documented that autoimmune NOD mice possess a number of DC deficiencies (whose inadequate function may in part further explain why in the absence of B cells NOD mice are permissive to transplant tolerance – Chapter II) it remains to be determined whether failures in the DC system further challenge the establishment of long-term transplant tolerance in the setting of autoimmunity, especially in light of recent data demonstrating that DCs can directly participate in allorecognition. Interestingly, B6 x NOD F1 recipients similarly resist anti-CD40L mediated transplant tolerance induction despite not progressing to diabetes[331]. Although these F1 mice possess restored NK and Macrophage function in comparison to WT NOD mice, these F1 offspring still retain deficiencies in their DC compartment. Specifically, DCs in both F1 and WT NOD mice possess abnormalities in maturation and express higher levels of the CD4 T cell co-stimulatory molecule CD86. Such DC deficiencies in the NOD

setting may in part render these mice resistant to transplant tolerance induction due to enhanced CD4 T cell activation. As G-CSF mediated diabetes prevention in NOD mice induces a DC dependent enhancement of islet-protective CD4 Tregs[332], these data warrant further exploration into the role of DCs in establishing transplant tolerance to allogeneic tissue grafts in NOD mice. In that I found a decreased propensity of conventional BMDCs to express the CD8 Treg survival factor IL-15 in NOD mice (Chapter III), such intrinsic DC failures may further perpetuate failures in generating those graft-protective regulatory necessary of instilling immunologic transplant tolerance in the context of autoimmunity.

Towards reliably achieving Clinical Operational Tolerance

Although permanent transplant tolerance is readily achieved in small animal models via a variety of agents (see *Pharmacologically Induced Transplant Tolerance)*, there currently exist no agents or protocols that reliably induce Clinical Operational Tolerance (COT) in human graft recipients. Accordingly, over the past two decades researchers have focused on advancing trials of preclinical agents to obtain COT. Some of the earliest trials included those exploring the efficacy of T cell costimulation blockade during transplantation using anti-CD40L therapy. Upon Kirk's discovery in 1999 that anti-CD40L monotherapy induced long-term renal allograft survival in Rhesus Macaques, as well as prevented any signs of acute rejection[333], a humanized form of anti-CD40L (BG9588) quickly entered clinical trials. Unfortunately however, Kirk's Phase I/II clinical was terminated early; not only did BG9588 monotherapy demonstrate poor efficacy in that 5/7 renal transplant recipients presented with signs of acute rejection, a number of recipients presented with life-threatening thromboembolic events[334]. Through a series of follow up experiments, it was later determined that human platelets, unlike their murine

counterparts, express the FcγRIIa through which bound anti-CD40L immune complexes induce platelet activation and aggregation[335]. Although these initial results came as a shock to the transplant community, these studies highlighted the challenge, importance, and necessity of discovering differences between the preclinical and human immune systems that may hinder safe and successful translation of novel transplant agents. In light of these results, a number of researchers are actively modifying humanized anti-CD40L antibodies via means of Fc elimination/modification with the overall goal of efficiently interrupting the CD40-CD40L axis while avoiding platelet aggregation[336].

Along similar lines, translational efforts to evaluate the clinical utility of the tolerance inducing agent ant-CD45RB raise additional challenges in the quest of realizing COT. In a fully-MHC mismatched renal allograft model between Cynomolgus monkeys, two-week monotherapy of a humanized form of anti-CD45RB (clone: 6G3) resulted in a 4-fold extension of MSTs (27 days) over untreated recipients as well as 2/8 recipients reaching >200 and >300 days of stable graft function[337]. In comparison, groups receiving FK506 monotherapy dosed until rejection all rejected with a MST of 27 days, a time that did not differ significantly from recipients receiving anti-CD45RB monotherapy. Although anti-CD45RB therapy never reached clinical transplant trials due to the untimely death of the study's Principal Investigator, studies such as these raise important questions concerning rational translation of new transplant agents. Specifically, what is the bar required for a new transplant agent to move to clinical trials? Does the new agent's efficacy need to meet or exceed that of currently utilized immunosuppression agents? Although the agent may not significantly exceed the efficacy of a currently available agent, how does one account for the potential reduction of side effects offered by the new agent in evaluating its

potential clinical utility? Although there is no specific answer to these questions, these issues represent ongoing challenges to translational transplant scientists.

In addition to anti-CD40L and anti-CD45RB therapy, numerous other preclinical tolerance-inducing agents have since entered non-human primate and clinical trials (including but not limited to anti-ICAM-1, anti-CD80/86, CTLA-4Ig, FTY720, and Tofacitinib)[334]. As informed from the anti-CD40L monotherapy trial however, these subsequent trials have switched to a design wherein these experimental agents (or a placebo control) are administered alongside conventional immunosuppression in order to maximize overall graft and patient survival and safety. Such trial design now raises the additional challenge of evaluating the individual efficacy of each trial agent. Does the confounding factor of generalized immunosuppression reduce or enhance the effects of the trial agent in question? To what extent must the trial agent improve graft and patient survival over administration of conventional regimens alone to justify its clinical utility? Are there additional benefits that the trial agent offers over conventional immunosuppression? Recent results from a 2016 report in the New England Journal of Medicine shed light on the challenge of answering these questions. Vincenti and colleagues demonstrated that administration of CTLA4-Ig (Belatacept) in place of Cyclosporine (alongside concomitant Basiliximab, MMF, and glucocorticoids) reduced overall risk of renal graft loss and death by 43% at 7 years after transplantation[338]. In assessing overall survival rates at 7 years, addition of Belatacept decreased risk of death from 14% to 9% and risk of graft loss from 10% to 5%. Interestingly however, patients receiving Belatacept in place of Cyclosporine also demonstrated significantly improved estimated Glomerular Filtration Rates (eGFR) at 7 years (65 vs. 45 mL/min/1.73m^2). Although addition of Belatacept statistically improved transplant outcomes, it remains to be determined whether these improvements justify its widespread clinical adoption at

an additional cost of $21,000 per year[339]. Ultimately, these studies may help usher in new agents that improve graft and patient survival odds. However, there now exists the additional challenge of parsing out the isolated immunological effects of these agents as well as determining whether or not they truly increase ones odds of achieving COT over standard therapy or rather, simply modify one's general state of immunosuppression.

Finally, it is interesting to note that within the approximately 100 cases of human renal transplant recipients demonstrating Clinical Operational Tolerance (COT) there now exist 8 documented COT transplant recipients with a history of autoimmune disease[340]. Although my data demonstrate that the murine autoimmune T1D and SLE environments represent a stringent barrier to anti-CD45RB induced transplant tolerance, it remains to be determined the degree to which clinical autoimmunity precludes COT or represents a modifiable barrier. Furthermore, it remains unknown whether specific types of clinical autoimmunity and their specific immune dysregulations represent unique or generalizable barriers to COT. In that past efforts have revealed a unique B cell signature in COT renal transplant recipients without any history of underlying autoimmune disease (see *Transplant Tolerance in the Clinic),* profiling this unique clinical cohort of COT transplant recipients with underlying autoimmunity represents an exciting opportunity to discover specific immune signatures that may differ 1) between COT transplant recipients with or without autoimmunity, 2) between COT transplant recipients and rejecting transplant recipient with autoimmunity, and 3) between COT transplant recipients with different forms of underlying autoimmunity. Overall, studying these unique cohorts of COT transplant recipients represents an unprecedented opportunity to enhance our understanding of which immune pathways and networks dictate immune tolerance to both self and transplanted tissue.

Conclusions

Achieving transplant tolerance in the autoimmune environment will require targeting multiple immunologic dysregulations in T-B cell collaboration that drive the aggressive anti-graft response. At a biologic level, my findings newly reveal the necessity of overcoming B lymphocyte mediated restriction of CD4 Treg function, failed HSC mobilization, and enhanced T cell metabolism in achieving transplant tolerance. At a cellular level, my dissertation demonstrates that specific failures in CD4 and CD8 Treg mediated suppression of the effector cell response in autoimmune T1D and SLE may contribute to a generalized resistance to transplant tolerance observed in these strains. Overall, identification of and surmounting the key dysregulations in T-B cell collaboration that permit loss of tolerance in autoimmunity may one day help realize the full clinical potential of transplantation as a cure for autoimmune disease.

CHAPTER VIII

MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University approved all procedures carried out during this study. Mice were housed in a specific-pathogen free facility at Vanderbilt University and maintained by Vanderbilt's Division of Animal Care (DAC). Specific mouse strains used in these studies, along with their MHC haplotypes, immunologic phenotypes, genetic alterations, and sources are listed in Table 4. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic Biosciences (Hudson, NY) or kindly donated by the investigators listed in Table 4. Prior to use, all mouse genotypes were confirmed by PCR using primers published by the strain's donating investigator.

Lymphoid cell preparation

Per IACUC standards, mice were euthanized via isoflurane overdose followed by cervical dislocation. Lymphoid organs of interest (spleens, thymuses, and pancreatic, inguinal, renal, and cervical lymph nodes) were surgically removed and then placed in ice-cold Phosphate Buffered Saline (PBS). To free lymphocytes from tissue their surrounding tissue stroma, lymphoid organs were crushed with the plunger end of a 1cc tuberculin syringe (BD, San Jose, CA) through 70μm pore size cell sieves (Thermo-Fisher Scientific, Waltham, MA) into 50mL conical tubes. Cell sieves were then washed three times with ice-cold PBS into the same 50mL conical tube to ensure maximal cell recovery. Isolated cells were then spun at 550g for 3 minutes and the

Strain (Shortened Strain Name)	MHC Haplotype	Immunologic Phenotype/Purpose for Study	Source/Stock Number
BALB/cJ (BALB/c)	d	MHC Mismatched Islet Donor	JAX - 000651
C3H/HeJ (C3H)	k	MHC Mismatched Islet Donor	JAX - 000659
C57BL6/J (B6)	b	Non-autoimmune (Control)	JAX - 000664
C57BL/6NTac-IL15tm1Imx N5 (B6.IL-15$^{-/-}$)	b	CD8 Treg Deficient (Non-autoimmune Control)	Taconic - 4269
B6.129S7-Rag1tm1Mom/J (B6.RAG)	b	T and B Cell Deficient (Non-autoimmune Control)	JAX - 002216
B6.NZM-Sle1NZM2410/Aeg Sle2NZM2410/Aeg Sle3NZM2410/Aeg/LmoJ (B6.SLE123)	b	Fully Penetrant SLE	JAX - 007228
B6.NZM-Sle1NZM2410/Aeg/LmoJ (B6.SLE1)	b	Partially Penetrant SLE	Donated by Edward Wakeland, UTSW
B6.NZM-Sle2NZM2410/Aeg/LmoJ (B6.SLE2)	b	Partially Penetrant SLE	Donated by Edward Wakeland, UTSW
B6.NZM-Sle3NZM2410/Aeg/LmoJ (B6.SLE3)	b	Partially Penetrant SLE	Donated by Edward Wakeland, UTSW
NOD/ShiLtJ (NOD)	g7	T1D Prone	JAX - 001976
NOD.129S7(B6)-Rag1tm1Mom/J (NOD.RAG)	g7	T and B Cell Deficient T1D Prone	JAX - 003729
NOD.129S2-Ighmtm1Cgn/Dvs (NODuMT)	g7	B Cell Deficient T1D Prone	JAX - 003903
NOD/ShiLtJ-H2-Ab1em1Ygch/J (NOD.H2-Ab$^{-/-}$)	g7	MHC Class II Deficient T1D Prone	JAX - 027057

Table 4. Animal strains used in dissertation.

resulting cell pellet was lysed of contaminating Red Blood Cells via ACK Lysis Buffer for 5 minutes. The lysis buffer was then quenched via dilution with PBS. Isolated cells were then centrifuged, resuspended in 10 mL of PBS, and counted via an Automated Cell Counter (BioRad, Hercules, CA). Cells were stored on ice until further analysis.

Bone marrow preparation

Following euthanasia, murine tibias and femurs were removed and surrounding muscle tissue was dissected away. Tibial and femural epiphyses were then cut with bone cutting scissors and bone marrow was flushed from the resulting long-bone diaphysis using a 22-gauge needle attached to a 5mL syringe filled with ice-cold PBS. The isolated bone marrow was then filtered over a 70μm cell sieve and cells were further processed as described in Lymphoid Organ Preparation.

Peripheral Blood Mononuclear Cell (PBMC) preparation

Mice were first anesthetized with a single intraperitoneal (i.p.) injection of a Ketamine/Xylazine solution diluted in 0.9% normal saline (15mg/dL Ketamine, 3mg/mL Xylazine) dosed at 7.5μl/g. Upon confirmation of full anesthesia via toe-pinch, mice were bled retro-orbitally using a heparinized Natelson blood collecting tube. 200μL of the blood sample was then mixed with 3mL of room temperature HBSS (without calcium or magnesium) and placed into a 15mL conical tube. Samples were then underlaid with 2mL of a 1.077 g/mL Histopaque solution (Sigma Aldrich, St. Louis, MO) via a Pastuer Pipette and then spun at 1800rpm for 30 minutes. Cells floating above the Histopaque layer (PBMCs at the buffy coat) were then removed via pipette and stored on ice until further analysis.

Conventional flow cytometry

Lymphocytes were plated at a concentration of 1 million cells per well in 96 well V-bottom plates. Plates were then centrifuged at 550g for 3 minutes, supernatants discarded, and cells were stained in a 50ul volume of FACS Buffer (PBS + 0.1% sodium azide + 3% Fetal Bovine Serum) composed of various panels of fluorophore-conjugated antibodies purchased from either BD Biosciences, San Jose, CA or eBioscience, San Diego, CA. Extracellular fluorophore-conjugated antibodies used included: B220(RA3-6B2), CD3ε(145-2C11), CD4(RM4-5), CD8α(53-6.7), CD25(7D4), CD122(TM-B1), CD279/PD-1(J43), CD11b(M1/70), CD11c(N418), CD34(RAM34), CD44(IM7), CD45(30-F11), CD45RB(C363.16A), CD49b(DX5), CD19(6D5), CD95/Fas(Jo2), CD254/RANKL(IK22/5), CXCR4(2B11), CXCR5(2G8), c-Kit(2B8), F4/80(BM8), GITR(DTA-1), H2-Kb(AF6-88.5.5.3), H-2Kd(SF1-1.1.1), H-2Kk(36-7-5), I-Ab(KH74), I-Ak/g7(10-3.6), ICOS (C398.4A), IgM(II/41), IL-15Ra(DNT15Ra), Ly6C/G(RB6-8C5), Ly49C/F/I/H(14B11), Ly49E/F(CM4), Ly49C/F/I/H(14B11), Ly49F(HBF-719), Ly-49G2(4D11), Osteocalcin(polyclonal), Sca-1(D7), TCRβ(H57-597), TCRVβ2(B20.6), TCRVβ3(KJ25), TCRVβ4(KT4), TCRVβ5.1/2(MR9-4), TCRVβ6(RR4-7), TCRVβ7(TR310), TCRVβ8.1/2(MR5-2), TCRVβ8.3(1B3.3), TCRVβ9(MR10-2), TCRVβ10b(B21.5), TCRVβ11(RR3-15), TCRVβ12(MR11-1), TCRVβ13(MR12-3), TCRVβ14(14-2), TCRVβ17a(KJ23), TER-119. Lymphocytes were stained at 4°C for 30 minutes, washed with FACS buffer, transferred to 5mL round bottom tubes, and kept at 4°C until analysis. For intracellular protein analysis, cells were fixed and permeabilized prior to staining via a 30 minutes soak at 4°C in a saponin/PFA based buffer purchased from eBioscience (Foxp3/Transcription Factor Staining Buffer Set). The following intracellular fluorophore-conjugated antibodies were used to stain cells for 30mins at 4°C after fixation/permeabilization:

Bcl-6(K112-91), Foxp3(FJK-16s), Helios(22F6), Ki67(SolA15), pSMAD2/3(D27F4), pSMAD1/5/8(D5B10). All samples were acquired on a BD LSRFortessa Flow Cytometer and analyzed by FlowJo software (TreeStar, Ashland, OR).

Cytokine stimulation assays and phosphoflow cytometry

For *ex vivo* signaling assays, splenocytes were exposed to increasing concentrations of either the IL-15C superagonist (1, 10, 100, 1000, 10000 pM, eBioscience, San Diego, CA) or recombinant IL-6 (0.2, 2, 20 ng/mL, eBioscience, San Diego, CA) for various periods of time (0, 5, 10, 15, 30, 60 minutes) in Cell Culture Media (DMEM + 10%FBS + 1% Penicillin/Streptomycin)[341]. For some conditions, cells were preincubated for 30 minutes at 37°C with either the JAK3 specific inhibitor Tofacitinib (1μM, SelleckChem, Houston, TX) or an anti-IL-6R antibody (10μg/mL, BioXCell, West Lebanon, NH) to inhibit JAK mediated phosphorylation of either STAT5 or STAT3. Cells were then fixed with 1% PFA, permeabilized with 100% ice cold methanol, and pSTAT levels were assessed by staining cells with a primary anti-pSTAT5[Y694](C11C5) or anti-pSTAT3[Y705](D3A7) rabbit antibody, followed by a secondary anti-rabbit Fab2-Alexa647 conjugate (Cell Signaling Technologies Danvers, MA). Specific CD4 and CD8 T cell populations were further identified by staining with various combinations of the extracellular fluorophore-conjugated antibodies listed above in the Conventional Flow Cytometry methods section. For *in vivo* signaling assays, mice were i.v. injected with 1μg of an *in vivo* stable IL-15C complex or saline as a control[207]. To generate an *in vivo* stable IL-15C complex, carrier free IL-15 (eBioscience, San Diego, CA) was incubated with the high affinity IL-15 Receptor alpha chain-Fc complex (R&D Systems, Minneapolis, MN). One hour after IL-15C or saline injection, spleens were immediately fixed with 1% PFA, permeabilized with methanol, and pSTAT5

signaling assessed[342]. As previously described, all samples were then acquired on a BD LSRFortessa Flow Cytometer (BD, San Jose, CA) and analyzed by FlowJo software (TreeStar, Ashland, OR).

