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Autoimmune Diabetes and Transplantation Tolerance Induced by Costimulation Blockade in NOD Mice: a Dissertation

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AUTOIMMUNE DIABETES AND TRANSPLANTATION TOLERANCE INDUCED BY COSTIMULATION BLOCKADE IN NOD MICE

A Dissertation Presented

By

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ABSTRACT

NOD mice model human type 1diabetes and have been used to investigate tolerance induction protocols for islet transplantation in a setting of autoimmunity. Costimulation blockade-based tolerance protocols that induce prolonged skin and permanent islet allograft survival in non-autoimmune mice have failed in NOD mice. To investigate the underlying mechanisms, we generated NOD hematopoietic chimeras. We were able to show that dendritic cell maturation defects seen in NOD mice are partially corrected in mixed hematopoietic chimeras. Furthermore, skin allograft survival was dependent upon the phenotype of the bone marrow donor, demonstrating that in the NOD the resistance to tolerance induction resides in the hematopoietic compartment. In addition, we studied congenic NOD mice bearing insulin dependent diabetes (*Idd*) loci that reduce diabetes incidence. The incidence of diabetes is reduced in NOD.B6 *Idd3* mice, and virtually absent in NOD.B6 *Idd3Idd5* mice. Islet allograft survival in NOD.B6 *Idd3* mice is prolonged as compared to NOD mice, and in NOD.B6 *Idd3Idd5* mice islet allograft survival is similar to that achieved in C57BL/6 mice. Alloreactive CD8 T cell depletion in NOD mice treated with costimulation blockade is impaired, but is partially restored in NOD.B6 *Idd3* mice, and completely restored in NOD.B6 *Idd3Idd5* mice. *Idd3* results from variations in *Il2* gene transcription. We hypothesized insufficient levels of IL-2 in NOD mice contributes to impaired deletion of alloreactive CD8 T cells and shortened islet allograft survival. We observed using synchimeric mice that co-

administration of exogenous IL-2 to NOD mice treated with costimulation blockade led to deletion of alloreactive CD8 T cells comparable to that in C57BL/6 mice and prolonged islet allograft survival. However, some *Idd* loci impaired the induction of transplantation tolerance. These data suggest that *Idd* loci can facilitate or impair induction of transplantation tolerance by costimulation blockade, and that *Idd3* (IL-2) is critical component in this process.

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Abbreviations

T1D	Type 1 Diabetes
ICA	islet cell antigens
GAD	glutamic acid decarboxylase
IA-2	islet autoantigen tyrosine phosphatase
JDRF	juvenile diabetes research foundation
MHC	major histocompatibility complex
APC	antigen presenting cell
TCR	T cell receptor
Th	helper T cell
MØ	macrophage
DTH	delayed-type hypersensitivity
CTL	cytotoxic T lymphocyte
Ig	immunoglobulin
IL	interleukin
IFNγ	interferon gamma
Treg	CD4+CD25+ regulatory T cell
mAb	monoclonal antibody
FasL	fas ligand
NK	natural killer cell
DC	dendritic cell
BCR	B cell receptor
ADCC	antibody dependent cellular cytotoxicity
Ts	suppressor T cell
AICD	activation induced cell death
ICOS	inducible costimulatory molecule
CTLA-4	cytotoxic T lymphocyte antigen-4
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
PD-1	programmed death 1
NOD	non obese diabetic
rIL-4	recombinant interleukin 4
Idd	insulin dependent diabetes
SNP	single nucleotide polymorphism

INTRODUCTION

Overview of diabetes mellitus

Diabetes mellitus is a disease characterized by hyperglycemia [1]. Most cases are classified as either type-1, insulin-dependent diabetes mellitus (T1D), or type-2 non-insulin-dependent diabetes mellitus [2]. Type-2 diabetes, the more common syndrome, is generally seen in adults, though its prevalence in children is rising [3]. It is associated with obesity and can often be controlled with diet and exercise. The pathology associated with type-2 diabetes is due to either an acquired insulin resistance or a relative deficiency in insulin. In most cases insulin secretion is altered but not totally lost [2].

T1D is much less common and usually presents during childhood. Unlike type-2 diabetes, T1D is caused by the autoimmune mediated destruction of insulin-producing pancreatic beta cells and results in absolute insulin deficiency. There is a substantial clinical dataset that supports the autoimmune hypothesis of T1D. Patients suffering from T1D often present with concurrent autoimmune diseases including Addison's disease, vitiligo, celiac disease and lymphocytic thyroiditis [4, 5]. In addition, T1D is characterized by the presence of autoantibodies directed against islet cell antigens (ICA), insulin autoantibodies (IAA), glutamic acid decarboxylase (GAD), and tyrosine phosphatase (IA-2) among others [5]. In fact, the presence of combinations of these autoantibodies can be predictive of disease onset in euglycemic children [6]. Additional support for the autoimmune hypothesis was obtained from transplantation studies involving the transplant of a pancreas from a nondiabetic donor to a diabetic

monozygotic twin. Inflammation of the transplanted islets and eventual β cell destruction developed, which suggested the recurrence of a tissue-specific autoimmune process [7].

Patients with T1D require the administration of exogenous insulin for survival. Even with patient compliance, insulin therapy does not achieve perfect glucose regulation and complications such as cardiovascular disease, blindness and kidney failure develop. The difficulty in managing this disease, along with the severity of the associated complications, has made the search for a cure paramount. Cyclosporine-based immunosuppressive therapy was found to prolong endogenous insulin production in diabetic children, but the toxicity of the drug was too high to be used for acceptable curative therapy in patients [8]. The JDRF currently lists numerous trials with other agents that are under way and involve strategies based upon immunosuppression, immunomodulation and oral tolerance [9]. However, at present there are no interventions documented to be both safe and efficacious in preventing or reversing T1D.

The only curative treatment presently available for patients with T1D is the transplantation of insulin-producing islet cells. In a landmark study, Scharp and colleagues published a successful report of one month of insulin independence in a TID patient following islet transplantation [10]. Unfortunately, technical limitations in islet isolation and complications from immunosuppression prevented the wide-scale application of this technique. Enthusiasm for using islet transplantation as a curative therapy for T1D declined until report of the Edmonton Islet Transplantation Protocol. Developed by Shapiro and colleagues in Edmonton, Canada, this transplantation protocol has achieved promising results through better islet isolation techniques and improved

immunosuppressive regimes [11, 12]. More than 70 patients have now undergone islet transplantation with this protocol, including three patients transplanted by the Diabetes Division at UMass.

Though islet transplantation can achieve short-term normal glucose regulation, the perennial hope that it will result in long-term freedom from the need for exogenous insulin has failed to materialize. This is partly due to the recurrence of autoimmunity. Patients suffering from TID are programmed for autoimmunity, and there is reason to believe that the transplanted islets will become the target of a smoldering autoimmune environment. In addition, there are many difficulties inherent with the toxic side effects of the requisite immunosuppressive drugs and the complications associated with generalized immune suppression. These side effects include oral ulcers, anemia, diarrhea, weight loss, fatigue, LDL (low-density lipoprotein) elevation, hypertension, renal dysfunction, peripheral edema and increased risk of infection [13]. Excellent outcomes notwithstanding, contemporary immunosuppressive medications are toxic, are often not taken by patients [14], and pose long-term risks of infection and malignancy [15]. An alternative to immunosuppressive therapy is to achieve donor islet allograft survival by inducing transplantation tolerance.

Solid Organ Transplantation

Direct and indirect pathways of allorecognition

All tissues express cell surface proteins collectively referred to as major histocompatibility complex (MHC). The encoded region of the MHC, while structurally homologous, is extremely polymorphic at the peptide binding region. It is the disparity,

or *allogenicity*, of MHC between individuals that constitutes immune recognition of "self" versus "non self". The more closely matched a donor and host are for MHC the greater the likelihood is that the donor graft will be accepted. The MHC is further subdivided into two classes designated MHC class I and MHC class II. Class I and class II molecules are responsible for presenting antigen to T cells in the generation of an immune response. Generally speaking, MHC class I molecules present self-antigen derived from the degradation of intracellular proteins. MHC class II molecules present extracellular, or foreign, antigens.

A targeted immune response to a transplanted graft occurs in two phases. In the first, donor antigens are presented to recipient T lymphocytes, which become activated, proliferate and secrete cytokines for growth and differentiation into a number of other cell types. The second phase, or effector phase, occurs when peripheral T cells are recruited into the site of the graft where they can recognize and destroy the foreign tissue.

As mentioned above, antigen presentation takes place within the context of MHC/peptide complexes. This recognition can either be direct or indirect. In the indirect pathway of allorecognition, host antigen presenting cells (APC) endocytose and process foreign antigen for expression in the extracellular domain of the class II MHC [16, 17]. Within the context of transplantation, this host TCR/MHC class II interaction would involve recognition of the foreign antigen and lead to the initiation of an immune response to the foreign tissue. While foreign donor antigens are usually presented to T cells by MHC class II molecules, presentation of foreign alloantigen by MHC class I molecules has also been proposed [17].

Alternatively, the direct pathway of alloantigen presentation involves the immediate recognition of MHC class I or class II on the surface of donor APC by host CD8+ and CD4+ T cells, respectively. This is thought to occur because of the high precursor frequency of T cells present in the host that have allospecific cross reactivity [18]. In this pathway, a TCR that is selected for restriction to a particular self-MHC is able to directly recognize and respond to allo-MHC-peptide complexes.