Magnetic Activated Cell Sorting (MACS)

In general, depletion or enrichment of B, CD4 T effector, CD4 Treg, or CD8α T cell populations was achieved by following the kit instructions provided by Miltenyi Biotec (San Diego, CA). For positive selection, isolated lymphocytes were resuspended in MACS Buffer (PBS + 0.5%FBS + 1%Pen/Strep) and then incubated with the following monoclonal antibodies pre-conjugated to magnetic microbeads [B cells: anti-B220(RA3-6B2), CD4 T cells: anti-CD4(L3T4), CD8 T cells: anti-CD8(Ly-2)]. For untouched negative selection, cells were incubated with the following biotin-conjugated antibody cocktails pre-mixed and supplied by Miltenyi [B cells: anti-CD43(Ly48), anti-CD4(L3T4), anti-Ter-119; CD4 T cells: anti-CD8α, anti-CD11b, anti-CD11c, anti-CD19, anti-B220, anti-CD49b, anti-CD105, anti-IAβ, anti-Ter119, and anti-TCRγ/δ; CD8α T cells: anti-CD4, anti-CD11b, anti-CD11c, anti-CD19, anti-B220, anti-CD49b, anti-CD105, anti-IAβ, anti-Ter119, and anti-TCRγ/δ]. Cells were washed and then mixed with magnetic microbeads pre-conjugated to an anti-biotin antibody. All magnetically labeled cell populations were then run over an LS column attached to a QuadroMACS Miltenyi Magnetic Stand and flow-throughs of interest were collected and counted. Isolation of CD4 T effector cells (CD4+CD25-) and/or CD4 T Regulatory cells (CD4+CD25+) was achieved by further labeling negatively selected CD4 T cells with an anti-CD25-PE antibody followed by incubation with anti-PE magnetic microbeads. Purity of the MACS sorted cell populations ranged from 90-95% as determined by flow cytometry.

Fluorescence Activated Cell Sorting (FACS)

Immediately following lymphocyte preparation, cells were resuspended in MACS Buffer and then stained with various panels of fluorophore-conjugated extracellular antibodies (listed in Conventional Flow Cytometry methods) to identify cell populations of interest. After staining, cells were resuspended at a concentration of 20 million cells per mL in MACS Buffer and then sorted via a BDFACSAriaIII (BD, San Jose, CA) housed, maintained, and operated by the Vanderbilt Flow Cytometry Core. T lymphocytes were sorted using a 70μm nozzle at a rate of 20 million cells per hour. Purity of the FACS sorted cell populations ranged from 97-100%.

Histology

For Formalin-Fixed, Paraffin Embedded (FFPE) histology, all samples were fixed overnight in a 10% formalin solution. Samples were then submitted to the Vanderbilt Translational Pathology Shared Resource (TPSR) for paraffin embedding, sectioning, and staining of tissue samples. In most cases, core services were provided for H&E, insulin, CD4, and Foxp3 staining of tolerant islet allografts using protocols developed and carried out by the TPSR. For fluorescent IHC, excised islet grafts were first fixed in a 4% PFA/PBS solution for 2 hours at room temperature and then soaked overnight at 4°C in a 30% sucrose/PBS mixture. Tissues were then frozen in OCT, cut into 6μm thick sections using a CryoStat, and then mounted on slides. Sections were fixed and permeabilized with a 1%FCS/1%Triton-X100 solution and islet graft tissue was identified using a primary rabbit polyclonal anti-insulin antibody followed by an anti-rabbit Fab2-Alexa488 (Cell Signaling Technologies, Danvers, MA) and infiltrating lymphocyte populations were identified using pre-conjugated anti-CD3-PE(145-2C11, BD), anti-CD4-eFluor570(4SM95, eBio), and anti-CD25-APC(PC61.5, eBio) antibodies. After staining, all

sections were mounted with Fluoromount-G with DAPI (eBio) and imaged using a Nikon Epi-fluorescence upright optical microscope at 20x.

Anti-Insulin IgG ELISA

To detect circulating anti-insulin IgG, 96-well MaxiSORP plates (eBioscience, Sand Diego, CA) were coated overnight at 4°C with 1 mg/mL human insulin (Sigma Aldrich, St. Lious, MO) diluted in Borate Buffered Saline. Mouse serum was diluted 1/100 in ELISA blocking buffer (PBS + 10%FBS + 0.05% Tween-20). Supernatant from the hybridoma mAb125, which produces anti-insulin IgG (kindly provided by Tom Thomas, Vanderbilt, Nashville, TN) was used as a positive control. Specific binding was verified by competition with 100μg/mL of free, unbound human insulin[343]. All samples were then counter-stained using a total anti-mouse IgG-biotin conjugated antibody mixed with Streptavidin-HRP (SouthernBiotech, Birmingham, AL) and developed with Pierce 1-Step Ultra TMB ELISA solution (ThermoFisher Scientific, Waltham, MA) followed by quenching with 2M HCl. Plates were then read at 450nm absorbance using a BioTek Automated Plate Reader (Winooski, VT).

***Ex vivo* Mixed Lymphocyte Reaction (MLR)**

One million splenocytes from B6 or B6.SLE123 mice (H-2b) were resuspended with one million Mitomycin-C pre-treated (50μg/mL, Sigma-Aldrich, St. Louis, MO) C3H splenocytes (H-2k) in CCM and incubated for 5 days in 96-well flat-bottom, tissue-cultured treated plates. Supernatants were then isolated and IL-6 and IFNγ levels were assessed as described in the Cytokine ELISAs section.

Cytokine ELISAs

IL-6 and IFNγ ELISA kits were purchased from BD Biosciences (San Jose, CA) and their instructions followed. Briefly, 96-well MaxiSORP plates (eBioscience, San Diego, CA) were coated overnight at 4°C with either an anti-IL-6 or anti-IFNγ capture antibody diluted at 1:250 in a freshly prepared Sodium Carbonate Buffer, pH 10. Plates were then blocked with ELISA blocking buffer (PBS + 10%FBS + 0.05% Tween-20), incubated with serum samples (neat), and counterstained with anti-IL-6 or anti-IFNγ biotin conjugated antibodies mixed with Streptavidin-HRP. Plates were then developed and read following the methods described in the anti-insulin ELISA methods section.

2-NBDG glucose uptake assay

Whole splenocytes from B6 and B6.SLE123 mice were plated at a concentration of 1 million cells per well in 96-well V-bottom plates in glucose-free CCM and cultured for 1 hour at 37°C. Cells were then supplemented with 30μM of the fluorescent glucose analogue 2-NBDG (Thermo-Fisher Scientific, Waltham, MA) and cultured for a further 30 minutes at 37°C. Splenocytes were then stained for antibodies directed against CD4 and analyzed by the methods described in the Conventional Flow Cytometry section.

Colony Forming Cell assay

Methylcellulose colony-forming (CFC) assays were performed via standard methods using materials from R&D Biosystems (Minneapolis, MN)(10). In brief, 5000 bone marrow cells from B6 and NDO mice were resuspended in IMDM/2%FBS at a 1:10 ratio with methylcellulose complete media containing human Epo (5IU/mL) and mouse IL-3 (10ng/mL), IL-6 (10ng/mL),

and SCF (50ng/mL). Cells were cultured for 8 days and colonies were counted by a blinded observer.

Osteoblast isolation, culture, and functional analysis by flow and RT-PCR

Bone marrow cells were differentiated to osteoblasts as previously described using 50μg/mL ascorbic acid and 5mM beta-glycerophosphate (Sigma Aldrich, St. Louis, MO). Cultured osteoblasts were exposed to 100ng/mL rmG-CSF (R&D), 50μg/mL anti-CD45RB (BioXCell, West Lebanon, NH), or media alone (control) for 24 hours. Cells were analyzed by flow cytometry or RT-PCR (for primer sets used see Table 2). Individual gene expression was normalized to GAPDH and relative gene expression changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

***Ex vivo* CD4 Treg suppression assay**

CD4+CD25- effector T cells and CD4+CD25+ Tregs were isolated from B6 or B6.SLE123 spleens using a CD4+CD25+ regulatory T cell isolation kit (Miltenyi, San Diego, CA). Effector CD4 T cells and CD4 Tregs were resuspended in Cell Culture Media (DMEM + 10%FCS + 1%Pen/Strep) and plated in 96 well tissue-culture treated, round-bottom plates (Corning Inc., Corning, NY) at Treg:T effector ratios ranging from 1:2 to 1:8[258]. Cells were stimulated with anti-CD3 (1 μg/ml, 145-2C11, BD, San Jose, CA) and anti-CD28 (1 μg/ml, 37.51, BD, San Jose, CA) and incubated for 72h. For TGFβ suppression assays, splenocytes were incubated with anti-CD3/CD28 (1 mg/ml) +/- 10 mg/ml TGFβ (R&D Systems, Minneapolis, MN) for 72h. Phosphorylation of the TGFβ signaling components SMAD within TGFβ stimulated CD4 T cells was assessed using antibodies directed at pSMAD2/3(D27F4) or pSMAD1/5/8(D5B10) (Cell

Signaling Technologies, Danvers, MA). Supernatants were collected and analyzed for IFNγ by ELISA (BD, San Jose, CA). The percent suppression was calculated as follows: [(IFNγ production in each condition/IFNγ T effector cells only)*100].

***In vivo* CD8 Treg suppression assay**

CD8 Treg donor mice were injected intraperitoneally (i.p.) with 100 mg of NP_{33}-KLH (BioSearch Technologies, Petaluma, CA) emulsified in Complete Freund's Adjuvant (Sigma Aldrich, St. Louis, MO). Seven days later, splenic CD8+ T cells were isolated magnetically (MACS) (Ly-2, Miltenyi, San Diego, CA) and then sorted fluorescently (FACS) to select for CD8 Treg (CD8+CD122+Ly49+) and non-CD8 Treg (CD8+CD122+Ly49-) populations (BD FACsAria III). In some cases, classically-defined CD8 Tregs (CD8+CD122+) and non-CD8 Tregs (CD8+CD122-) were also sorted via FACS for other analyses. FACS-sorted CD8 Tregs or non-CD8 Tregs were then i.v. injected into recipient RAG mice. Reconstituted RAG mice then received MACS purified splenic B Cells (2 million) and CD4+CD25- T Cells (1 million) from naïve, unimmunized donors. Mice were injected i.p. with 100 mg of NP_{33}-KLH/CFA. Ten days after initial immunization, mice were boosted with 50 mg of NP_{33}-KLH emulsified in Incomplete Freund's Adjuvant (Sigma Aldrich, St. Louis, MO). Seven days after boosting, the anti-NP_8 IgG response was measured via ELISA (NP_8-BSA, BioSearch Technologies, Petaluma, CA; anti-IgG [goat anti-mouse polyclonal] was purchased from Southern Biotech (Birmingham, AL) over a Log_2 serum dilution. This experimental assay was adapted from work published by the Cantor group (Harvard, MA) and is depicted in Figure 3.1A[124].

Alloimmunization and alloantibody titer analysis

Twenty million splenocytes from Major Histocompatibility Complex (MHC) mismatched C3H mice (H-2k) were intravenously injected (i.v.) into recipient B6 and B6.SLE123 mice (H-2b) or NOD mice (H2-g7). Sera was isolated on days 0, 7, 14, 21, and 28 and incubated with target C3H splenocytes at a 1:25 dilution. Splenocytes were stained with antibodies directed at CD3e(145-2C11), IgM(II/41), IgG1(A85-1), IgG2a(m2a-15F8), IgG2b(m2b-25G4), and IgG3 (SB76b) (Southern Biotech, Birmingham, AL). The Median Fluorescence Intensity (MFI) of bound mouse Ig on target C3H CD3 T cells was used to assess relative alloantibody titer.

Adoptive transfer of diabetes

Immunodeficient NOD.RAG mice received i.v. injections of 5-10 million CD8 T cell depleted MACS splenocytes from diabetic NOD mice (blood sugars >300mg/dL). Blood glucose levels of recipient NOD.RAG mice were recorded every 3 days via an AccuChek Blood Glucose Monitor (Roche Diagnostics, Basel Switzerland). Two consecutive blood glucose levels > 250mg/dL were used to denote diabetes onset.

Streptozotocin induced diabetes

Fasting mice were injected with at a single dose of 180-225mg/kg Streptozotocin (Sigma Aldrich, St. Louis, MO) dissolved in ice-cold Sodium Citrate Buffer (pH 4.5), as previously described[344]. Two days after injection, blood glucose levels were checked via an AccuChek Blood Glucose Monitor (Roche Diagnostics, Basel Switzerland). Only those mice with blood glucose levels > 300mg/dL were included in islet transplantation studies.

Donor islet harvest

Eight to ten week old female Balb/c (H-2d) or C3H (H-2k) donor mice were euthanized via isoflurane overdose followed by cervical dislocation (of note, only female islets donors were used to avoid reactivity to the male H-Y antigen)[345]. A V-shaped incision through the skin and muscle layers was then made on the ventral side of the mice starting at the pubis and extending toward the axilla. Using cotton-tipped swabs, the intestines were then reflected to the mouse's left side to reveal the posterior aspect of the stomach and duodenum. Under a Nikon dissecting microscope, a curve-tipped hemostat was then clamped across the duodenum over the Ampulla of Vater through which the Common Bile Duct empties its contents. A 0.5inch, 30 gauge needle attached to 5mL syringe filled with 3-4mL of an ice cold 0.5mg/mL Collagenase P (Roche, Basel, Switzerland) solution diluted in HBSS (with calcium and magnesium) was then inserted into the Common Bile Duct at the confluence of the Cystic Duct and Hepatic Duct. Upon visual confirmation of successful catheterization, 3-4mLs of the HBSS/Collagenase P solution was injected to inflate the pancreas. The inflated pancreatic tissue was then carefully dissected away from the surrounding tissue and place in a 15mL conical tube containing 7mL of the HBSS/Collagenase P solution. Inflated pancreases were then manually disrupted via a 6-minute hand shake in a 37°C water bath followed by a 2-minute hand shake at room temperature. The Collagenase-P solution was then quenched with HBSS (with calcium and magnesium) supplemented with 3%FCS, washed three times with the same solution, underlaid with 2.5mL of a 1.077 g/mL Histopaque solution (Sigma Aldrich, St. Lious, MO) via a Pastuer Pipette, and then centrifuged at 1850rpm for 16 minutes. Islets floating above the Histopaque layer (the dissociated acinar tissue having spun to the bottom of the conical tube) were then removed via transfer pipette, hand picked by pipette, placed into Islet Culture Media (RPMI1640 w/

glutamine + 10%FBS + 1% Pen/Step), and cultured overnight at 37°C/5%CO2 in a cell culture incubator.

Islet transplantation and anti-CD45RB mediated tolerance induction

Sub-capsular renal islet transplantation was carried out as previously described[133]. Briefly, chemically diabetic mice (on either the B6[H2-b] or NOD[H2-g7] backgrounds) were transplanted with at minimum 400 female MHC-mismatched C3H(H2-k) or Balb/c(H2-d) islets under their left kidney capsule. Cultured islets suspended in 37°C Islet Culture Media were delivered to anesthetized mice using a 1cc tuberculin syringe fitted with a beveled PE50 tube. Treated mice were injected with 100μg of anti-CD45RB antibody (BioXCell, West Lebanon, NH) on days 0, 1, 3, 5, and 7 after transplantation. In some cases, mice also received the following agents to alter tolerance induction: the CD4 Treg depleting anti-CD25 (PC61, BioXCell, West Lebanon, NH, 500μg spaced 5 days apart), the HSC-depleting antibody ACK2 (Tocris Biosciences, Bristol, UK, 500μg on day -1), an anti-IL-6R blocking antibody (BioXCell, West Lebanon, NH, 500μg on days -3, -1, 1, and 3), the CXCR4 antagonist AMD3100 (Sigma-Aldrich, St. Louis, MO, 100μg on days -4, -3, -2, -1, 0), the anti-sympathetic agent 6-OHD (Sigma-Aldrich, St. Louis, MO, 100mg/kg on day -4 and 250mg/kg on day -2), the JAK3 inhibitor Tofacitinib (SelleckChem, Houston, TX), delivered by a subcutaneous Alzet Osmotic Pump implanted on day -5 (Model AP2002; 14 day delivery at 0.5μl/hr, filled with a 50% water / 50% DMSO Tofacitinib solution at 50mg/mL), or drinking water supplemented with the anti-metabolic agents 2-deoxy-D-gluocse (5mg/mL, Sigma Aldrich, St. Lious, MO) and Metformin (3mg/mL, Enzo Life Sciences, Farmingdale, NY) given from days -7 to 14. Successful islet engraftment was confirmed by a blood glucose reading of <140 mg/dL the day after

transplantation and graft rejection was recorded when recipient mice demonstrated glucose readings above 250 mg/dL on 2 consecutive days. Recipient blood glucose readings were recorded every 3 days.