Allografts can be rejected by all three mechanisms just described. In addition, the tempo of acute humoral, acute allograft and chronic rejections are mediated by different populations of cells and employ different mechanisms. Because of this, it is important to consider the individual and collaborative roles certain subsets of immune cells have in transplantation.

Role of T cells

Helper T cells (Th) express the coreceptor CD4 and bind to MHC class II/peptide complexes. CD4+ T cells can recognize alloantigen via both the direct recognition of the allo-MHC on the graft or through the indirect presentation of alloantigen within the context of self-MHC by the host APC. Th responses are subdivided into two categories, Th1 and Th2 [19]. Th1 CD4+ cells produce the cytokines IL-2, IFN-γ and TNF-β, which can activate both macrophages (Mø) and T cells. These responses are proinflammatory and promote targeted cellular immunity such as delayed type hypersensitivity (DTH) and CTL activity that serve as an important effector mechanism in allograft rejection. Th2 CD4+ T cells, on the other hand, polarize an immune response toward humoral IgE-mediated allergic and mucosal immune responses through the production of IL-4, IL-5

and IL-10 [20]. Although Th2 type cells are thought to have an immunosuppressive effect on the tempo of allograft rejection, some studies have shown that in the absence of CD8+ T cells, Th2-type cytokines can contribute to graft rejection [21]. Depending on the cytokine milieu produced, immune responses can be polarized toward either a Th1 or a Th2 response. Allograft recognition by CD4+ cells in the presence of IL-4 skews the response to a Th2-type, while antigen recognition in the presence of IFN-γ directs a more Th1-type response. IFN-γ directs this polarization by preventing the differentiation of naïve CD4+ cells into Th2-type cells.

There is also support of a role for a subset of CD4+ T cells in allograft survival. Depletion of CD4+ T cells during the maintenance phase of allograft survival has leads to rapid rejection of the transplanted tissue, and long-term survival of skin allografts in response to costimulation blockade-induced tolerance requires the presence of a CD4+ population [22, 23]. This is probably due to the ability of a subset of CD4+ CD25+ regulatory T cell (Treg) to regulate the activity of CD8+ T cells in alloresponses [24]. Interestingly, injection of anti-CD25 mAb does not prevent the deletion of alloreactive CD8+ T cells, though skin graft survival was nonetheless brief. In contrast, CD4+CD25+ cell deletion did not effect islet allograft survival [25]. This suggests that tissues have differential requirements in tolerance and therapies aimed at inducing tolerance may need to be modulated depending on the tissue being targeted.

CD8+, or cytotoxic T lymphocytes (CTL), are restricted to recognition of MHC class I/peptide and have the ability to directly lyse a target cell. In addition, upon receiving help from CD4+ cells, naïve CD8+ T cells can become activated and release

perforin and granzymes, two cytotoxic granules that damage the offending cell membrane and lead to death. CTLs also express CD95 (Fas ligand), which can lead to target cell destruction via capsase-induced apoptosis on CD90 (Fas)-expressing cells [26]. CTLs are capable of rejecting allografts both through the release of cytotoxic granules and via FasL mechanisms [27]. Direct recognition of the allo-MHC is the main way CTL exert their effect in acute rejection, and eliminating alloreactive CD8+ T cells during the peri transplant period is a critical component of many tolerance induction protocols [22, 28, 29].

Naïve T cells are cells that have emigrated from the thymus and have not encountered antigen. They have a stringent requirement of TCR engagement for their peripheral survival. Memory T cells, however, are antigen experienced and retain memory of that exposure. Naïve T cells have a higher requirement of antigen, need stronger costimulatory signals and rely on the presence of enhancing cytokines much more heavily than memory T cells for their activation [30]. Because of the differential requirements naïve T cells and memory T cells have for activation, tolerance induction protocols must be developed that both prevent the activation of allospecific naïve cells while eliminating the preexisting memory T cells able to respond to alloantigen.

Role of NK cells

Natural killer cells (NK) do not require pre-sensitization and can directly lyse tumor and virus-infected target cells without T cell help. Unlike T cells, which rely on recognition of the appropriate peptide/MHC complex, NK cell recognition of malignant or infected targets is not restricted by the MHC. NK cell effector mechanisms, much like

CTL, involve perforin and granzymes. They can also drive antibody dependent cellular cytotoxicity (ADCC) of antibody coated targets through the expression of Fc receptors. Within the context of transplantation, NK cells can respond to allogeneic donor tissue that bears a different complement of MHC molecules [31]. In cardiac and skin allograft transplantation animal models, depletion of different lymphocyte populations showed that NK cells were neither necessary nor sufficient for acute allograft rejection [32, 33]. Evidence in favor of a role for allogeneic NK cells in allograft rejection comes from studies involving cardiac allografts in CD28 knockout mice [34, 35]. In these experiments, MHC haploidentical cardiac transplants were tolerated without immunosuppression. Fully MHC mismatched grafts were rejected acutely. This rejection was prevented with the depletion of NK cells.

Role of dendritic cells

Dendritic cells (DC) are sentinels of the immune system residing in interstitial tissues and can induce and regulate T cell activity through their role as an APC. They are uniquely well equipped because they can process and present antigen to naïve and memory T cells while providing the necessary costimulation molecules and cytokines required by T cells to become activated. Costimulation and its role in transplantation tolerance will be discussed in more detail in subsequent sections.

Several studies suggest that DC are involved in both the direct and indirect pathways of allorecognition. In the indirect pathway, host DC continuously traffic through an allograft, where they uptake soluble antigen and cellular debris and present it within the context of self-MHC class II to host CD4+ T cells in peripheral lymphoid

organs [36]. This form of indirect allorecognition is similar to the process that occurs during pathogen recognition as DC process and present foreign antigen within the context of self-MHC.

Alternatively, the direct pathway of allorecognition is mediated by passenger DC that migrate from the donor graft into the spleens of graft recipients, where they initiate immune responses with host CD4+ and CD8+ T cells [37]. Support for the role of DC in direct allorecognition has been shown *in vitro*. In primary mixed leukocyte cultures (MLC), the direct pathway is responsible for the proliferation of responder T cells following allogeneic APC stimulation and resulted from the high number of T cells able to recognize allogeneic MHC [38].

Because DC are such potent initiators of the allograft rejection response, therapies that either eliminate or block DC have been shown to result in prolonged allograft survival [39]. According to the "danger hypothesis", a pro-inflammatory Th1-type environment leads to increased surface expression of MHC and costimulatory molecules on the DC and the production of proinflammatory factors that collectively induce a potent T cell response [40].

In addition to their role in initiating allograft rejection, DC can also serve a suppressive function. DC that have gobbled apoptotic debris are tolerogenic [41] and the injection of apoptotic cells improves the acceptance of allogeneic bone marrow grafts, presumably through the generation of tolerogenic DC [42]. The uptake of antigens by immature DC that express low levels of MHC and costimulatory molecules may induce tolerance to the peptide presented. This hypothesis is based upon the fact that binding of

the TCR on a naïve T cell to MHC-peptide complexes on a DC, either in the absence of or with low levels of costimulation leads to either anergy or apoptosis of the T cell [43] or to the generation of Treg [44].

Role of B cells

B cells are the effector cells of humoral immunity and secrete antibodies [45]. The B cell receptor (BCR) is a membrane-bound immunoglobulin (Ig) that is the same specificity as the Ig secreted by that particular B cell. A B cell activation program includes class-switching and affinity maturation of the Ig genes and results in high affinity, antigen-specific antibody responses. In addition, B cells express MHC class II and function as APC to T cells, efficiently presenting antigenic determinants that are specific for the BCR while concomitantly providing costimulation to the responding T cell [46].

Although T cells alone are sufficient to reject an allograft, it is possible that the humoral activity of B cells could participate in this process either by synergizing with T cells or antagonizing them to prevent rejection [27]. Antibodies directed against the allograft could target the graft for destruction by Mø and neutrophils. Additionally, allografts coated with antibody could be recognized by NK cells via membrane-bound Fc receptors and targeted for ADCC.

Role of Mø

Mø are among the first APC to infiltrate a graft and can initiate the inflammatory process while scavenging debris from necrotic graft tissue. Like other APC, they process and present alloantigen to the immune system with concomitant release of

proinflammatory cytokines and chemokines. Experimentally, mixed lymphocyte cultures performed in the absence of B7/CD28 interaction leads to the generation of Mø that have suppressive function, implicating a potential tolerogenic role for Mø in costimulation blockade-induced tolerance [47].

Central and peripheral tolerance

Transplantation tolerance is defined functionally as the survival of foreign tissue in a host in the absence of immunosuppression. T cell tolerance may be accomplished through many mechanisms, but is most broadly divided into two categories: central tolerance and peripheral tolerance [48].

Central tolerance

Central tolerance is achieved in the thymus during T cell development. During positive selection, T cells undergo a highly selective process that ensures that the T cells bearing functional antigen receptors are selected for survival. For many developing T cells this is the end of their career. Most of the randomly rearranged T cell receptors (TCR) are useless because they cannot bind the MHC expressed in the individual. Positive selection is a crucial first step that ensures the selection of T cell progenitors that are MHC restricted. It allows only cells that express a TCR that interacts with self-peptide-MHC to differentiate further.

In contrast, clonal deletion ensures that self-reactive T cells that have high avidity for self MHC-peptide are deleted and do not immigrate to the periphery [49]. Within the context of transplantation tolerance, it follows that alloantigen expression in the thymus should likewise lead to the clonal deletion of alloreactive T cells and prevent peripheral

allograft rejection. Intrathymic injection of alloantigen, along with deletion of preexisting peripheral alloreactive T cells has shown to be effective for the tolerance to many different tissues [27]. These results are cautionary though, and have failed to work in other animal models or non-human primates.