Survival nephrectomy and islet re-transplantation

Mice demonstrating greater than 100 days of tolerance to islet allografts were anesthetized with a Ketamine/Xylazine solution and anesthesia was confirmed by toe-pinch. A dorsal incision was made over left flank and an incision was made through both skin and muscle layers using fine tipped scissors. Surgical adhesions formed from the initial transplantation surgery were carefully dissected away using micro-vannas scissors until the islet-graft containing kidney was fully free from surrounding tissue. A pair of straight-tipped hemostats was then clamped across the renal vessels of the kidney and a 6-0 silk suture was used to permanently ligate the renal vessels using 4 alternating surgeon-knots under the hemostats. The graft kidney was then cut away using a number 11 scalpel blade above the hemostat, which was then stored in a 10% formalin solution for histology. The hemostats were then unclamped slowly to ensure successful ligation prior to closure. A 5-0 absorbable suture was then used to close the muscle layer using a continuous running pattern and the skin incision closed via dermal staples. A rapid return to hyperglycemia (blood glucose > 250mg/dL) by day 2 was used to confirm islet graft function vs. endogenous beta cell regeneration. To test whether animals were in fact tolerant to the original allograft, mice were retransplanted in their contralateral kidney with either a MHC-matched [C3H(H2-k)] or 3rd party MHC-mismatched [Balb/c(H2-d)] islet allograft in the absence of any further treatment following the methods described above. Blood sugars were recorded every 3 days and blood glucose readings >250mg/dL on 2 consecutive days were used to denote rejection.

Generation of chimeric mice and bone marrow transplantation

Seven days prior to irradiation, recipient NOD.RAG mice were given drinking water supplemented with 100μg/mL Neomycin and Polymyxin B antibiotic solution (XGEN Pharmaceuticals, Horseheads, NY). On the day of bone marrow transplantation, recipient NOD.RAG mice were whole body-irradiated with a low-dose of 400 rads using a Radioactive Cesium source. 6-8 hours after irradiation, recipient mice were anesthetized with a Ketamine/Xylazine solution and then received an i.v. injection of a total of 5 million T and B cell MACS depleted bone marrow cells. Recipient groups included NOD marrow only, NODμMT marrow only, NODμMT (80%) + NOD (20%) marrow, or NODμMT (80%) + NOD.H2-$Ab^{-/-}$ (20%) marrow[177]. Eight weeks after bone marrow transplantation, PBMCs from these transplanted mice were analyzed via flow cytometry to evaluate reconstitution efficiency (Figure 2.9). Only fully reconstituted mice were used in further islet transplantation experiments.

Statistics

Statistical analysis was performed with GraphPad Prism V6 (La Jolla, CA), using the student's t-test for comparison of two normally-distributed conditions. One or two-way ANOVA followed by Bonferroni post-test was used to compare multiple groups. In cases of non-normally distributed data, data was compared by a Mann-Whitney Test for two conditions or by a Kruskal-Wallis Test for multiple groups. Analysis of the anti-NP response was analyzed via performing a semi-logarithmic linear regression analysis followed by y-intercept and slope curve comparison. Diabetes onset or islet graft rejection was graphed as Kaplan Meier curve and compared by Log-rank statistical analysis. Error bars denote the Standard Error of the Mean. Statistical comparisons with p-values <0.05 were deemed significant.

REFERENCES

1. Starzl, T. E. The Landmark Identical Twin Case. *JAMA* **251,** 2572–2573 (1984).
2. Joseph E Murray, M. D. *Surgery of the Soul*. (Science History Publications, 2004).
3. Joseph E. Murray - Nobel Lecture: The First Successful Organ Transplants in Man. http://www.nobelprize.org/nobel_prizes/medicine/laureates/1990/murray-lecture.html
4. Starzl, T. E. & Zinkernagel, R. M. Transplantation tolerance from a historical perspective. *Nat. Rev. Immunol.* **1,** 233–239 (2001).
5. Mathe, G., Amiel, J.L., Schwarzenberg, L., Cattan, A. & Schneider, M. Heamatopoietic chimera in man after allogenic (homologous) bone-marrow transplantation. *Br Med J* **2,** 1633–1635 (1963).
6. Schwartz, R. & Dameshek, W. Drug-induced immunological tolerance. *Nature* **183,** 1682–1683 (1959).
7. Murray, J. E., Merrill, J. P., Harrison, J. H., WILSON, R. E. & DAMMIN, G. J. Prolonged survival of human-kidney homografts by immunosuppressive drug therapy. *N. Engl. J. Med.* **268,** 1315–1323 (1963).
8. Starzl, T. E., Marchioro, T. L. & Waddell, W. R. The reversal of rejection in human renal homografts with subsequent development of homograft tolerance. *Surg Gynecol Obstet* **117,** 385–395 (1963).
9. Starzl, T. L. The use of heterologous antilymphoid agents in canine renal and liver homotransplantation and in human renal homotransplantation. *Surg Gynecol Obstet* **124,** 301 (1967).
10. Terasaki, P. I., Mickey, M. R. & Singal, D. P. Serotyping for Homotransplantation: Selection of Recipients for Cadaver Donor Transplants. *NEJM* (1968).
11. Sheldon, S. & Poulton, K. HLA typing and its influence on organ transplantation. *Methods Mol. Biol.* **333,** 157–174 (2006).
12. Starzl, T. L. Homotransplantation of the liver in humans. *Surg Gynecol Obstet* **117,** 659 (1963).
13. Kelly, W. D., Lillehei, R. C., Merkel, F. K., Idezuki, Y. & Goetz, F. C. Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. *Surgery* **61,** 827–837 (1967).
14. Barnard, C. N. The operation. A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town. *S. Afr. Med. J.* **41,** 1271–1274 (1967).
15. Cosimi, A. B., Shield, C. F., Peters, C., Burton, R. C. & Scott, G. Prolongation of allograft survival by cyclosporin A. *Surgical Forum* **30**, 287 (1979).
16. Calne, R. Y., White, D. J., Thiru, S. & Evans, D. B. Cyclosporin A in clinical kidney grafting from cadaver donors. *Proc. Eur. Dial. Transplant Assoc.* **16,** 3051 (1979).
17. Schreiber, S. L. & Crabtree, G. R. The mechanism of action of cyclosporin A and FK506. *Immunology Today* **13,** 136–142 (1992).
18. Goto, T., Kino, T., Hatanaka, H., Nishiyama, M., Okuhara, M., Kohsaka, M., Aoki, H. & Imanaka, H. Discovery of FK-506, a novel immunosuppressant isolated from Streptomyces tsukubaensis. *Transplant. Proc.* **19,** 4–8 (1987).
19. Fulton, B. & Markham, A. Mycophenolate mofetil. A review of its pharmacodynamic and pharmacokinetic properties and clinical efficacy in renal transplantation. *Drugs* **51,**

278–298 (1996).
20. Dumont, F. J. & Su, Q. Mechanism of action of the immunosuppressant rapamycin. *Life sciences* (1995).
21. Nashan, B., Moore, R., Amlot, P., Schmidt, A. G., Abeywickrama, K. & Soulillou, J.-P. Randomised trial of basiliximab versus placebo for control of acute cellular rejection in renal allograft recipients. CHIB 201 International Study Group. *The Lancet* **350,** 1193–1198 (1997).
22. Friend, P. J., Hale, G., Waldmann, H., Gore, S., Thiru, S., Joysey, V., Evans, D. B. & Calne, R. Y. Campath-1M--prophylactic use after kidney transplantation. A randomized controlled clinical trial. *Transplantation* **48,** 248–253 (1989).
23. Medawar, P. B. The behaviour and fate of skin autografts and skin homografts in rabbits: A report to the War Wounds Committee of the Medical Research Council. *J. Anat*. **78,** 176–199 (1944).
24. Gorer, P. A., Lyman, S. & Snell, G. D. Studies on the Genetic and Antigenic Basis of Tumour Transplantation. Linkage between a Histocompatibility Gene and 'Fused' in Mice. *Proceedings of the Royal Society B: Biological Sciences* **135,** 499–505 (1948).
25. Brent, L. *A History of Transplantation Immunology*. (Academic Press, 1996).
26. Mitchison, N. A. Passive Transfer of Transplantation Immunity. *Proceedings of the Royal Society B: Biological Sciences* **142,** 72–87 (1954).
27. McDevitt, H. O. & Chinitz, A. Genetic control of the antibody response: relationship between immune response and histocompatibility (H-2) type. *Science* **163,** 1207–1208 (1969).
28. Zinkernagel, R. M. & Doherty, P. C. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248,** 701–702 (1974).
29. Cerottini, J. C., Nordin, A. A. & Brunner, K. T. In vitro cytotoxic activity of thymus cells sensitized to alloantigens. *Nature* **227,** 72–73 (1970).
30. Rogers, N. J. & Lechler, R. I. Allorecognition. *Am. J. Transplant*. **1,** 97–102 (2001).
31. Suchin, E. J., Langmuir, P. B., Palmer, E., Sayegh, M. H., Wells, A. D. & Turka, L. A. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J. Immunol*. **166,** 973–981 (2001).
32. Lechler, R. & Lombardi, G. The structural basis of alloreactivity. *Immunol. Res*. **9,** 135–146 (1990).
33. Pietra, B. A., Wiseman, A., Bolwerk, A., Rizeq, M. & Gill, R. G. CD4 T cell–mediated cardiac allograft rejection requires donor but not host MHC class II. *J. Clin. Invest*. **106,** 1003–1010 (2000).
34. Brennan, T. V., Jaigirdar, A., Hoang, V., Hayden, T., Liu, F. C., Zaid, H., Chang, C. K., Bucy, R. P., Tang, Q. & Kang, S.-M. Preferential Priming of Alloreactive T Cells with Indirect Reactivity. *American Journal of Transplantation* **9,** 709–718 (2009).
35. Auchincloss, H., Lee, R., Shea, S., Markowitz, J. S., Grusby, M. J. & Glimcher, L. H. The role of 'indirect' recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proceedings of the National Academy of Sciences* **90,** 3373–3377 (1993).
36. Mulder, A., Eijsink, C., Kardol, M. J., Franke-van Dijk, M. E. I., van der Burg, S. H., Kester, M., Doxiadis, I. I. N. & Claas, F. H. J. Identification, Isolation, and Culture of HLA-A2-Specific B Lymphocytes Using MHC Class I Tetramers. *The Journal of*

Immunology **171,** 6599–6603 (2003).
37. Chen, J., Yin, H., Xu, J., Wang, Q., Edelblum, K. L., Sciammas, R. & Chong, A. S. Reversing Endogenous Alloreactive B Cell GC Responses With Anti-CD154 or CTLA-4Ig. *American Journal of Transplantation* **13,** 2280–2292 (2013).
38. Mulder, A., Kardol, M. J. & Kamp, J. Determination of the frequency of HLA antibody secreting B-lymphocytes in alloantigen sensitized individuals *Clinical & Experimental Immunology*. **124,** 9-15 (2001).
39. Colvin, R. B. & Smith, R. N. Antibody-mediated organ-allograft rejection. *Nat. Rev. Immunol*. **5,** 807–817 (2005).
40. Zeng, Q., Ng, Y.-H., Singh, T., Jiang, K., Sheriff, K. A., Ippolito, R., Zahalka, S., Li, Q., Randhawa, P., Hoffman, R. A., Ramaswami, B., Lund, F. E. & Chalasani, G. B cells mediate chronic allograft rejection independently of antibody production. *J. Clin. Invest*. **124,** 1052–1056 (2014).
41. Springer, T. A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* (1994).
42. Shulman, Z., Shinder, V., Klein, E., Grabovsky, V. & Yeger, O. Lymphocyte Crawling and Transendothelial Migration Require Chemokine Triggering of High-Affinity LFA-1 Integrin. *Immunity* (2009).
43. Setoguchi, K., Schenk, A. D., Ishii, D., Hattori, Y., Baldwin, W. M., III, Tanabe, K. & Fairchild, R. L. LFA-1 Antagonism Inhibits Early Infiltration of Endogenous Memory CD8 T Cells into Cardiac Allografts and Donor-Reactive T Cell Priming. *American Journal of Transplantation* **11,** 923–935 (2011).
44. Kitchens, W. H., Haridas, D., Wagener, M. E., Song, M., Kirk, A. D., Larsen, C. P. & Ford, M. L. Integrin Antagonists Prevent Costimulatory Blockade-Resistant Transplant Rejection by CD8+ Memory T Cells. *American Journal of Transplantation* **12,** 69–80 (2011).
45. Hancock, W. W., Gao, W., Faia, K. L. & Csizmadia, V. Chemokines and their receptors in allograft rejection. *Curr. Opin. Immunol*. **12,** 511–516 (2000).
46. Halloran, P. F. & Fairchild, R. L. The Puzzling Role of CXCR3 and Its Ligands in Organ Allograft Rejection. *American Journal of Transplantation* **8,** 1578–1579 (2008).
47. Oberbarnscheidt, M. H., Walch, J. M., Li, Q., Williams, A. L., Walters, J. T., Hoffman, R. A., Demetris, A. J., Gerard, C., Camirand, G. & Lakkis, F. G. Memory T Cells Migrate to and Reject Vascularized Cardiac Allografts Independent of the Chemokine Receptor CXCR3. *Transplantation* **91,** 827–832 (2011).
48. Walch, J. M., Zeng, Q., Li, Q., Oberbarnscheidt, M. H., Hoffman, R. A., Williams, A. L., Rothstein, D. M., Shlomchik, W. D., Kim, J. V., Camirand, G. & Lakkis, F. G. Cognate antigen directs CD8+ T cell migration to vascularized transplants. *J. Clin. Invest*. **123,** 2663–2671 (2013).
49. Delfs, M. W., Furukawa, Y., Mitchell, R. N. & Lichtman, A. H. CD8+ T Cell subets Tc1 and Tc2 cause different histopathologic forms of murine cardiac allograft rejection. *Transplantation* **71,** 606 (2001).
50. Jones, N.D.T-cell activation, proliferation, and memory after cardiac transplantation in vivo. *Annals of Surgery* **229,** 570 (1999).
51. Diamond, A. S. & Gill, R. G. An Essential Contribution by IFN- to CD8+ T Cell-Mediated Rejection of Pancreatic Islet Allografts. *The Journal of Immunology* **165,** 247–255 (2000).

52. Nagano, H., Mitchell, R. N., Taylor, M. K., Hasegawa, S., Tilney, N. L. & Libby, P. Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J. Clin. Invest.* **100,** 550–557 (1997).
53. Kapoor, A., Morita, K., Engeman, T. M., Koga, S., Vapnek, E. M., Hobart, M. G. & Fairchild, R. L. Early expression of interferon-γ inducible protein 10 and monokine induced interferon-γ in cardiac allografts is mediated by CD8+ T cells. *Transplantation* **69,** 1147 (2000).
54. Strehlau, J., Pavlakis, M., Lipman, M., Shapiro, M., Vasconcellos, L., Harmon, W. & Strom, T. B. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *PNAS,* **94**, 695-700 (1997).
55. Kamoun, M. Cellular and molecular parameters in human renal allograft rejection. *Clinical biochemistry* (2001).
56. Zimmerer, J. M., Pham, T. A., Wright, C. L., Tobin, K. J., Sanghavi, P. B., Elzein, S. M., Sanders, V. M. & Bumgardner, G. L. Alloprimed CD8 +T Cells Regulate Alloantibody and Eliminate Alloprimed B Cells Through Perforin- and FasL-Dependent Mechanisms. *American Journal of Transplantation* **14,** 295–304 (2014).
57. Bueno, V. & Pestana, J. O. M. The role of CD8+ T cells during allograft rejection. *Braz J Med Biol Res* **35,** (2002).
58. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136,** 2348–2357 (1986).
59. Liu, Z., Fan, H. & Jiang, S. CD4(+) T-cell subsets in transplantation. *Immunol. Rev.* **252,** 183–191 (2013).
60. Jiang, S., Herrera, O. & Lechler, R. I. New spectrum of allorecognition pathways: implications for graft rejection and transplantation tolerance. *Curr. Opin. Immunol.* **16,** 550–557 (2004).
61. Waaga, A. M., Gasser, M., Kist-van Holthe, J. E., Najafian, N., Müller, A., Vella, J. P., Womer, K. L., Chandraker, A., Khoury, S. J. & Sayegh, M. H. Regulatory functions of self-restricted MHC class II allopeptide-specific Th2 clones in vivo. *J. Clin. Invest.* **107,** 909–916 (2001).
62. Zelenika, D., Adams, E., Mellor, A., Simpson, E., Chandler, P., Stockinger, B., Waldmann, H. & Cobbold, S. P. Rejection of H-Y Disparate Skin Grafts by Monospecific CD4+ Th1 and Th2 Cells: No Requirement for CD8+ T Cells or B Cells. *The Journal of Immunology*. **161,** 1868-1874 (1998).
63. Barbara, J. A., Turvey, S. E., Kingsley, C. I., Spriewald, B. M., Hara, M., Witzke, O., Morris, P. J. & Wood, K. J. Islet allograft rejection can be mediated by CD4+, alloantigen experienced, direct pathway T cells of TH1 and TH2 cytokine phenotype. *Transplantation* **70,** 1641–1649 (2000).
64. Sabet-Baktach, M., Eggenhofer, E., Rovira, J., Renner, P., Lantow, M., Farkas, S. A., Malaisé, M., Edtinger, K., Shaotang, Z., Koehl, G. E., Dahlke, M. H., Schlitt, H. J., Geissler, E. K. & Kroemer, A. Double deficiency for RORγt and T-bet drives Th2-mediated allograft rejection in mice. *The Journal of Immunology* **191,** 4440–4446 (2013).
65. Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A. & Cua, D. J. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201,** 233–240 (2005).