Central tolerance mechanisms are not perfect and some self-reactive T cells do escape into the periphery [50, 51]. Multiple peripheral tolerance mechanisms evolved to ensure that these thymic emigrant renegade autoreactive T cells are prevented from becoming inappropriately activated. These mechanisms include ignorance, apoptosis, anergy and the active suppression by CD4+CD25+ regulatory T cells (Tregs) and suppressor T cells (Ts) [52]. In fact, experimental approaches to the induction of tolerance have relied heavily on the mechanisms of peripheral tolerance to induce long-term survival of allografts. We will discuss these mechanisms in the next section within the context of the multiple positive and negative signaling pathways available to a T cell. The signaling events propagated through these pathways collectively determine whether or not a T cell is permitted to become activated and gain effector function, remain lethargic and ignorant, or is destined to die in the periphery.

Peripheral tolerance and costimulation blockade-based tolerance induction

The holy grail of inducing tolerance to foreign tissue is to find a way to prevent an alloimmune response against the grafted tissue without chronic generalized, nonspecific immune suppression. A promising area of research has been to target the various signaling pathways that lead to complete T cell activation or, conversely, activation induced cell death (AICD) or anergy. These pathways are triggered in response to antigen

recognition by the TCR and costimulation provided by an APC or in some cases epithelial cells. Modulation of these positive and negative signals triggered in response to allorecognition by the TCR can lead to targeted immune suppression that is allospecific.

The concept of costimulation was first introduced in the 1970s with the statement "induction and paralysis of an immune response involve the recognition of one and two determinants on an antigen respectively."[53]. This was soon expanded to become the "two signal hypothesis" of complete T cell activation [54]. This postulates that in order to become fully activated, a T cell needs to recognize a cognate MHC-antigen complex. Antigen recognition by the TCR generates *signal 1* while the simultaneously delivered *signal 2*, or costimulation, completes the activation of the T cell. Furthermore, T cells that encounter cognate MHC-antigen and receive signal 1 in the absence of costimulatory signal 2 are either rendered anergic or undergo apoptosis. A more complete understanding of the two signal hypothesis within the context of allospecific T cells suggests that in order for a T cell to become fully activated, three receptor-ligand interactions must occur. The first follows TCR recognition of antigen/MHC complexes (signal 1). As a consequence, CD154 (CD40L) on the T cell interacts with CD40 on the APC (coactivation). Finally, CD80/86 (B7-1/2) on the participating APC provides costimulation (signal 2) to the responding T cell through CD28. These three interactions characterize a classic T cell response to foreign antigen, though in reality there are many more receptor/ligand pairs that participate or compete. Antibody-mediated blockade of these receptor-ligand interactions lead to functional differences in T cell activation. The different efficiencies of costimulation blockade-based modulation of T cell activation is

likely due to the crosstalk and competition that occurs between these various receptor-ligand pairs and the downstream signaling events they initiate.

While signal 1 is provided by the antigen and is responsible for the specificity of the response, costimulation is not antigen specific and relies on the interactions between many cell surface receptors and their ligands. These costimulatory receptors are not restricted to T cell-APC interactions and have been found to be involved in cross talk between T cells with each other, interaction between T cells and B cells and T cell signaling from peripheral nonlymphoid cells including endothelial cells [55]. In contrast to the development of activation and effector function provided by positive costimulatory signals, negative costimulation results in anergy, apoptosis or the induction of Tregs. It therefore follows that the functional outcome of an alloimmune response is mediated by interactions between T cells and many other cell subsets and involves both positive and negative signaling events shared by multiple pathways. Modulating destructive alloimmune responses through costimulation blockade may therefore require the concomitant targeting of multiple costimulatory pathways.

CD28/B7

Costimulatory molecules are grouped into two broad families based upon structural homologies, the CD28/B7 family and the tumor necrosis factor/tumor necrosis factor receptor (TNF/TNFR) family. CD28 is constitutively expressed on the surface of naïve T cells and as mentioned above has two known ligands expressed on APCs, CD80 (B7-1) and CD86 (B7-2). Upon activation through the TCR, T cells upregulate CD28 and CTLA-4. Though structurally similar, CTLA-4 binds with much higher avidity to CD80

and CD86 and results in a negative signal to the T cell that inhibits an immune response [56]. In contrast, costimulation through CD28 enhances T cell activation. Therefore, the competition between CD28 and CTLA-4 is one factor that determines the net result of a TCR signaling event.

The role these receptor/ligand pairs have in transplantation has been investigated using animal models and monoclonal antibodies that are able to block downstream signaling events. Interestingly, CD80/CD86 double knockout mice reject skin and islet allografts but not cardiac allografts [57, 58]. In addition, blocking the interaction between CD28 and CD80/CD86 with the recombinant fusion protein CTLA4Ig has been shown to be tolorogenic [59, 60].

CD40/CD154

Another positive signaling costimulatory pathway involves interaction between the TNF superfamily members CD40 and its ligand CD154. CD40 is constitutively expressed on APC, fibroblasts and endothelial cells while CD154 is found on activated T cells, B cells, dendritic cells and platelets [61, 62]. Unlike the CD28 pathway discussed above, signaling through CD40/CD154 results in indirect costimulation of the responding T cell. CD40/CD154 interaction augments the ability of the APC to present antigen, thus indirectly strengthening a T cell response.

The *in vivo* use of an antibody directed against CD154 (anti-CD154) has been shown to block the CD40/CD154 ligation and prevent allograft rejection in mouse models of skin, islet, bone marrow and cardiac allografts and non-human primate models of kidney allografts [63-66]. It is important to note that while these studies showed the

utility of a blocking anti-CD154 antibody for use in preventing acute allograft rejection, long-term prevention of chronic allograft rejection requires additional factors. Co-administration of bone marrow or a donor specific transfusion (DST) with the anti-CD154 antibody results in durable, long-term allograft survival and donor-specific tolerance and will be discussed in more detail later [23, 67, 68].

ICOS/B7h

A third costimulatory pathway that promotes complete T cell activation involves inducible costimulatory molecule (ICOS) and its ligand B7h. Unlike its homolog CD28, ICOS is not constitutively expressed but, as its name suggests, is induced upon TCR engagement on naïve T cells and on resting memory cells [69]. Its ligand, B7h is expressed on APCs, B cells and non-lymphoid endothelial cells at low levels, but upon cytokine triggered cellular activation is rapidly upregulated [70]. In a classic example of the crosstalk that occurs between various signaling pathways, ICOS/B7h ligation results in the increased expression of CD154, which upregulates CD80/CD86 expression and in turn increases CD28 signaling and T cell activation [71]. The ability of ICOS/B7h to buttress the strength of CD28 signaling illustrates how the amplitude and timing of various signaling pathways together maintain a balanced immune response.

In addition to being a regulator of T cell activation and effector function, ICOS signaling provides an important boost to activated and resting memory T cells and aids in their production of the effector cytokines IL-2, IL-4, IL-5 and IFNγ [72]. It has also been shown to have a role in transplantation tolerance. Like the studies involving αCD154, ICOS blockade alone can prolong allograft survival, though to a lesser extent than

αCD154, but cannot prevent chronic allograft rejection [73]. When used in combination with αCD154, ICOS/B7h blockade resulted in the prolongation of cardiac allograft survival by preventing both acute and chronic allograft rejection [74].

OX40/OX40L

A fourth pathway involved in the provision of costimulation to T cells and recognized to have a role in allograft tolerance is the pathway involving CD134 (OX40) and its ligand, CD134L (OX40L). Expressed on activated T cells, engagement of OX40 with OX40L on APCs results in a costimulatory signal to the T cell as potent as that generated by CD28 in gaining Th effector function and Treg development [74].

The role of OX40 in transplantation tolerance has been examined using rodent fully mismatched MHC cardiac and skin transplant models. While blockade of OX40L alone did not prolong allograft survival, when used in combination with CTLA-4Ig it was effective in achieving prolonged allograft survival [75]. Most interesting, however, is that memory T cell-mediated skin allograft rejection that is refractory to CD28/CD154 blockade may be sensitive to OX40 blockade [76]. In this model, memory T cells generated by donor antigen priming quickly rejected skin allografts in mice treated with OX40 blockade alone, but when used in combination with CD154 blockade resulted in long term skin allograft survival. Once again, this illustrates that concomitant targeting of multiple costimulatory pathways may be necessary to achieve donor specific allograft tolerance and prevent both acute and chronic graft rejection.

41-BB/41-BBL

CD137 (4-1BB) is a TNFR family member like CD40 and CD154. It is expressed on activated T cells, DC and NK cells, while its ligand, 4-1BBL, is found on Mø, DCs and B cells [77-79]. 4-1BB engagement provides positive costimulatory signals to both CD4+ and CD8+ T cells, though its role in CD8+ T cell survival following activation seems to be critical [80]. It is perhaps the role of 4-1BB in circumventing activation induced cell death (AICD) in recently activated allospecific CD8+ T cells that explains the accelerated allograft rejection in mouse cardiac and skin transplant models in response to *in vivo* administration of a signaling 4-1BB mAb during the peri transplant period [81, 82].

To extend these observations, some groups have shown in rejection-resistant mouse models the importance of CD8+ and NK cells as mediators of allograft rejection. In one study using CD28-deficient/CTLA-4-deficient mice with fully allogeneic cardiac grafts, CD86 blockade actually prolonged allograft survival, indicating that CD86 has an additional receptor that can provide a positive costimulatory signal to T cells [83]. In addition, another group investigated the effect of an OX40L blocking antibody on T cell activation in CD28-deficient mice and demonstrated that B cells can provide CD28-independent costimulation to T cells [84]. These studies bolster the idea that targeting multiple positive costimulation pathways, including 4-1BB and OX40, may be important in modulating CD8+ mediated allograft rejection in stringent transplantation models.