66. Van Kooten, C., Boonstra, J. G., Paape, M. E., Fossiez, F., Banchereau, J., Lebecque, S., Bruijn, J. A., De Fijter, J. W., Van Es, L. A. & Daha, M. R. Interleukin-17 activates human renal epithelial cells in vitro and is expressed during renal allograft rejection. *J. Am. Soc. Nephrol.* **9,** 1526–1534 (1998).
67. Snell, G. I., Levvey, B. J., Zheng, L., Bailey, M., Orsida, B., Williams, T. J. & Kotsimbos, T. C. Interleukin-17 and Airway Inflammation: A Longitudinal Airway Biopsy Study After Lung Transplantation. *The Journal of Heart and Lung Transplantation* **26,** 669–674 (2007).
68. Fábrega, E., López-Hoyos, M., San Segundo, D., Casafont, F. & Pons-Romero, F. Changes in the serum levels of interleukin-17/interleukin-23 during acute rejection in liver transplantation. *Liver Transpl.* **15,** 629–633 (2009).
69. Yuan, X., Paez-Cortez, J., Schmitt-Knosalla, I., D'Addio, F., Mfarrej, B., Donnarumma, M., Habicht, A., Clarkson, M. R., Iacomini, J., Glimcher, L. H., Sayegh, M. H. & Ansari, M. J. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J. Exp. Med.* **205,** 3133–3144 (2008).
70. Miura, M., El-Sawy, T. & Fairchild, R. L. Neutrophils Mediate Parenchymal Tissue Necrosis and Accelerate the Rejection of Complete Major Histocompatibility Complex-Disparate Cardiac Allografts in the Absence of Interferon-γ. *The American journal of pathology* (2003).
71. Faust, S. M., Lu, G., Marini, B. L., Zou, W., Gordon, D., Iwakura, Y., Laouar, Y. & Bishop, D. K. Role of T cell TGFbeta signaling and IL-17 in allograft acceptance and fibrosis associated with chronic rejection. *The Journal of Immunology* **183,** 7297–7306 (2009).
72. Fan, L., Benson, H. L., Vittal, R., Mickler, E. A., Presson, R., Fisher, A. J., Cummings, O. W., Heidler, K. M., Keller, M. R., Burlingham, W. J. & Wilkes, D. S. Neutralizing IL-17 prevents obliterative bronchiolitis in murine orthotopic lung transplantation. *Am. J. Transplant.* **11,** 911–922 (2011).
73. Shi, G., Field, D. J., Ko, K.-A., Ture, S., Srivastava, K., Levy, S., Kowalska, M. A., Poncz, M., Fowell, D. J. & Morrell, C. N. Platelet factor 4 limits Th17 differentiation and cardiac allograft rejection. *J. Clin. Invest.* **124,** 543–552 (2014).
74. Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M. & Förster, R. Follicular B Helper T Cells Express Cxc Chemokine Receptor 5, Localize to B Cell Follicles, and Support Immunoglobulin Production. *J. Exp. Med.* **192,** 1545–1552 (2000).
75. Conlon, T. M., Saeb-Parsy, K., Cole, J. L., Motallebzadeh, R., Qureshi, M. S., Rehakova, S., Negus, M. C., Callaghan, C. J., Bolton, E. M., Bradley, J. A. & Pettigrew, G. J. Germinal Center Alloantibody Responses Are Mediated Exclusively by Indirect-Pathway CD4 T Follicular Helper Cells. *The Journal of Immunology* **188,** 2643–2652 (2012).
76. Petrelli, A., Carvello, M., Vergani, A., Lee, K. M., Tezza, S., Du, M., Kleffel, S., Chengwen, L., Mfarrej, B. G., Hwu, P., Secchi, A., Leonard, W. J., Young, D., Sayegh, M. H., Markmann, J. F., Zajac, A. J. & Fiorina, P. IL-21 Is an Antitolerogenic Cytokine of the Late-Phase Alloimmune Response. *Diabetes* (2011).
77. de Graav, G. N., Dieterich, M., Hesselink, D. A., Boer, K., Clahsen-van Groningen, M. C., Kraaijeveld, R., Litjens, N. H. R., Bouamar, R., Vanderlocht, J., Tilanus, M., Houba, I., Boonstra, A., Roelen, D. L., Claas, F. H. J., Betjes, M. G. H., Weimar, W. & Baan, C.

C. Follicular T helper cells and humoral reactivity in kidney transplant patients. *Clin. Exp. Immunol.* **180,** 329–340 (2015).
78. Brändle, D., Joergensen, J., Zenke, G., Bürki, K. & Hof, R. P. Contribution of donor-specific antibodies to acute allograft rejection: Evidence from B Cell-Deficient Mice. *Transplantation* **65,** 1489 (1998).
79. Thaunat, O., Patey, N., Caligiuri, G., Gautreau, C., Mamani-Matsuda, M., Mekki, Y., Dieu-Nosjean, M. C., Eberl, G., Ecochard, R., Michel, J. B., Graff-Dubois, S. & Nicoletti, A. Chronic Rejection Triggers the Development of an Aggressive Intragraft Immune Response through Recapitulation of Lymphoid Organogenesis. *The Journal of Immunology* **185,** 717–728 (2010).
80. Noorchashm, H., Reed, A. J., Rostami, S. Y., Mozaffari, R., Zekavat, G., Koeberlein, B., Caton, A. J. & Naji, A. B Cell-Mediated Antigen Presentation Is Required for the Pathogenesis of Acute Cardiac Allograft Rejection. *The Journal of Immunology* **177,** 7715–7722 (2006).
81. Vo, A. A., Lukovsky, M., Toyoda, M., Wang, J., Reinsmoen, N. L., Lai, C.-H., Peng, A., Villicana, R. & Jordan, S. C. Rituximab and intravenous immune globulin for desensitization during renal transplantation. *N. Engl. J. Med.* **359,** 242–251 (2008).
82. DiLillo, D. J., Griffiths, R., Seshan, S. V., Magro, C. M., Ruiz, P., Coffman, T. M. & Tedder, T. F. B Lymphocytes Differentially Influence Acute and Chronic Allograft Rejection in Mice. *The Journal of Immunology* **186,** 2643–2654 (2011).
83. Carvello, M., Petrelli, A., Vergani, A., Lee, K. M., Tezza, S., Chin, M., Orsenigo, E., Staudacher, C., Secchi, A., Dunussi-Joannopoulos, K., Sayegh, M. H., Markmann, J. F. & Fiorina, P. Inotuzumab ozogamicin murine analog-mediated B-cell depletion reduces anti-islet allo- and autoimmune responses. *Diabetes* **61,** 155–165 (2012).
84. Lee, K. M., Yeh, H., Zhao, G., Wei, L., O'Connor, M., Stott, R. T., Soohoo, J., Dunussi, K., Fiorina, P., Deng, S., Markmann, J. F. & Kim, J. I. B cell depletion improves islet allograft survival with anti-CD45RB. *Cell Transplant* (2012).
85. Parsons, R. F., Yu, M., Vivek, K., Zekavat, G., Rostami, S. Y., Ziaie, A. S., Luo, Y., Koeberlein, B., Redfield, R. R., Ward, C. D., Migone, T.-S., Cancro, M. P., Naji, A. & Noorchashm, H. Murine Islet Allograft Tolerance Upon Blockade of the B-Lymphocyte Stimulator, BLyS/BAFF. *Transplantation* **93,** 676–685 (2012).
86. van den Hoogen, M. W. F., Kamburova, E. G., Baas, M. C., Steenbergen, E. J., Florquin, S., M Koenen, H. J. P., Joosten, I. & Hilbrands, L. B. Rituximab as Induction Therapy After Renal Transplantation: A Randomized, Double-Blind, Placebo-Controlled Study of Efficacy and Safety. *American Journal of Transplantation* **15,** 407–416 (2015).
87. Peter Medawar - Nobel Lecture: Immunological Tolerance. http://www.nobelprize.org/nobel_prizes/medicine/laureates/1960/medawar-lecture.html
88. Billingham, R. E., Brent, L. & Medawar, P. B. Actively acquired tolerance of foreign cells. *Nature* **172,** 603–606 (1953).
89. Main, J. M. & Prehn, R. T. Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow. *J. Natl. Cancer Inst.* **15,** 1023–1029 (1955).
90. Cosimi, A. B., Burton, R. C., Colvin, R. B., Goldstein, G., Delmonico, F. L., LaQuaglia, M. P., Tolkoff-Rubin, N., Rubin, R. H., Herrin, J. T. & Russell, P. S. Treatment of acute renal allograft rejection with OKT3 monoclonal antibody. *Transplantation* **32,** 535–539 (1981).

91. Mackie, J. D., Pankewycz, O. G., Bastos, M. G., Kelley, V. E. & Strom, T. B. Dose-related mechanisms of immunosuppression mediated by murine anti-CD3 monoclonal antibody in pancreatic islet cell transplantation and delayed-type hypersensitivity. *Transplantation* **49,** 1150–1154 (1990).
92. Isobe, M., Yagita, H., Okumura, K. & Ihara, A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* **255,** 1125–1127 (1992).
93. Larsen, C. P., Elwood, E. T., Alexander, D. Z., Ritchie, S. C., Hendrix, R., Tucker-Burden, C., Cho, H. R., Aruffo, A., Hollenbaugh, D., Linsley, P. S., Winn, K. J. & Pearson, T. C. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* **381,** 434–438 (1996).
94. Lazarovits, A. I., Poppema, S., Zhang, Z., Khandaker, M., Le Feuvre, C. E., Singhal, S. K., Garcia, B. M., Ogasa, N., Jevnikar, A. M., White, M. H., Singh, G., Stiller, C. R. & Zhong, R. Z. Prevention and reversal of renal allograft rejection by antibody against CD45RB. *Nature* **380,** 717–720 (1996).
95. Gao, Z., Zhong, R., Jiang, J., Garcia, B., Xing, J. J., White, M. J. & Lazarovits, A. I. Adoptively transferable tolerance induced by CD45RB monoclonal antibody. *J. Am. Soc. Nephrol.* **10,** 374–381 (1999).
96. Changelian, P. S., Flanagan, M. E., Ball, D. J., Kent, C. R., Magnuson, K. S., Martin, W. H., Rizzuti, B. J., Sawyer, P. S., Perry, B. D., Brissette, W. H., McCurdy, S. P., Kudlacz, E. M., Conklyn, M. J., Elliott, E. A., Koslov, E. R., Fisher, M. B., Strelevitz, T. J., Yoon, K., Whipple, D. A., Sun, J., Munchhof, M. J., Doty, J. L., Casavant, J. M., Blumenkopf, T. A., Hines, M., Brown, M. F., Lillie, B. M., Subramanyam, C., Shang-Poa, C., Milici, A. J., Beckius, G. E., Moyer, J. D., Su, C., Woodworth, T. G., Gaweco, A. S., Beals, C. R., Littman, B. H., Fisher, D. A., Smith, J. F., Zagouras, P., Magna, H. A., Saltarelli, M. J., Johnson, K. S., Nelms, L. F., Etages, Des, S. G., Hayes, L. S., Kawabata, T. T., Finco-Kent, D., Baker, D. L., Larson, M., Si, M.-S., Paniagua, R., Higgins, J., Holm, B., Reitz, B., Zhou, Y.-J., Morris, R. E., O'Shea, J. J. & Borie, D. C. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. *Science* **302,** 875–878 (2003).
97. Newell, K. A., Asare, A., Kirk, A. D., Gisler, T. D., Bourcier, K., Suthanthiran, M., Burlingham, W. J., Marks, W. H., Sanz, I., Lechler, R. I., Hernandez-Fuentes, M. P., Turka, L. A., Seyfert-Margolis, V. L.Immune Tolerance Network ST507 Study Group. Identification of a B cell signature associated with renal transplant tolerance in humans. *J. Clin. Invest.* **120,** 1836–1847 (2010).
98. Li, Y., Koshiba, T., Yoshizawa, A., Yonekawa, Y., Masuda, K., Ito, A., Ueda, M., Mori, T., Kawamoto, H., Tanaka, Y., Sakaguchi, S., Minato, N., Wood, K. J. & Tanaka, K. Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am. J. Transplant.* **4,** 2118–2125 (2004).
99. Martínez-Llordella, M., Puig-Pey, I., Orlando, G., Ramoni, M., Tisone, G., Rimola, A., Lerut, J., Latinne, D., Margarit, C., Bilbao, I., Brouard, S., Hernández-Fuentes, M., Soulillou, J.-P. & Sánchez-Fueyo, A. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am. J. Transplant.* **7,** 309–319 (2007).
100. Mazariegos, G. V., Zahorchak, A. F., Reyes, J., Ostrowski, L., Flynn, B., Zeevi, A. & Thomson, A. W. Dendritic cell subset ratio in peripheral blood correlates with successful withdrawal of immunosuppression in liver transplant patients. *Am. J. Transplant.* **3,** 689–696 (2003).
101. Gao, Z., McAlister, V. C. & Williams, G. M. Repopulation of liver endothelium by

bone-marrow-derived cells. *The Lancet* **357,** 932–933 (2001).
102. Chandrasekharan, D., Issa, F. & Wood, K. J. Achieving operational tolerance in transplantation: how can lessons from the clinic inform research directions? *Transpl. Int.* **26,** 576–589 (2013).
103. Qin, S., Cobbold, S. P., Pope, H., Elliott, J., Kioussis, D., Davies, J. & Waldmann, H. 'Infectious' transplantation tolerance. *Science* **259,** 974–977 (1993).
104. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **155,** 1151–1164 (1995).
105. Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F. & Ramsdell, F. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* **27,** 68–73 (2001).
106. Liu, Y., Zhang, P., Li, J., Kulkarni, A. B., Perruche, S. & Chen, W. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat. Immunol.* **9,** 632–640 (2008).
107. Chen, W., Jin, W., Hardegen, N., Lei, K.-J., Li, L., Marinos, N., McGrady, G. & Wahl, S. M. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* **198,** 1875–1886 (2003).
108. Zhou, X., Bailey-Bucktrout, S. L., Jeker, L. T., Penaranda, C., Martinez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W. & Bluestone, J. A. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* **10,** 1000–1007 (2009).
109. Camirand, G., Wang, Y., Lu, Y., Wan, Y. Y., Lin, Y., Deng, S., Guz, G., Perkins, D. L., Finn, P. W., Farber, D. L., Flavell, R. A., Shlomchik, W. D., Lakkis, F. G., Rudd, C. E. & Rothstein, D. M. CD45 ligation expands Tregs by promoting interactions with DCs. *J. Clin. Invest.* **124,** 4603–4613 (2014).
110. Komatsu, N., Okamoto, K., Sawa, S., Nakashima, T., Oh-hora, M., Kodama, T., Tanaka, S., Bluestone, J. A. & Takayanagi, H. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat. Med.* **20,** 62–68 (2014).
111. Vu, M. D., Xiao, X., Gao, W., Degauque, N., Chen, M., Kroemer, A., Killeen, N., Ishii, N. & Li, X. C. OX40 costimulation turns off Foxp3+ Tregs. *Blood* **110,** 2501–2510 (2007).
112. Wei, J., Duramad, O., Perng, O. A., Reiner, S. L., Liu, Y.-J. & Qin, F. X.-F. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. *Proceedings of the National Academy of Sciences* **104,** 18169–18174 (2007).
113. Haxhinasto, S., Mathis, D. & Benoist, C. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J. Exp. Med.* **205,** 565–574 (2008).
114. Deng, S., Moore, D. J., Huang, X., Mohiuddin, M., Lee, M. K., Velidedeoglu, E., Lian, M.-M., Chiaccio, M., Sonawane, S., Orlin, A., Wang, J., Chen, H., Caton, A., Zhong, R. & Markmann, J. F. Antibody-induced transplantation tolerance that is dependent on thymus-derived regulatory T cells. *J. Immunol.* **176,** 2799–2807 (2006).
115. Li, X. C. & Turka, L. A. An update on regulatory T cells in transplant tolerance and

rejection. *Nat Rev Nephrol* **6,** 577–583 (2010).
116. Lee, M. K., Moore, D. J., Jarrett, B. P., Lian, M.-M., Deng, S., Huang, X., Markmann, J. W., Chiaccio, M., Barker, C. F., Caton, A. J. & Markmann, J. F. Promotion of allograft survival by CD4+CD25+ regulatory T cells: evidence for in vivo inhibition of effector cell proliferation. *J. Immunol.* **172,** 6539–6544 (2004).
117. Zhao, G., Moore, D. J., Kim, J. I., Lee, K. M., O'Connor, M., Yang, M., Marshall, A. F., Lei, J., Schuetz, C., Markmann, J. F. & Deng, S. An immunosufficient murine model for the study of human islets. *Xenotransplantation* **21,** 567–573 (2014).
118. Zhang, N., Schröppel, B., Lal, G., Jakubzick, C., Mao, X., Chen, D., Yin, N., Jessberger, R., Ochando, J. C., Ding, Y. & Bromberg, J. S. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity* **30,** 458–469 (2009).
119. Kim, H.-J., Barnitz, R. A., Kreslavsky, T., Brown, F. D., Moffett, H., Lemieux, M. E., Kaygusuz, Y., Meissner, T., Holderried, T. A. W., Chan, S., Kastner, P., Haining, W. N. & Cantor, H. Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. *Science* **350,** 334–339 (2015).
120. Guillonneau, C., Picarda, E. & Anegon, I. CD8+ regulatory T cells in solid organ transplantation. *Curr Opin Organ Transplant* **15,** 751–756 (2010).
121. Hu, D., Ikizawa, K., Lu, L., Sanchirico, M. E., Shinohara, M. L. & Cantor, H. Analysis of regulatory CD8 T cells in Qa-1-deficient mice. *Nat. Immunol.* **5,** 516–523 (2004).
122. Leavenworth, J. W., Tang, X., Kim, H.-J., Wang, X. & Cantor, H. Amelioration of arthritis through mobilization of peptide-specific CD8+ regulatory T cells. *J. Clin. Invest.* **123,** 1382–1389 (2013).
123. Kim, H.-J., Verbinnen, B., Tang, X., Lu, L. & Cantor, H. Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance. *Nature* **467,** 328–332 (2010).
124. Kim, H.-J., Wang, X., Radfar, S., Sproule, T. J., Roopenian, D. C. & Cantor, H. CD8+ T regulatory cells express the Ly49 Class I MHC receptor and are defective in autoimmune prone B6-Yaa mice. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 2010–2015 (2011).
125. Dai, Z., Zhang, S., Xie, Q., Wu, S., Su, J., Li, S., Xu, Y. & Li, X. C. Natural CD8+CD122+ T Cells Are More Potent in Suppression of Allograft Rejection Than CD4+CD25+ Regulatory T Cells. *Am. J. Transplant.* (2013). doi:10.1111/ajt.12515
126. Dai, H., Wan, N., Zhang, S., Moore, Y., Wan, F. & Dai, Z. Cutting edge: programmed death-1 defines CD8+CD122+ T cells as regulatory versus memory T cells. *J. Immunol.* **185,** 803–807 (2010).
127. Koehn, B. H., Ford, M. L., Ferrer, I. R., Borom, K., Gangappa, S., Kirk, A. D. & Larsen, C. P. PD-1-dependent mechanisms maintain peripheral tolerance of donor-reactive CD8+ T cells to transplanted tissue. *The Journal of Immunology* **181,** 5313–5322 (2008).
128. Izawa, A., Yamaura, K., Albin, M. J., Jurewicz, M., Tanaka, K., Clarkson, M. R., Ueno, T., Habicht, A., Freeman, G. J., Yagita, H., Abdi, R., Pearson, T., Greiner, D. L., Sayegh, M. H. & Najafian, N. A novel alloantigen-specific CD8+PD1+ regulatory T cell induced by ICOS-B7h blockade in vivo. *J. Immunol.* **179,** 786–796 (2007).
129. Shi, Z., Rifa'i, M., Lee, Y. H., Shiku, H., Isobe, K.-I. & Suzuki, H. Importance of CD80/CD86-CD28 interactions in the recognition of target cells by CD8+CD122+ regulatory T cells. *Immunology* **124,** 121–128 (2008).
130. Shi, Z., Okuno, Y., Rifa'i, M., Endharti, A. T., Akane, K., Isobe, K.-I. & Suzuki, H.

Human CD8+CXCR3+ T cells have the same function as murine CD8+CD122+ Treg. *Eur. J. Immunol.* **39,** 2106–2119 (2009).
131. Sho, M., Kishimoto, K., Harada, H., Livak, M., Sanchez-Fueyo, A., Yamada, A., Zheng, X. X., Strom, T. B., Basadonna, G. P., Sayegh, M. H. & Rothstein, D. M. Requirements for induction and maintenance of peripheral tolerance in stringent allograft models. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 13230–13235 (2005).
132. Mauri, C. & Blair, P. A. Regulatory B cells in autoimmunity: developments and controversies. *Nat Rev Rheumatol* **6,** 636–643 (2010).
133. Deng, S., Moore, D. J., Huang, X., Lian, M.-M., Mohiuddin, M., Velededeoglu, E., Lee, M. K., Sonawane, S., Kim, J., Wang, J., Chen, H., Corfe, S. A., Paige, C., Shlomchik, M., Caton, A. & Markmann, J. F. Cutting edge: transplant tolerance induced by anti-CD45RB requires B lymphocytes. *J. Immunol.* **178,** 6028–6032 (2007).
134. Ding, Q., Yeung, M., Camirand, G., Zeng, Q., Akiba, H., Yagita, H., Chalasani, G., Sayegh, M. H., Najafian, N. & Rothstein, D. M. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *J. Clin. Invest.* **121,** 3645–3656 (2011).
135. Kim, J. I., Lee, K. M., Stott, R., O'Connor, M. R., Yeh, H., Zhao, G., Eliades, P., Soohoo, J., Fox, C., Cheng, N., Deng, S. & Markmann, J. F. Anti-CD45RB/Anti-TIM-1-Induced Tolerance Requires Regulatory B Cells. *Am. J. Transplant.* **12,** 53–53 (2012).
136. Lee, K. M., Stott, R. T., Zhao, G., Soohoo, J., Xiong, W., Lian, M.-M., Fitzgerald, L., Shi, S., Akrawi, E., Lei, J., Deng, S., Yeh, H., Markmann, J. F. & Kim, J. I. TGF-β-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. *Eur. J. Immunol.* **44,** 1728–1736 (2014).
137. Walters, S., Webster, K. E., Sutherland, A., Gardam, S., Groom, J., Liuwantara, D., Mariño, E., Thaxton, J., Weinberg, A., Mackay, F., Brink, R., Sprent, J. & Grey, S. T. Increased CD4+Foxp3+ T cells in BAFF-transgenic mice suppress T cell effector responses. *J. Immunol.* **182,** 793–801 (2009).
138. Zhao, G., Moore, D. J., Lee, K. M., Kim, J. I., Duff, P. E., O'Connor, M. R., Hirohashi, T., Lei, J., Yang, M., Markmann, J. F. & Deng, S. An unexpected counter-regulatory role of IL-10 in B-lymphocyte-mediated transplantation tolerance. *Am. J. Transplant.* **10,** 796–801 (2010).
139. Lal, G., Kulkarni, N., Nakayama, Y., Singh, A. K., Sethi, A., Burrell, B. E., Brinkman, C. C., Iwami, D., Zhang, T., Hehlgans, T. & Bromberg, J. S. IL-10 from marginal zone precursor B cells controls the differentiation of Th17, Tfh and Tfr cells in transplantation tolerance. *Immunol. Lett.* **170,** 52–63 (2016).
140. Silva, H. M., Takenaka, M. C. S., Moraes-Vieira, P. M. M., Monteiro, S. M., Hernandez, M. O., Chaara, W., Six, A., Agena, F., Sesterheim, P., Barbé-Tuana, F. M., Saitovitch, D., Lemos, F., Kalil, J. & Coelho, V. Preserving the B-cell compartment favors operational tolerance in human renal transplantation. *Mol. Med.* **18,** 733–743 (2012).
141. Danger, R., Pallier, A., Giral, M., Martinez-Llordella, M., Lozano, J. J., Degauque, N., Sanchez-Fueyo, A., Soulillou, J.-P. & Brouard, S. Upregulation of miR-142-3p in peripheral blood mononuclear cells of operationally tolerant patients with a renal transplant. *J. Am. Soc. Nephrol.* **23,** 597–606 (2012).
142. Piemonti, L., Everly, M. J., Maffi, P., Scavini, M., Poli, F., Nano, R., Cardillo, M., Melzi, R., Mercalli, A., Sordi, V., Lampasona, V., Espadas de Arias, A., Scalamogna, M., Bosi, E., Bonifacio, E., Secchi, A. & Terasaki, P. I. Alloantibody and autoantibody

monitoring predicts islet transplantation outcome in human type 1 diabetes. *Diabetes* **62,** 1656–1664 (2013).
143. Markees, T. G., Serreze, D. V., Phillips, N. E., Sorli, C. H., Gordon, E. J., Shultz, L. D., Noelle, R. J., Woda, B. A., Greiner, D. L., Mordes, J. P. & Rossini, A. A. NOD mice have a generalized defect in their response to transplantation tolerance induction. *Diabetes* **48,** 967–974 (1999).
144. Moore, D. J., Huang, X., Lee, M. K., Lian, M.-M., Chiaccio, M., Chen, H., Koeberlein, B., Zhong, R., Markmann, J. F. & Deng, S. Resistance to anti-CD45RB-induced tolerance in NOD mice: mechanisms involved. *Transpl. Int.* **17,** 261–269 (2004).
145. Prochazka, M., Serreze, D. V., Worthen, S. M. & Leiter, E. H. Genetic control of diabetogenesis in NOD/Lt mice. Development and analysis of congenic stocks. *Diabetes* **38,** 1446–1455 (1989).
146. Wicker, L. S., Todd, J. A., Prins, J. B., Podolin, P. L., Renjilian, R. J. & Peterson, L. B. Resistance alleles at two non-major histocompatibility complex-linked insulin-dependent diabetes loci on chromosome 3, Idd3 and Idd10, protect nonobese diabetic mice from diabetes. *J. Exp. Med.* **180,** 1705–1713 (1994).
147. Mangada, J., Pearson, T., Brehm, M. A., Wicker, L. S., Peterson, L. B., Shultz, L. D., Serreze, D. V., Rossini, A. A. & Greiner, D. L. Idd loci synergize to prolong islet allograft survival induced by costimulation blockade in NOD mice. *Diabetes* **58,** 165–173 (2009).
148. Bird, J. J., Brown, D. R., Mullen, A. C., Moskowitz, N. H., Mahowald, M. A., Sider, J. R., Gajewski, T. F., Wang, C. R. & Reiner, S. L. Helper T cell differentiation is controlled by the cell cycle. *Immunity* **9,** 229–237 (1998).
149. Noorchashm, H., Moore, D. J., Noto, L. E., Noorchashm, N., Reed, A. J., Reed, A. L., Song, H. K., Mozaffari, R., Jevnikar, A. M., Barker, C. F. & Naji, A. Impaired CD4 T cell activation due to reliance upon B cell-mediated costimulation in nonobese diabetic (NOD) mice. *J. Immunol.* **165,** 4685–4696 (2000).
150. Kupfer, T., Beilke, J. N., Pham, K. & Buhrman, J. 'Indirect' Acute Islet Allograft Destruction in Nonobese Diabetic Mice Is Independent of Donor Major Histocompatibility Complex and Requires Host B Lymphocytes. *Transplantation* (2008).
151. Makhlouf, L. Allorecognition and Effector Pathways of Islet Allograft Rejection in Normal versus Nonobese Diabetic Mice. *J. Am. Soc. Nephrol.* **14,** 2168–2175 (2003).
152. Mariño, E., Walters, S. N., Villanueva, J. E., Richards, J. L., Mackay, C. R. & Grey, S. T. BAFF regulates activation of self-reactive T cells through B-cell dependent mechanisms and mediates protection in NOD mice. *Eur. J. Immunol.* **44,** 983–993 (2014).
153. Börjesson, A., Andersson, A. K. & Sandler, S. Survival of an islet allograft deficient in iNOS after implantation into diabetic NOD mice. *Cell Transplant* **15,** 769–775 (2006).
154. Callewaert, H. I., Gysemans, C. A., Ladrière, L., D'Hertog, W., Hagenbrock, J., Overbergh, L., Eizirik, D. L. & Mathieu, C. Deletion of STAT-1 pancreatic islets protects against streptozotocin-induced diabetes and early graft failure but not against late rejection. *Diabetes* **56,** 2169–2173 (2007).
155. Fousteri, G., Jofra, T., Di Fonte, R. & Battaglia, M. Combination of an Antigen-Specific Therapy and an Immunomodulatory Treatment to Simultaneous Block Recurrent Autoimmunity and Alloreactivity in Non-Obese Diabetic Mice. *PLoS ONE* **10,**

e0127631 (2015).
156. Ansari, M., Fiorina, P., Dada, S., Guleria, I. & Ueno, T. Role of ICOS pathway in autoimmune and alloimmune responses in NOD mice. *Clinical Immunology*. **126,** 140-147 (2008).
157. Molano, R. D., Pileggi, A., Berney, T., Poggioli, R., Zahr, E., Oliver, R., Malek, T. R., Ricordi, C. & Inverardi, L. Long-term islet allograft survival in nonobese diabetic mice treated with tacrolimus, rapamycin, and anti-interleukin-2 antibody. *Transplantation* **75,** 1812–1819 (2003).
158. Zhang, Y. C., Pileggi, A., Molano, R. D., Wasserfall, C., Campbell-Thompson, M., Ricordi, C., Atkinson, M. A. & Inverardi, L. Systemic Overexpression of Interleukin-10 Fails to Protect Allogeneic Islet Transplants in Nonobese Diabetic Mice. *Transplantation* **80,** 530–533 (2005).
159. Rønn, S. G., Börjesson, A., Bruun, C., Heding, P. E., Frobøse, H., Mandrup-Poulsen, T., Karlsen, A. E., Rasschaert, J., Sandler, S. & Billestrup, N. Suppressor of cytokine signalling-3 expression inhibits cytokine-mediated destruction of primary mouse and rat pancreatic islets and delays allograft rejection. *Diabetologia* **51,** 1873–1882 (2008).
160. Lee, K., Nguyen, V., Lee, K. M., Kang, S.-M. & Tang, Q. Attenuation of donor-reactive T cells allows effective control of allograft rejection using regulatory T cell therapy. *Am. J. Transplant.* **14,** 27–38 (2014).
161. Stanescu, D. E., Lord, K. & Lipman, T. H. The epidemiology of type 1 diabetes in children. *Endocrinol. Metab. Clin. North Am.* **41,** 679–694 (2012).
162. Brown, A., Reynolds, L. R. & Bruemmer, D. Intensive glycemic control and cardiovascular disease: an update. *Nat Rev Cardiol* **7,** 369–375 (2010).
163. Donnelly, L. A., Morris, A. D., Frier, B. M., Ellis, J. D., Donnan, P. T., Durrant, R., Band, M. M., Reekie, G., Leese, G. P.DARTS/MEMO Collaboration. Frequency and predictors of hypoglycaemia in Type 1 and insulin-treated Type 2 diabetes: a population-based study. *Diabet. Med.* **22,** 749–755 (2005).
164. Sherry, N. A., Tsai, E. B. & Herold, K. C. Natural history of beta-cell function in type 1 diabetes. *Diabetes* **54 Suppl 2,** S32–9 (2005).
165. Barton, F. B., Rickels, M. R., Alejandro, R., Hering, B. J., Wease, S., Naziruddin, B., Oberholzer, J., Odorico, J. S., Garfinkel, M. R., Levy, M., Pattou, F., Berney, T., Secchi, A., Messinger, S., Senior, P. A., Maffi, P., Posselt, A., Stock, P. G., Kaufman, D. B., Luo, X., Kandeel, F., Cagliero, E., Turgeon, N. A., Witkowski, P., Naji, A., O'Connell, P. J., Greenbaum, C., Kudva, Y. C., Brayman, K. L., Aull, M. J., Larsen, C., Kay, T. W. H., Fernandez, L. A., Vantyghem, M.-C., Bellin, M. & Shapiro, A. M. J. Improvement in outcomes of clinical islet transplantation: 1999-2010. *Diabetes Care* **35,** 1436–1445 (2012).
166. D'Addio, F., Maffi, P., Vezzulli, P., Vergani, A., Mello, A., Bassi, R., Nano, R., Falautano, M., Coppi, E., Finzi, G., D'Angelo, A., Fermo, I., Pellegatta, F., La Rosa, S., Magnani, G., Piemonti, L., Falini, A., Folli, F., Secchi, A. & Fiorina, P. Islet transplantation stabilizes hemostatic abnormalities and cerebral metabolism in individuals with type 1 diabetes. *Diabetes Care* **37,** 267–276 (2014).
167. Rickels, M. R., Kong, S. M., Fuller, C., Dalton-Bakes, C., Ferguson, J. F., Reilly, M. P., Teff, K. L. & Naji, A. Improvement in insulin sensitivity after human islet transplantation for type 1 diabetes. *J. Clin. Endocrinol. Metab.* **98,** E1780–5 (2013).
168. Rickels, M. R., Liu, C., Shlansky-Goldberg, R. D., Soleimanpour, S. A., Vivek, K.,