CTLA-4

In addition to the positive costimulation pathways involved in T cell activation, there exist multiple negative signaling pathways that serve to downregulate a T cell allorecognition event. As mentioned above, CTLA-4 expression is upregulated on T cells following the initiation of an activation program that includes CD40-CD154 engagement. Functionally, CTLA-4 competes with CD28 for CD80 and CD86 ligation and has a 10-100 fold higher affinity for binding than does CD28 [56, 85]. The role of CTLA-4 in delivering a negative signal to the T cell and downregulating an immune response has been shown with CTLA-4 knockout mice. These animals develop a severe lymphoproliferative disease that can be prevented with the use of a CTLA-4Ig fusion protein that blocks CD28 interaction with CD80/86 [86, 87].

The use of a CTLA-4Ig fusion protein that competitively antagonizes CD28 binding with CD80/86 has shed light on the role of CTLA-4 in allograft tolerance [88]. In one study using a rat cardiac allograft model, treatment of the animals with CTLA-4Ig during the transplant period prevented rejection of allografts compared to control rats [89]. In another study using rat renal allografts, the ability of CTLA-4Ig to prevent allograft rejection was based upon the timing of administration relative to transplantation [90]. This study also showed that the tolerance to allografts induced by CTLA-4Ig was donor specific, as third party allografts were quickly rejected. These observations have been extended to non-human primate studies, where CTLA-4Ig or CD80/86 blocking mAb prolongs both renal and islet allograft survival [91-93]. Interestingly, the effect seen

in some of these studies was dependent upon the coadministration of CTLA-4Ig and αCD154.

PD-1

Like CTLA-4, programmed death-1 (PD-1) also has a role in downregulating a T cell response by providing a negative signal. It is expressed on activated T cells, B cells, NK cells and Mø [94, 95]. The ligands for PD-1, PDL1 (B7-H1) and PDL2 (B7-DC) are tissue specific. PDL1 is promiscuously expressed in both the hematopoietic and non-hematopoietic compartments, while PDL2 expression is restricted to DC and Mø [96-98]. Unlike the CTLA-4:CD80/86 pathway discussed above that is responsive to CD28 engagement, PD-1 seems to have a role in the downregulation of CD4+ T cells independent of CD28 signaling [99].

Previous work in transplantation models has shown that ligation of PD-1 with PD-L1.Ig prolonged cardiac allograft survival in conjunction with cyclosporine and was associated with decreased IFNγ production in both CD28 knockout and wild-type (WT) recipients [100]. In another study, PD-L1.Ig and αCD154 mAb synergized to promote long-term islet allograft survival [101]. It was also recently shown that donor cardiac allografts deficient in PD-L1 were quickly rejected in a partially mismatched mouse model where WT grafts survive indefinitely without any intervention, demonstrating that PD-L1 expression on allograft tissue serves as an important negative regulator of alloimmune responses *in vivo* [102].

It is likely that the clinical application of successful donor specific tolerance induction will depend upon a more complete understanding of the balance between the

positive and negative signaling pathways that affect the activation of alloreactive T cells. The scope and breadth of an alloimmune response is a balance between positive and negative regulation, and harnessing negative signaling pathways while blocking positive costimulatory receptors may lead to effective new therapies in inducing transplantation tolerance.

The tolerance induction strategy used throughout this thesis is based upon a two-element protocol that includes a donor specific transfusion (DST) to deliver a source of alloantigen and activate the immune response while blocking CD40-CD154 to induce peripheral transplantation tolerance [27] (Figure 1). As described in a previous section, alloantigen recognition by the TCR of the MHC-antigen complex on an APC results in the engagement of the TCR, generating signal 1. This induces CD154 expression on the responding T cell. The second step, upregulation of CD154, permits the engagement of CD40 on the APC and is termed coactivation. Triggering through CD40 leads to the subsequent upregulation of the costimulatory molecules CD80 and CD86 on the APC. The APC then reciprocally activates the T cell via interaction between CD80/86 and CD28 on the T cell in a process called signal 2, or costimulation. The activated allospecific T cell then becomes an effector cell capable of mediating allograft rejection. Blocking signal 2 with the anti-CD154 mAb while introducing the alloantigen via the DST prevents activation of the responding T cell and promotes non-responsiveness in the host to donor alloantigen. This protocol has prolonged allograft survival to both islets and skin in a mouse model [66, 103, 104] and concordant rat to mouse xenograft model [105, 106]. Mechanistic studies have shown that allograft survival in response to costimulation

blockade is dependent upon both the presence of CD4+ cells and the deletion of recipient alloreactive CD8+ T cells [22, 23, 28]. Interestingly, the survival of skin and islet allografts is differentially regulated and appears to be mediated by different CD4+ subsets [25].