Kamoun, M., Min, Z., Markmann, E., Palangian, M., Dalton-Bakes, C., Fuller, C., Chiou, A. J., Barker, C. F., Luning Prak, E. T. & Naji, A. Improvement in β-cell secretory capacity after human islet transplantation according to the CIT07 protocol. *Diabetes* **62,** 2890–2897 (2013).
169. McCall, M. & Shapiro, A. M. J. Update on islet transplantation. *Cold Spring Harb Perspect Med* **2,** a007823 (2012).
170. Barlow, A. D., Nicholson, M. L. & Herbert, T. P. Evidence for rapamycin toxicity in pancreatic β-cells and a review of the underlying molecular mechanisms. *Diabetes* **62,** 2674–2682 (2013).
171. Reed, J. C. & Herold, K. C. Thinking bedside at the bench: the NOD mouse model of T1DM. *Nat Rev Endocrinol* **11,** 308–314 (2015).
172. Redondo, M. J., Jeffrey, J., Fain, P. R., Eisenbarth, G. S. & Orban, T. Concordance for islet autoimmunity among monozygotic twins. *N. Engl. J. Med.* **359,** 2849–2850 (2008).
173. Wen, L., Ley, R. E., Volchkov, P. Y., Stranges, P. B., Avanesyan, L., Stonebraker, A. C., Hu, C., Wong, F. S., Szot, G. L., Bluestone, J. A., Gordon, J. I. & Chervonsky, A. V. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* **455,** 1109–1113 (2008).
174. Oldstone, M. B. Prevention of type I diabetes in nonobese diabetic mice by virus infection. *Science* **239,** 500–502 (1988).
175. Ziegler, A. G., Rewers, M., Simell, O., Simell, T., Lempainen, J., Steck, A., Winkler, C., Ilonen, J., Veijola, R., Knip, M., Bonifacio, E. & Eisenbarth, G. S. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. *JAMA* **309,** 2473–2479 (2013).
176. Serreze, D. V., Chapman, H. D., Varnum, D. S., Hanson, M. S., Reifsnyder, P. C., Richard, S. D., Fleming, S. A., Leiter, E. H. & Shultz, L. D. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new 'speed congenic' stock of NOD.Ig mu null mice. *J. Exp. Med.* **184,** 2049–2053 (1996).
177. Noorchashm, H., Lieu, Y. K., Noorchashm, N., Rostami, S. Y., Greeley, S. A., Schlachterman, A., Song, H. K., Noto, L. E., Jevnikar, A. M., Barker, C. F. & Naji, A. I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet beta cells of nonobese diabetic mice. *J. Immunol.* **163,** 743–750 (1999).
178. Serreze, D. V., Fleming, S. A., Chapman, H. D., Richard, S. D., Leiter, E. H. & Tisch, R. M. B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J. Immunol.* **161,** 3912–3918 (1998).
179. Silveira, P. A., Dombrowsky, J., Johnson, E., Chapman, H. D., Nemazee, D. & Serreze, D. V. B cell selection defects underlie the development of diabetogenic APCs in nonobese diabetic mice. *J. Immunol.* **172,** 5086–5094 (2004).
180. Hulbert, C., Riseili, B., Rojas, M. & Thomas, J. W. B cell specificity contributes to the outcome of diabetes in nonobese diabetic mice. *J. Immunol.* **167,** 5535–5538 (2001).
181. Pescovitz, M. D., Greenbaum, C. J., Krause-Steinrauf, H., Becker, D. J., Gitelman, S. E., Goland, R., Gottlieb, P. A., Marks, J. B., McGee, P. F., Moran, A. M., Raskin, P., Rodriguez, H., Schatz, D. A., Wherrett, D., Wilson, D. M., Lachin, J. M., Skyler, J. S.Type 1 Diabetes TrialNet Anti-CD20 Study Group. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. *N. Engl. J. Med.* **361,** 2143–2152

(2009).
182. Greeley, S. A., Moore, D. J., Noorchashm, H., Noto, L. E., Rostami, S. Y., Schlachterman, A., Song, H. K., Koeberlein, B., Barker, C. F. & Naji, A. Impaired activation of islet-reactive CD4 T cells in pancreatic lymph nodes of B cell-deficient nonobese diabetic mice. *J. Immunol.* **167,** 4351–4357 (2001).
183. Mariño, E., Villanueva, J., Walters, S., Liuwantara, D., Mackay, F. & Grey, S. T. CD4(+)CD25(+) T-cells control autoimmunity in the absence of B-cells. *Diabetes* **58,** 1568–1577 (2009).
184. Ellis, J. S., Wan, X. & Braley-Mullen, H. Transient depletion of CD4+ CD25+ regulatory T cells results in multiple autoimmune diseases in wild-type and B-cell-deficient NOD mice. *Immunology* **139,** 179–186 (2013).
185. Brode, S., Raine, T., Zaccone, P. & Cooke, A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+CD25+Foxp3+ regulatory T cells. *J. Immunol.* **177,** 6603–6612 (2006).
186. Bluestone, J. A. & Tang, Q. How do CD4+CD25+ regulatory T cells control autoimmunity? *Curr. Opin. Immunol.* **17,** 638–642 (2005).
187. Lee, K. M., O'Connor, M., Yang, M. & Marshall, A. F. An immunosufficient murine model for the study of human islets. *Xenotransplantation. **21,** 567-573* (2014).
188. Vong, A. M., Daneshjou, N., Norori, P. Y., Sheng, H., Braciak, T. A., Sercarz, E. E. & Gabaglia, C. R. Spectratyping analysis of the islet-reactive T cell repertoire in diabetic NOD Igμ(null) mice after polyclonal B cell reconstitution. *J Transl Med* **9,** 101 (2011).
189. Krishnamurthy, B., Chee, J., Jhala, G., Trivedi, P., Catterall, T., Selck, C., Gurzov, E. N., Brodnicki, T. C., Graham, K. L., Wali, J. A., Zhan, Y., Gray, D., Strasser, A., Allison, J., Thomas, H. E. & Kay, T. W. H. BIM Deficiency Protects NOD Mice From Diabetes by Diverting Thymocytes to Regulatory T Cells. *Diabetes* **64,** 3229–3238 (2015).
190. Trani, J., Moore, D. J., Jarrett, B. P., Markmann, J. W., Lee, M. K., Singer, A., Lian, M.-M., Tran, B., Caton, A. J. & Markmann, J. F. CD25+ immunoregulatory CD4 T cells mediate acquired central transplantation tolerance. *J. Immunol.* **170,** 279–286 (2003).
191. Hu, C.-Y., Rodriguez-Pinto, D., Du, W., Ahuja, A., Henegariu, O., Wong, F. S., Shlomchik, M. J. & Wen, L. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *J. Clin. Invest.* **117,** 3857–3867 (2007).
192. Silveira, P. A., Johnson, E., Chapman, H. D., Bui, T., Tisch, R. M. & Serreze, D. V. The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors. *Eur. J. Immunol.* **32,** 3657–3666 (2002).
193. Chiu, P. P., Jevnikar, A. M. & Danska, J. S. Genetic control of T and B lymphocyte activation in nonobese diabetic mice. *J. Immunol.* **167,** 7169–7179 (2001).
194. Herold, K. C., Gitelman, S. E., Ehlers, M. R., Gottlieb, P. A., Greenbaum, C. J., Hagopian, W., Boyle, K. D., Keyes-Elstein, L., Aggarwal, S., Phippard, D., Sayre, P. H., McNamara, J., Bluestone, J. A.AbATE Study Team. Teplizumab (Anti-CD3 mAb) Treatment Preserves C-Peptide Responses in Patients With New-Onset Type 1 Diabetes in a Randomized Controlled Trial: Metabolic and Immunologic Features at Baseline Identify a Subgroup of Responders. *Diabetes* **62,** 3766–3774 (2013).
195. Orban, T., et al. Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. *The Lancet* **378,**

412–419 (2011).
196. Craft, J. E. Follicular helper T cells in immunity and systemic autoimmunity. *Nat Rev Rheumatol* **8,** 337–347 (2012).
197. Kenefeck, R., Wang, C. J., Kapadi, T., Wardzinski, L., Attridge, K., Clough, L. E., Heuts, F., Kogimtzis, A., Patel, S., Rosenthal, M., Ono, M., Sansom, D. M., Narendran, P. & Walker, L. S. K. Follicular helper T cell signature in type 1 diabetes. *J. Clin. Invest.* **125,** 292–303 (2015).
198. Lim, H. W., Hillsamer, P., Banham, A. H. & Kim, C. H. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J. Immunol.* **175,** 4180–4183 (2005).
199. Okamura, T., Sumitomo, S., Morita, K., Iwasaki, Y., Inoue, M., Nakachi, S., Komai, T., Shoda, H., Miyazaki, J.-I., Fujio, K. & Yamamoto, K. TGF-β3-expressing CD4+CD25(-)LAG3+ regulatory T cells control humoral immune responses. *Nat Commun* **6,** 6329 (2015).
200. Linterman, M. A., Pierson, W., Lee, S. K., Kallies, A., Kawamoto, S., Rayner, T. F., Srivastava, M., Divekar, D. P., Beaton, L., Hogan, J. J., Fagarasan, S., Liston, A., Smith, K. G. C. & Vinuesa, C. G. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat. Med.* **17,** 975–982 (2011).
201. Herold, K. C., Vignali, D. A. A., Cooke, A. & Bluestone, J. A. Type 1 diabetes: translating mechanistic observations into effective clinical outcomes. *Nat. Rev. Immunol.* **13,** 243–256 (2013).
202. Kim, H.-J. & Cantor, H. Regulation of self-tolerance by Qa-1-restricted CD8(+) regulatory T cells. *Semin. Immunol.* **23,** 446–452 (2011).
203. Lu, L., Kim, H.-J., Werneck, M. B. F. & Cantor, H. Regulation of CD8+ regulatory T cells: Interruption of the NKG2A-Qa-1 interaction allows robust suppressive activity and resolution of autoimmune disease. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 19420–19425 (2008).
204. Leavenworth, J. W., Schellack, C., Kim, H.-J., Lu, L., Spee, P. & Cantor, H. Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F(ab')2. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 2562–2567 (2010).
205. Ping, Y., Bamford, R. & Waldmann, T. A. IL-15-dependent CD8(+) CD122(+) T cells ameliorate experimental autoimmune encephalomyelitis by modulating IL-17 production by CD4(+) T cells. *Eur. J. Immunol.* (2014). doi:10.1002/eji.201444675
206. Stoklasek, T. A., Schluns, K. S. & Lefrançois, L. Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo. *J. Immunol.* **177,** 6072–6080 (2006).
207. Rubinstein, M. P., Kovar, M., Purton, J. F., Cho, J.-H., Boyman, O., Surh, C. D. & Sprent, J. Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 9166–9171 (2006).
208. Belanger, S., Tai, L.-H., Anderson, S. K. & Makrigiannis, A. P. Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome. *Genes Immun.* **9,** 509–521 (2008).
209. Mortier, E., Woo, T., Advincula, R., Gozalo, S. & Ma, A. IL-15Ralpha chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. *J. Exp. Med.* **205,** 1213–1225 (2008).
210. Waldmann, T. A. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat. Rev. Immunol.* **6,** 595–601 (2006).

211. Gomez-Nicola, D., Spagnolo, A., Guaza, C. & Nieto-Sampedro, M. Aggravated experimental autoimmune encephalomyelitis in IL-15 knockout mice. *Exp. Neurol.* **222,** 235–242 (2010).
212. Wu, Y., Zheng, Z., Jiang, Y., Chess, L. & Jiang, H. The specificity of T cell regulation that enables self-nonself discrimination in the periphery. *Proceedings of the National Academy of Sciences* **106,** 534–539 (2009).
213. Sundstrom, M. & Lejon, K. The prolonged and enhanced immune response in the non-obese diabetic mouse is dependent on genes in the Idd1/24, Idd12 and Idd18 regions. *J. Autoimmun.* **35,** 375–382 (2010).
214. Carnaud, C., Gombert, J., Donnars, O., Garchon, H. & Herbelin, A. Protection against diabetes and improved NK/NKT cell performance in NOD.NK1.1 mice congenic at the NK complex. *J. Immunol.* **166,** 2404–2411 (2001).
215. Arndt, B., Witkowski, L., Ellwart, J. & Seissler, J. CD8+ CD122+ PD-1- effector cells promote the development of diabetes in NOD mice. *J. Leukoc. Biol.* **97,** 111–120 (2015).
216. Suwanai, H., Wilcox, M. A., Mathis, D. & Benoist, C. A defective Il15 allele underlies the deficiency in natural killer cell activity in nonobese diabetic mice. *PNAS.* **107,** 9305-9310 (2010).
217. Bobbala, D., Chen, X.-L., Leblanc, C., Mayhue, M., Stankova, J., Tanaka, T., Chen, Y.-G., Ilangumaran, S. & Ramanathan, S. Interleukin-15 plays an essential role in the pathogenesis of autoimmune diabetes in the NOD mouse. *Diabetologia* **55,** 3010–3020 (2012).
218. Chen, J., Feigenbaum, L., Awasthi, P., Butcher, D. O., Anver, M. R., Golubeva, Y. G., Bamford, R., Zhang, X., St Claire, M. B., Thomas, C. J., Discepolo, V., Jabri, B. & Waldmann, T. A. Insulin-dependent diabetes induced by pancreatic beta cell expression of IL-15 and IL-15Rα. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 13534–13539 (2013).
219. Xia, J., Liu, W., Hu, B., Tian, Z. & Yang, Y. IL-15 promotes regulatory T cell function and protects against diabetes development in NK-depleted NOD mice. *Clin. Immunol.* **134,** 130–139 (2010).
220. Jiang, H., Canfield, S. M., Gallagher, M. P., Jiang, H. H., Jiang, Y., Zheng, Z. & Chess, L. HLA-E-restricted regulatory CD8(+) T cells are involved in development and control of human autoimmune type 1 diabetes. *J. Clin. Invest.* **120,** 3641–3650 (2010).
221. Ablamunits, V., Bisikirska, B. & Herold, K. C. Acquisition of regulatory function by human CD8(+) T cells treated with anti-CD3 antibody requires TNF. *Eur. J. Immunol.* **40,** 2891–2901 (2010).
222. Guo, Y., Luan, L., Rabacal, W., Bohannon, J. K., Fensterheim, B. A., Hernandez, A. & Sherwood, E. R. IL-15 Superagonist-Mediated Immunotoxicity: Role of NK Cells and IFN-γ. *The Journal of Immunology* **195,** 2353–2364 (2015).
223. Gill, R. G. NK cells: elusive participants in transplantation immunity and tolerance. *Curr. Opin. Immunol.* (2010).
224. Yu, G., Xu, X., Vu, M. D., Kilpatrick, E. D. & Li, X. C. NK cells promote transplant tolerance by killing donor antigen-presenting cells. *J. Exp. Med.* **203,** 1851–1858 (2006).
225. Beilke, J. N., Kuhl, N. R., Kaer, L. V. & Gill, R. G. NK cells promote islet allograft tolerance via a perforin-dependent mechanism. *Nat. Med.* **11,** 1059–1065 (2005).
226. Pearson, T., Markees, T. G., Wicker, L. S., Serreze, D. V., Peterson, L. B., Mordes, J. P., Rossini, A. A. & Greiner, D. L. NOD congenic mice genetically protected from autoimmune diabetes remain resistant to transplantation tolerance induction. *Diabetes*

52, 321–326 (2003).
227. Seung, E., Iwakoshi, N., Woda, B. A., Markees, T. G., Mordes, J. P., Rossini, A. A. & Greiner, D. L. Allogeneic hematopoietic chimerism in mice treated with sublethal myeloablation and anti-CD154 antibody: absence of graft-versus-host disease, induction of skin allograft tolerance, and prevention of recurrent autoimmunity in islet-allografted NOD/Lt mice. *Blood* **95,** 2175–2182 (2000).
228. Ikehara, S., Ohtsuki, H., Good, R. A., Asamoto, H., Nakamura, T., Sekita, K., Muso, E., Tochino, Y., Ida, T. & Kuzuya, H. Prevention of type I diabetes in nonobese diabetic mice by allogenic bone marrow transplantation. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 7743–7747 (1985).
229. Li, H., Kaufman, C. L. & Ildstad, S. T. Allogeneic chimerism induces donor-specific tolerance to simultaneous islet allografts in nonobese diabetic mice. *Surgery* **118,** 192–7– discussion 197–8 (1995).
230. Voltarelli, J. C., Couri, C. E. B., Stracieri, A. B. P. L., Oliveira, M. C., Moraes, D. A., Pieroni, F., Coutinho, M., Malmegrim, K. C. R., Foss-Freitas, M. C., Simões, B. P., Foss, M. C., Squiers, E. & Burt, R. K. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *JAMA* **297,** 1568–1576 (2007).
231. Couri, C. E. B., Oliveira, M. C. B., Stracieri, A. B. P. L., Moraes, D. A., Pieroni, F., Barros, G. M. N., Madeira, M. I. A., Malmegrim, K. C. R., Foss-Freitas, M. C., Simões, B. P., Martinez, E. Z., Foss, M. C., Burt, R. K. & Voltarelli, J. C. C-peptide levels and insulin independence following autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *JAMA* **301,** 1573–1579 (2009).
232. Mineo, D., Ricordi, C., Xu, X., Pileggi, A., Garcia-Morales, R., Khan, A., Baidal, D. A., Han, D., Monroy, K., Miller, J., Pugliese, A., Froud, T., Inverardi, L., Kenyon, N. S. & Alejandro, R. Combined islet and hematopoietic stem cell allotransplantation: a clinical pilot trial to induce chimerism and graft tolerance. *Am. J. Transplant.* **8,** 1262–1274 (2008).
233. Sykes, M. Immune tolerance: mechanisms and application in clinical transplantation. *J. Intern. Med.* **262,** 288–310 (2007).
234. Baldridge, M. T., King, K. Y., Boles, N. C., Weksberg, D. C. & Goodell, M. A. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* **465,** 793–797 (2010).
235. King, K. Y. & Goodell, M. A. Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. *Nat. Rev. Immunol.* **11,** 685–692 (2011).
236. Boulais, P. E. & Frenette, P. S. Making sense of hematopoietic stem cell niches. *Blood* **125,** 2621–2629 (2015).
237. Katayama, Y., Battista, M., Kao, W.-M., Hidalgo, A., Peired, A. J., Thomas, S. A. & Frenette, P. S. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* **124,** 407–421 (2006).
238. Albiero, M., Poncina, N., Tjwa, M., Ciciliot, S., Menegazzo, L., Ceolotto, G., Vigili de Kreutzenberg, S., Moura, R., Giorgio, M., Pelicci, P., Avogaro, A. & Fadini, G. P. Diabetes causes bone marrow autonomic neuropathy and impairs stem cell mobilization via dysregulated p66Shc and Sirt1. *Diabetes* **63,** 1353–1365 (2014).
239. Solders, G., Thalme, B., Aguirre-Aquino, M., Brandt, L., Berg, U. & Persson, A. Nerve

conduction and autonomic nerve function in diabetic children. A 10-year follow-up study. *Acta Paediatr*. **86,** 361–366 (1997).
240. Fadini, G. P., Fiala, M., Cappellari, R., Danna, M., Park, S., Poncina, N., Menegazzo, L., Albiero, M., DiPersio, J., Stockerl-Goldstein, K. & Avogaro, A. Diabetes Limits Stem Cell Mobilization Following G-CSF but Not Plerixafor. *Diabetes* **64,** 2969–2977 (2015).
241. Moraes, O. A., Colucci, J. A., Souza, L. E., Scapini, K. B., Moraes-Silva, I. C., Mostarda, C., De Angelis, K., Casarini, D. E. & Irigoyen, M. C. Cardiovascular autonomic dysfunction in non-obese diabetic mice. *Auton Neurosci* **177,** 143–147 (2013).
242. Tsui, H., Chan, Y., Tang, L., Winer, S., Cheung, R. K., Paltser, G., Selvanantham, T., Elford, A. R., Ellis, J. R., Becker, D. J., Ohashi, P. S. & Dosch, H.-M. Targeting of pancreatic glia in type 1 diabetes. *Diabetes* **57,** 918–928 (2008).
243. Winer, S., Astsaturov, I., Cheung, R., Gunaratnam, L., Kubiak, V., Cortez, M. A., Moscarello, M., O'Connor, P. W., McKerlie, C., Becker, D. J. & Dosch, H. M. Type I diabetes and multiple sclerosis patients target islet plus central nervous system autoantigens; nonimmunized nonobese diabetic mice can develop autoimmune encephalitis. *J. Immunol*. **166,** 2831–2841 (2001).
244. Winer, S., Tsui, H., Lau, A., Song, A., Li, X., Cheung, R. K., Sampson, A., Afifiyan, F., Elford, A., Jackowski, G., Becker, D. J., Santamaria, P., Ohashi, P. & Dosch, H.-M. Autoimmune islet destruction in spontaneous type 1 diabetes is not beta-cell exclusive. *Nat. Med*. **9,** 198–205 (2003).
245. Leng, Q., Nie, Y., Zou, Y. & Chen, J. Elevated CXCL12 expression in the bone marrow of NOD mice is associated with altered T cell and stem cell trafficking and diabetes development. *BMC Immunol*. **9,** 51 (2008).
246. Fiorina, P., Jurewicz, M., Vergani, A., Petrelli, A., Carvello, M., D'Addio, F., Godwin, J. G., Law, K., Wu, E., Tian, Z., Thoma, G., Kovarik, J., La Rosa, S., Capella, C., Rodig, S., Zerwes, H.-G., Sayegh, M. H. & Abdi, R. Targeting the CXCR4-CXCL12 axis mobilizes autologous hematopoietic stem cells and prolongs islet allograft survival via programmed death ligand 1. *The Journal of Immunology* **186,** 121–131 (2011).
247. Chilton, P. M., Rezzoug, F., Ratajczak, M. Z., Fugier-Vivier, I., Ratajczak, J., Kucia, M., Huang, Y., Tanner, M. K. & Ildstad, S. T. Hematopoietic stem cells from NOD mice exhibit autonomous behavior and a competitive advantage in allogeneic recipients. *Blood* **105,** 2189–2197 (2005).
248. Somers, E. C., Marder, W., Cagnoli, P., Lewis, E. E., DeGuire, P., Gordon, C., Helmick, C. G., Wang, L., Wing, J. J., Dhar, J. P., Leisen, J., Shaltis, D. & McCune, W. J. Population-based incidence and prevalence of systemic lupus erythematosus: the Michigan Lupus Epidemiology and Surveillance program. *Arthritis Rheumatol* **66,** 369–378 (2014).
249. Harley, J. B., Kelly, J. A. & Kaufman, K. M. Unraveling the genetics of systemic lupus erythematosus. *Springer Semin. Immunopathol*. **28,** 119–130 (2006).
250. Deapen, D., Escalante, A., Weinrib, L., Horwitz, D., Bachman, B., Roy-Burman, P., Walker, A. & Mack, T. M. A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis Rheum*. **35,** 311–318 (1992).
251. De Groof, A., Ducreux, J., Humby, F., Nzeusseu Toukap, A., Badot, V., Pitzalis, C., Houssiau, F. A., Durez, P. & Lauwerys, B. R. Higher expression of TNFα-induced genes in the synovium of patients with early rheumatoid arthritis correlates with disease

activity, and predicts absence of response to first line therapy. *Arthritis Res. Ther.* **18,** 19 (2016).
252. Contreras, G., Mattiazzi, A., Guerra, G., Ortega, L. M., Tozman, E. C., Li, H., Tamariz, L., Carvalho, C., Kupin, W., Ladino, M., LeClercq, B., Jaraba, I., Carvalho, D., Carles, E. & Roth, D. Recurrence of lupus nephritis after kidney transplantation. *J. Am. Soc. Nephrol.* **21,** 1200–1207 (2010).
253. Ford, M. L., Adams, A. B. & Pearson, T. C. Targeting co-stimulatory pathways: transplantation and autoimmunity. *Nat Rev Nephrol* **10,** 14–24 (2014).
254. Morel, L. Genetics of SLE: evidence from mouse models. *Nat Rev Rheumatol* **6,** 348–357 (2010).
255. Chavele, K.-M. & Ehrenstein, M. R. Regulatory T-cells in systemic lupus erythematosus and rheumatoid arthritis. *FEBS Lett.* **585,** 3603–3610 (2011).
256. Hayashi, T., Hasegawa, K. & Adachi, C. Elimination of CD4(+)CD25(+) T cell accelerates the development of glomerulonephritis during the preactive phase in autoimmune-prone female NZB x NZW F mice. *Int J Exp Pathol* **86,** 289–296 (2005).
257. Scalapino, K. J., Tang, Q., Bluestone, J. A., Bonyhadi, M. L. & Daikh, D. I. Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. *J. Immunol.* **177,** 1451–1459 (2006).
258. Wilhelm, A. J., Rhoads, J. P., Wade, N. S. & Major, A. S. Dysregulated CD4+ T cells from SLE-susceptible mice are sufficient to accelerate atherosclerosis in LDLr-/- mice. *Ann. Rheum. Dis.* **74,** 778–785 (2015).
259. D'Alise, A. M., Auyeung, V., Feuerer, M., Nishio, J., Fontenot, J., Benoist, C. & Mathis, D. The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 19857–19862 (2008).
260. Venigalla, R. K. C., Tretter, T., Krienke, S., Max, R., Eckstein, V., Blank, N., Fiehn, C., Ho, A. D. & Lorenz, H.-M. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127 -/low regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum.* **58,** 2120–2130 (2008).
261. Sfikakis, P. P., Souliotis, V. L., Fragiadaki, K. G., Moutsopoulos, H. M., Boletis, J. N. & Theofilopoulos, A. N. Increased expression of the FoxP3 functional marker of regulatory T cells following B cell depletion with rituximab in patients with lupus nephritis. *Clin. Immunol.* **123,** 66–73 (2007).
262. Dinesh, R. K., Skaggs, B. J., La Cava, A., Hahn, B. H. & Singh, R. P. CD8+ Tregs in lupus, autoimmunity, and beyond. *Autoimmun Rev* **9,** 560–568 (2010).
263. Morel, L., Croker, B. P., Blenman, K. R., Mohan, C., Huang, G., Gilkeson, G. & Wakeland, E. K. Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 6670–6675 (2000).
264. Linterman, M. A., Rigby, R. J., Wong, R. K., Yu, D., Brink, R., Cannons, J. L., Schwartzberg, P. L., Cook, M. C., Walters, G. D. & Vinuesa, C. G. Follicular helper T cells are required for systemic autoimmunity. *J. Exp. Med.* **206,** 561–576 (2009).
265. Zhang, B., Zhang, X., Tang, F., Zhu, L. & Liu, Y. Reduction of forkhead box P3 levels in CD4+CD25high T cells in patients with new-onset systemic lupus erythematosus. *Clin. Exp. Immunol.* **153,** 182–187 (2008).
266. Gracie, J. A. & Bradley, J. A. Interleukin-12 induces interferon-gamma-dependent switching of IgG alloantibody subclass. *Eur. J. Immunol.* **26,** 1217–1221 (1996).

267. Nakamura, K., Kitani, A. & Strober, W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J. Exp. Med.* **194,** 629–644 (2001).
268. Takimoto, T., Wakabayashi, Y., Sekiya, T., Inoue, N., Morita, R., Ichiyama, K., Takahashi, R., Asakawa, M., Muto, G., Mori, T., Hasegawa, E., Saika, S., Shizuya, S., Hara, T., Nomura, M. & Yoshimura, A. Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. *J. Immunol.* **185,** 842–855 (2010).
269. Parietti, V., Monneaux, F., Décossas, M. & Muller, S. Function of CD4+,CD25+ Treg cells in MRL/lpr mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. *Arthritis Rheum.* **58,** 1751–1761 (2008).
270. Wong, E. B., Khan, T. N., Mohan, C. & Rahman, Z. S. M. The lupus-prone NZM2410/NZW strain-derived Sle1b sublocus alters the germinal center checkpoint in female mice in a B cell-intrinsic manner. *J. Immunol.* **189,** 5667–5681 (2012).
271. Smith, R. N. & Colvin, R. B. Chronic alloantibody mediated rejection. *Semin. Immunol.* **24,** 115–121 (2012).
272. Hagberg, N., Theorell, J., Hjorton, K., Spee, P., Eloranta, M.-L., Bryceson, Y. T. & Rönnblom, L. Functional Anti-CD94/NKG2A and Anti-CD94/NKG2C Autoantibodies in Patients With Systemic Lupus Erythematosus. *Arthritis Rheumatol* **67,** 1000–1011 (2015).
273. Melzi, R., Sanvito, F., Mercalli, A., Andralojc, K., Bonifacio, E. & Piemonti, L. Intrahepatic islet transplant in the mouse: functional and morphological characterization. *Cell Transplant* **17,** 1361–1370 (2008).
274. Mohan, C., Alas, E., Morel, L., Yang, P. & Wakeland, E. K. Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/DNA subnucleosomes. *J. Clin. Invest.* **101,** 1362–1372 (1998).
275. Rahman, Z. S. M. & Manser, T. Failed up-regulation of the inhibitory IgG Fc receptor Fc gamma RIIB on germinal center B cells in autoimmune-prone mice is not associated with deletion polymorphisms in the promoter region of the Fc gamma RIIB gene. *J. Immunol.* **175,** 1440–1449 (2005).
276. Kumar, K. R., Li, L., Yan, M., Bhaskarabhatla, M., Mobley, A. B., Nguyen, C., Mooney, J. M., Schatzle, J. D., Wakeland, E. K. & Mohan, C. Regulation of B cell tolerance by the lupus susceptibility gene Ly108. *Science* **312,** 1665–1669 (2006).
277. Wan, S., Xia, C. & Morel, L. IL-6 produced by dendritic cells from lupus-prone mice inhibits CD4+CD25+ T cell regulatory functions. *J. Immunol.* **178,** 271–279 (2007).
278. Yoachim, S. D., Nuxoll, J. S., Bynoté, K. K. & Gould, K. A. Estrogen receptor alpha signaling promotes Sle1-induced loss of tolerance and immune cell activation and is responsible for sex bias in B6.Sle1 congenic mice. *Clin. Immunol.* **158,** 153–166 (2015).
279. Chen, Y., Cuda, C. & Morel, L. Genetic determination of T cell help in loss of tolerance to nuclear antigens. *J. Immunol.* **174,** 7692–7702 (2005).
280. Cuda, C. M., Wan, S., Sobel, E. S., Croker, B. P. & Morel, L. Murine lupus susceptibility locus Sle1a controls regulatory T cell number and function through multiple mechanisms. *J. Immunol.* **179,** 7439–7447 (2007).
281. Cuda, C. M., Zeumer, L., Sobel, E. S., Croker, B. P. & Morel, L. Murine lupus susceptibility locus Sle1a requires the expression of two sub-loci to induce inflammatory T cells. *Genes Immun.* **11,** 542–553 (2010).

282. Cuda, C. M., Li, S., Liang, S., Yin, Y., Potula, H. H. S. K., Xu, Z., Sengupta, M., Chen, Y., Butfiloski, E., Baker, H., Chang, L. J., Dozmorov, I., Sobel, E. S. & Morel, L. Pre-B Cell Leukemia Homeobox 1 Is Associated with Lupus Susceptibility in Mice and Humans. *The Journal of Immunology* **188,** 604–614 (2012).
283. Wandstrat, A. E., Nguyen, C., Limaye, N. & Chan, A. Y. Association of Extensive Polymorphisms in the SLAM/CD2 Gene Cluster with Murine Lupus. *Immunity* (2004).
284. Boackle, S. A., Holers, V. M., Chen, X., Szakonyi, G., Karp, D. R., Wakeland, E. K. & Morel, L. Cr2, a candidate gene in the murine Sle1c lupus susceptibility locus, encodes a dysfunctional protein. *Immunity* **15,** 775–785 (2001).
285. Perry, D. J., Yin, Y., Telarico, T., Baker, H. V., Dozmorov, I., Perl, A. & Morel, L. Murine Lupus Susceptibility Locus Sle1c2 Mediates CD4+ T Cell Activation and Maps to Estrogen-Related Receptor γ. *The Journal of Immunology*. **189**, 793-803 (2012).
286. Morel, L., Blenman, K. R., Croker, B. P. & Wakeland, E. K. The major murine systemic lupus erythematosus susceptibility locus, Sle1, is a cluster of functionally related genes. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 1787–1792 (2001).
287. Mohan, C., Morel, L., Yang, P. & Wakeland, E. K. Genetic dissection of systemic lupus erythematosus pathogenesis: Sle2 on murine chromosome 4 leads to B cell hyperactivity. *J. Immunol.* **159,** 454–465 (1997).
288. Xu, Z., Duan, B., Croker, B. P., Wakeland, E. K. & Morel, L. Genetic dissection of the murine lupus susceptibility locus Sle2: contributions to increased peritoneal B-1a cells and lupus nephritis map to different loci. *J. Immunol.* **175,** 936–943 (2005).
289. Li, J., Liu, Y., Xie, C., Zhu, J., Kreska, D., Morel, L. & Mohan, C. Deficiency of type I interferon contributes toSle2-associated component lupus phenotypes. *Arthritis Rheum.* **52,** 3063–3072 (2005).
290. Lantow, M., Sivakumar, R., Zeumer, L., Wasserfall, C., Zheng, Y.-Y., Atkinson, M. A. & Morel, L. The granulocyte colony stimulating factor pathway regulates autoantibody production in a murine induced model of systemic lupus erythematosus. *Arthritis Res. Ther.* **15,** R49 (2013).
291. Mohan, C., Yu, Y., Morel, L., Yang, P. & Wakeland, E. K. Genetic dissection of Sle pathogenesis: Sle3 on murine chromosome 7 impacts T cell activation, differentiation, and cell death. *J. Immunol.* **162,** 6492–6502 (1999).
292. Liu, K., Li, Q. Z., Yu, Y., Liang, C., Subramanian, S., Zeng, Z., Wang, H. W., Xie, C., Zhou, X. J., Mohan, C. & Wakeland, E. K. Sle3 and Sle5 can independently couple with Sle1 to mediate severe lupus nephritis. *Genes Immun.* **8,** 634–645 (2007).
293. Jacob, C. O., Zhu, J., Armstrong, D. L., Yan, M., Han, J., Zhou, X. J., Thomas, J. A., Reiff, A., Myones, B. L., Ojwang, J. O., Kaufman, K. M., Klein-Gitelman, M., McCurdy, D., Wagner-Weiner, L., Silverman, E., Ziegler, J., Kelly, J. A., Merrill, J. T., Harley, J. B., Ramsey-Goldman, R., Vila, L. M., Bae, S. C., Vyse, T. J., Gilkeson, G. S., Gaffney, P. M., Moser, K. L., Langefeld, C. D., Zidovetzki, R. & Mohan, C. Identification of IRAK1 as a risk gene with critical role in the pathogenesis of systemic lupus erythematosus. *Proceedings of the National Academy of Sciences* **106,** 6256–6261 (2009).
294. Liu, K., Li, Q.-Z., Delgado-Vega, A. M., Abelson, A.-K., Sánchez, E., Kelly, J. A., Li, L., Liu, Y., Zhou, J., Yan, M., Ye, Q., Liu, S., Xie, C., Zhou, X. J., Chung, S. A., Pons-Estel, B., Witte, T., de Ramón, E., Bae, S.-C., Barizzone, N., Sebastiani, G. D., Merrill, J. T., Gregersen, P. K., Gilkeson, G. G., Kimberly, R. P., Vyse, T. J., Kim, I., D'Alfonso,

S., Martin, J., Harley, J. B., Criswell, L. A., Profile Study Group, Italian Collaborative Group, German Collaborative Group, Spanish Collaborative Group, Argentinian Collaborative Group, SLEGEN Consortium, Wakeland, E. K., Alarcón-Riquelme, M. E. & Mohan, C. Kallikrein genes are associated with lupus and glomerular basement membrane-specific antibody-induced nephritis in mice and humans. *J. Clin. Invest.* **119,** 911–923 (2009).

295. Yin, Y., Choi, S.-C., Xu, Z., Perry, D. J., Seay, H., Croker, B. P., Sobel, E. S., Brusko, T. M. & Morel, L. Normalization of CD4+ T cell metabolism reverses lupus. *Sci Transl Med* **7,** 274ra18 (2015).
296. Jones, S. A., Scheller, J. & Rose-John, S. Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *J. Clin. Invest.* **121,** 3375–3383 (2011).
297. La Torre, De, M., Urra, J. M. & Blanco, J. Raised expression of cytokine receptor gp130 subunit on peripheral lymphocytes of patients with active lupus. A useful tool for monitoring the disease activity? *Lupus* **18,** 216–222 (2009).
298. Migita, K., Izumi, Y., Jiuchi, Y., Kozuru, H., Kawahara, C., Izumi, M., Sakai, T., Nakamura, M., Motokawa, S., Nakamura, T. & Kawakami, A. Effects of Janus kinase inhibitor tofacitinib on circulating serum amyloid A and interleukin-6 during treatment for rheumatoid arthritis. *Clin. Exp. Immunol.* **175,** 208–214 (2014).
299. Pawlus, M. R., Wang, L. & Hu, C.-J. STAT3 and HIF1α cooperatively activate HIF1 target genes in MDA-MB-231 and RCC4 cells. *Oncogene* **33,** 1670–1679 (2014).
300. Lee, C.-F., Lo, Y.-C., Cheng, C.-H., Furtmüller, G. J., Oh, B., Andrade-Oliveira, V., Thomas, A. G., Bowman, C. E., Slusher, B. S., Wolfgang, M. J., Brandacher, G. & Powell, J. D. Preventing Allograft Rejection by Targeting Immune Metabolism. *Cell Rep* **13,** 760–770 (2015).
301. Chen, L., Ahmed, E., Wang, T., Wang, Y., Ochando, J., Chong, A. S. & Alegre, M.-L. TLR signals promote IL-6/IL-17-dependent transplant rejection. *The Journal of Immunology* **182,** 6217–6225 (2009).
302. Wang, T., Ahmed, E. B., Chen, L., Xu, J., Tao, J., Wang, C. R., Alegre, M.-L. & Chong, A. S. Infection with the intracellular bacterium, Listeria monocytogenes, overrides established tolerance in a mouse cardiac allograft model. *Am. J. Transplant.* **10,** 1524–1533 (2010).
303. Riella, L. V., Yang, J., Chock, S., Safa, K., Magee, C. N., Vanguri, V., Elyaman, W., Lahoud, Y., Yagita, H., Abdi, R., Najafian, N., Medina-Pestana, J. O. & Chandraker, A. Jagged2-signaling promotes IL-6-dependent transplant rejection. *Eur. J. Immunol.* **43,** 1449–1458 (2013).
304. Yamamoto, M., Yokoyama, Y., Shimizu, Y., Yajima, H., Sakurai, N., Suzuki, C., Naishiro, Y. & Takahashi, H. Tofacitinib can decrease anti-DNA antibody titers in inactive systemic lupus erythematosus complicated by rheumatoid arthritis. *Mod Rheumatol* 1–2 (2015). doi:10.3109/14397595.2015.1069473
305. Vincenti, F., Tedesco Silva, H., Busque, S., O'Connell, P., Friedewald, J., Cibrik, D., Budde, K., Yoshida, A., Cohney, S., Weimar, W., Kim, Y. S., Lawendy, N., Lan, S.-P., Kudlacz, E., Krishnaswami, S. & Chan, G. Randomized phase 2b trial of tofacitinib (CP-690,550) in de novo kidney transplant patients: efficacy, renal function and safety at 1 year. *Am. J. Transplant.* **12,** 2446–2456 (2012).
306. Yang, R., Lirussi, D., Thornton, T. M., Jelley-Gibbs, D. M., Diehl, S. A., Case, L. K., Madesh, M., Taatjes, D. J., Teuscher, C., Haynes, L. & Rincón, M. Mitochondrial Ca^{2+}

and membrane potential, an alternative pathway for Interleukin 6 to regulate CD4 cell effector function. *Elife* **4,** (2015).
307. Sun, Y., Wang, W., Shan, B., Di, J., Chen, L., Ren, L., Li, W., Li, D.-J. & Lin, Y. FTY720-induced conversion of conventional Foxp3- CD4+ T cells to Foxp3+ regulatory T cells in NOD mice. *Am. J. Reprod. Immunol.* **66,** 349–362 (2011).
308. Gerriets, V. A., Kishton, R. J., Nichols, A. G., Macintyre, A. N., Inoue, M., Ilkayeva, O., Winter, P. S., Liu, X., Priyadharshini, B., Slawinska, M. E., Haeberli, L., Huck, C., Turka, L. A., Wood, K. C., Hale, L. P., Smith, P. A., Schneider, M. A., MacIver, N. J., Locasale, J. W., Newgard, C. B., Shinohara, M. L. & Rathmell, J. C. Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *J. Clin. Invest.* **125,** 194–207 (2015).
309. Shrestha, S., Yang, K., Guy, C., Vogel, P., Neale, G. & Chi, H. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat. Immunol.* **16,** 178–187 (2015).
310. Macintyre, A. N., Gerriets, V. A., Nichols, A. G., Michalek, R. D., Rudolph, M. C., Deoliveira, D., Anderson, S. M., Abel, E. D., Chen, B. J., Hale, L. P. & Rathmell, J. C. The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. *Cell Metab.* **20,** 61–72 (2014).
311. Lefaucheur, C., Loupy, A., Hill, G. S., Andrade, J., Nochy, D., Antoine, C., Gautreau, C., Charron, D., Glotz, D. & Suberbielle-Boissel, C. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. *J. Am. Soc. Nephrol.* **21,** 1398–1406 (2010).
312. Hussain, S. & Delovitch, T. L. Intravenous transfusion of BCR-activated B cells protects NOD mice from type 1 diabetes in an IL-10-dependent manner. *J. Immunol.* **179,** 7225–7232 (2007).
313. Wicker, L. S., Appel, M. C., Dotta, F., Pressey, A., Miller, B. J., DeLarato, N. H., Fischer, P. A., Boltz, R. C. & Peterson, L. B. Autoimmune syndromes in major histocompatibility complex (MHC) congenic strains of nonobese diabetic (NOD) mice. The NOD MHC is dominant for insulitis and cyclophosphamide-induced diabetes. *J. Exp. Med.* **176,** 67–77 (1992).
314. Kendall, P. L., Moore, D. J., Hulbert, C., Hoek, K. L., Khan, W. N. & Thomas, J. W. Reduced diabetes in btk-deficient nonobese diabetic mice and restoration of diabetes with provision of an anti-insulin IgH chain transgene. *The Journal of Immunology* **183,** 6403–6412 (2009).
315. Ziegler, A. I., Le Page, M. A., Maxwell, M. J., Stolp, J., Guo, H., Jayasimhan, A., Hibbs, M. L., Santamaria, P., Miller, J. F., Plebanski, M., Silveira, P. A. & Slattery, R. M. The CD19 signalling molecule is elevated in NOD mice and controls type 1 diabetes development. *Diabetologia* **56,** 2659–2668 (2013).
316. Yamano, T., Nedjic, J., Hinterberger, M., Steinert, M., Koser, S., Pinto, S., Gerdes, N., Lutgens, E., Ishimaru, N., Busslinger, M., Brors, B., Kyewski, B. & Klein, L. Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction. *Immunity* **42,** 1048–1061 (2015).
317. Walters, S. N., Webster, K. E., Daley, S. & Grey, S. T. A role for intrathymic B cells in the generation of natural regulatory T cells. *The Journal of Immunology* **193,** 170–176 (2014).
318. Herman, A. E., Freeman, G. J., Mathis, D. & Benoist, C. CD4+CD25+ T regulatory cells

dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J. Exp. Med.* **199,** 1479–1489 (2004).
319. Wu, Z., Bensinger, S. J., Zhang, J., Chen, C., Yuan, X., Huang, X., Markmann, J. F., Kassaee, A., Rosengard, B. R., Hancock, W. W., Sayegh, M. H. & Turka, L. A. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat. Med.* **10,** 87–92 (2004).
320. Wu, H., Chen, Y., Liu, H., Xu, L.-L., Teuscher, P., Wang, S., Lu, S. & Dent, A. L. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur. J. Immunol.* **46,** 1152–1161 (2016).
321. Molano, R. D., Pileggi, A., Berney, T., Poggioli, R., Zahr, E., Oliver, R., Ricordi, C., Rothstein, D. M., Basadonna, G. P. & Inverardi, L. Prolonged islet allograft survival in diabetic NOD mice by targeting CD45RB and CD154. *Diabetes* **52,** 957–964 (2003).
322. Vrtovec, B., Haddad, F., Pham, M., Deuse, T., Fearon, W. F., Schrepfer, S., Leon, S., Vu, T., Valantine, H. & Hunt, S. A. Granulocyte colony-stimulating factor therapy is associated with a reduced incidence of acute rejection episodes or allograft vasculopathy in heart transplant recipients. *Transplant. Proc.* **45,** 2406–2409 (2013).
323. Haller, M. J., Gitelman, S. E., Gottlieb, P. A., Michels, A. W., Rosenthal, S. M., Shuster, J. J., Zou, B., Brusko, T. M., Hulme, M. A., Wasserfall, C. H., Mathews, C. E., Atkinson, M. A. & Schatz, D. A. Anti-thymocyte globulin/G-CSF treatment preserves β cell function in patients with established type 1 diabetes. *J. Clin. Invest.* **125,** 448–455 (2015).
324. Konieczny, B. T., Dai, Z., Elwood, E. T., Saleem, S., Linsley, P. S., Baddoura, F. K., Larsen, C. P., Pearson, T. C. & Lakkis, F. G. IFN-gamma is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. *J. Immunol.* **160,** 2059–2064 (1998).
325. Yi, Z., Li, L., Garland, A., He, Q., Wang, H., Katz, J. D., Tisch, R. & Wang, B. IFN-γ receptor deficiency prevents diabetes induction by diabetogenic CD4+, but not CD8+, T cells. *Eur. J. Immunol.* **42,** 2010–2018 (2012).
326. Nguyen, H. D., Chatterjee, S., Haarberg, K. M. K., Wu, Y., Bastian, D., Heinrichs, J., Fu, J., Daenthanasanmak, A., Schutt, S., Shrestha, S., Liu, C., Wang, H., Chi, H., Mehrotra, S. & Yu, X.-Z. Metabolic reprogramming of alloantigen-activated T cells after hematopoietic cell transplantation. *J. Clin. Invest.* **126,** 1337–1352 (2016).
327. Erdbruegger, U., Scheffner, I., Mengel, M., Schwarz, A., Verhagen, W., Haller, H. & Gwinner, W. Impact of CMV infection on acute rejection and long-term renal allograft function: a systematic analysis in patients with protocol biopsies and indicated biopsies. *Nephrol. Dial. Transplant.* **27,** 435–443 (2012).
328. Adams, A. B., Williams, M. A., Jones, T. R., Shirasugi, N., Durham, M. M., Kaech, S. M., Wherry, E. J., Onami, T., Lanier, J. G., Kokko, K. E., Pearson, T. C., Ahmed, R. & Larsen, C. P. Heterologous immunity provides a potent barrier to transplantation tolerance. *J. Clin. Invest.* **111,** 1887–1895 (2003).
329. Miller, M. L., Daniels, M. D., Wang, T., Chen, J., Young, J., Xu, J., Wang, Y., Yin, D., Vu, V., Husain, A. N., Alegre, M.-L. & Chong, A. S. Spontaneous restoration of transplantation tolerance after acute rejection. *Nat Commun* **6,** 7566 (2015).
330. Mok, M. Y. Tolerogenic dendritic cells: role and therapeutic implications in systemic lupus erythematosus. *Int J Rheum Dis* **18,** 250–259 (2015).
331. Pearson, T., Weiser, P., Markees, T. G., Serreze, D. V., Wicker, L. S., Peterson, L. B.,

Cumisky, A.-M., Shultz, L. D., Mordes, J. P., Rossini, A. A. & Greiner, D. L. Islet allograft survival induced by costimulation blockade in NOD mice is controlled by allelic variants of Idd3. *Diabetes* **53,** 1972–1978 (2004).
332. Gaudreau, S., Guindi, C., Ménard, M., Besin, G., Dupuis, G. & Amrani, A. Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of CD4+CD25+ regulatory T cells. *J. Immunol.* **179,** 3638–3647 (2007).
333. Kirk, A. D., Burkly, L. C., Batty, D. S., Baumgartner, R. E., Berning, J. D., Buchanan, K., Fechner, J. H., Germond, R. L., Kampen, R. L., Patterson, N. B., Swanson, S. J., Tadaki, D. K., TenHoor, C. N., White, L., Knechtle, S. J. & Harlan, D. M. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat. Med.* **5,** 686–693 (1999).
334. Vincenti, F. What's in the Pipeline? New Immunosuppressive Drugs in Transplantation - Vincenti - 2002 - American Journal of Transplantation - Wiley Online Library. *American Journal of Transplantation* (2002).
335. Robles-Carrillo, L., Meyer, T., Hatfield, M., Desai, H., Dávila, M., Langer, F., Amaya, M., Garber, E., Francis, J. L., Hsu, Y.-M. & Amirkhosravi, A. Anti-CD40L immune complexes potently activate platelets in vitro and cause thrombosis in FCGR2A transgenic mice. *The Journal of Immunology* **185,** 1577–1583 (2010).
336. Xie, J. H., Yamniuk, A. P., Borowski, V., Kuhn, R., Susulic, V., Rex-Rabe, S., Yang, X., Zhou, X., Zhang, Y., Gillooly, K., Brosius, R., Ravishankar, R., Waggie, K., Mink, K., Price, L., Rehfuss, R., Tamura, J., An, Y., Cheng, L., Abramczyk, B., Ignatovich, O., Drew, P., Grant, S., Bryson, J. W., Suchard, S., Salter-Cid, L., Nadler, S. & Suri, A. Engineering of a novel anti-CD40L domain antibody for treatment of autoimmune diseases. *The Journal of Immunology* **192,** 4083–4092 (2014).
337. Chen, G., Luke, P. P. W., Yang, H., Visser, L., Sun, H., Garcia, B., Qian, H., Xiang, Y., Huang, X., Liu, W., Senaldi, G., Schneider, A., Poppema, S., Wang, H., Jevnikar, A. M. & Zhong, R. Anti-CD45RB monoclonal antibody prolongs renal allograft survival in cynomolgus monkeys. *Am. J. Transplant.* **7,** 27–37 (2007).
338. Vincenti, F., Rostaing, L., Grinyo, J., Rice, K., Steinberg, S., Gaite, L., Moal, M.-C., Mondragon-Ramirez, G. A., Kothari, J., Polinsky, M. S., Meier-Kriesche, H.-U., Munier, S. & Larsen, C. P. Belatacept and Long-Term Outcomes in Kidney Transplantation. *N. Engl. J. Med.* **374,** 333–343 (2016).
339. Heher, E. & Markmann, J. F. The Clearer BENEFITS of Belatacept. *N. Engl. J. Med.* **374,** 388–389 (2016).
340. Massart, A., Pallier, A., Pascual, J., Viklicky, O., Budde, K., Spasovski, G., Klinger, M., Sever, M. S., Sørensen, S. S., Hadaya, K., Oberbauer, R., Dudley, C., De Fijter, J. W., Yussim, A., Hazzan, M., Wekerle, T., Berglund, D., De Biase, C., Pérez-Sáez, M. J., Mühlfeld, A., Orlando, G., Clemente, K., Lai, Q., Pisani, F., Kandus, A., Baas, M., Bemelman, F., Ponikvar, J. B., Mazouz, H., Stratta, P., Subra, J.-F., Villemain, F., Hoitsma, A., Braun, L., Cantarell, M. C., Colak, H., Courtney, A., Frasca, G. M., Howse, M., Naesens, M., Reischig, T., Serón, D., Seyahi, N., Tugmen, C., Alonso Hernandez, A., Beňa, L., Biancone, L., Cuna, V., Díaz-Corte, C., Dufay, A., Gaasbeek, A., Garnier, A., Gatault, P., Gentil Govantes, M. A., Glowacki, F., Gross, O., Hurault de Ligny, B., Huynh-Do, U., Janbon, B., Jiménez Del Cerro, L. A., Keller, F., La Manna, G., Lauzurica, R., Le Monies De Sagazan, H., Thaiss, F., Legendre, C., Martin, S., Moal,

M.-C., Noël, C., Pillebout, E., Piredda, G. B., Puga, A. R., Sulowicz, W., Tuglular, S., Prokopova, M., Chesneau, M., Le Moine, A., Guérif, P., Soulillou, J.-P., Abramowicz, M., Giral, M., Racapé, J., Maggiore, U., Brouard, S. & Abramowicz, D. The DESCARTES-Nantes survey of kidney transplant recipients displaying clinical operational tolerance identifies 35 new tolerant patients and 34 almost tolerant patients. *Nephrol. Dial. Transplant.* **31,** 1002–1013 (2016).

341. Ring, A. M., Lin, J.-X., Feng, D., Mitra, S., Rickert, M., Bowman, G. R., Pande, V. S., Li, P., Moraga, I., Spolski, R., Ozkan, E., Leonard, W. J. & Garcia, K. C. Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat. Immunol.* **13,** 1187–1195 (2012).
342. Krutzik, P. O., Crane, J. M., Clutter, M. R. & Nolan, G. P. High-content single-cell drug screening with phosphospecific flow cytometry : Article : Nature Chemical Biology. *Nat. Chem. Biol.* (2008).
343. Henry, R. A., Kendall, P. L. & Thomas, J. W. Autoantigen-specific B-cell depletion overcomes failed immune tolerance in type 1 diabetes. *Diabetes* **61,** 2037–2044 (2012).
344. Deeds, M. C., Anderson, J. M., Armstrong, A. S., Gastineau, D. A., Hiddinga, H. J., Jahangir, A., Eberhardt, N. L. & Kudva, Y. C. Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models. *Lab. Anim.* **45,** 131–140 (2011).
345. Li, D.-S., Yuan, Y.-H., Tu, H.-J., Liang, Q.-L. & Dai, L.-J. A protocol for islet isolation from mouse pancreas. *Nat Protoc* **4,** 1649–1652 (2009).