Figure 1: T cell activation

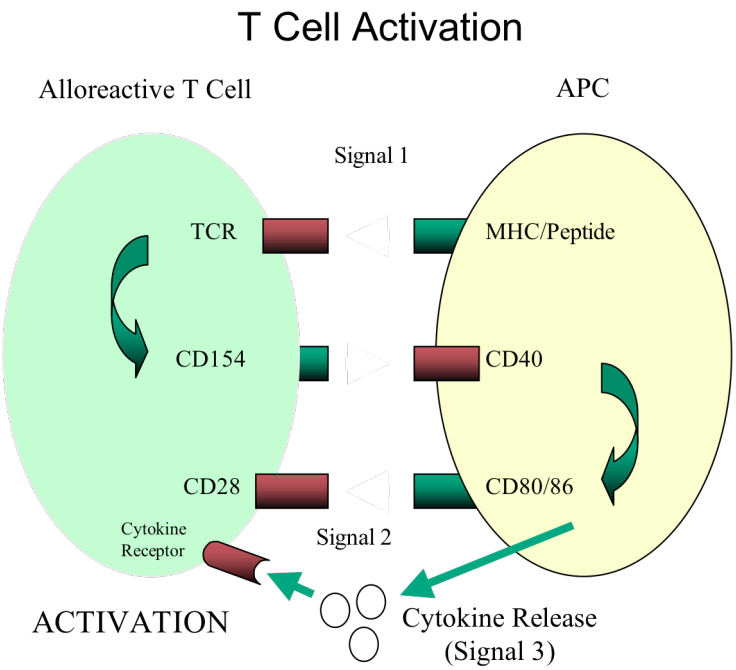


Figure 1 legend: In order for a T cell to become fully activated, three receptor-ligand interactions must occur. The first follows TCR recognition of antigen/MHC complexes (signal 1). As a consequence, CD154 (CD40L) on the T cell interacts with CD40 on the APC (coactivation). Finally, CD80/86 (B7-1/2) on the participating APC provides costimulation (signal 2) to the responding T cell through CD28.In the absence of signal 2, as is the case with administration of anti-CD154 mAb, T cell activation does not occur.

Modeling type 1 diabetes and Transplantation tolerance in NOD mice

We have chosen the Non-obese Diabetic (NOD) mouse to study transplantation tolerance within the context of autoimmunity. The ethical and technical pitfalls to studying diseases in human subjects make the development of animal models necessary. Animal models of diabetes have been bred to study modes of inheritance. They can be biopsied and autopsied. Their genome can be manipulated, or held constant by inbreeding. Furthermore, therapies developed to reverse or cure the disease can be tested in animal models. The two most common small animal models of type 1 diabetes are the BioBreeding (BB) rat and the Non-obese Diabetic (NOD) mouse [107]. The use of these two models has had a fundamental impact on our understanding of the genetics and pathogenesis of T1D. In addition, the NOD has proven invaluable as a tool for dissecting apart the pathways involved in tolerance, both to self and to alloantigens.

The NOD mouse as a model for diabetes

The NOD mouse is a model of type 1-like autoimmune diabetes mediated primarily by autoreactive T cells [107-109]. The kinetics of disease onset in the NOD closely resembles the tempo of disease progression in humans, with hyperglycemia being preceded by infiltration of mononuclear cells. Peri-insulitis is followed by more invasive infiltration directly into the islet. Development of diabetes proceeds from insulitis to an autoaggressive phase in which β cells are viciously destroyed and glucose homeostasis is disrupted. The mononuclear infiltrates include CD4+ T cells, CD8+ T cells, DC, NK cells, B cells and Mø [110]. Disease seems to be dependent upon CD4+ and CD8+ cells, as purified populations of these cells from overtly diabetic donors were able to transfer

disease into nondiabetic NOD recipients [111-113], and Class I and Class II-restricted T cell clones derived from NOD islets can transfer disease to nondiabetic recipients [114].

NOD immunological defects

NK cells

NOD mice express a number of immune defects that may contribute to the pathogenesis of T1D. NK cell-mediated cytotoxicity is deficient in NOD mice when assayed against NK-sensitive targets [115-117]. A possible explanation for the reduced cell number and function of NOD NK cells is that these cells co-express the NK cell receptor NKG2D and its ligand, RAE-1 [118]. It is hypothesized that the dysregulated expression of RAE-1 causes NKG2D to be internalized, thus leading to reduced NK cell activity. In addition, NK cells have been associated with disease severity in some TCR transgenic NOD models, and NK cell depletion leads to reduced pathology [119]. The exact role NK cell defects play in the pathogenesis of diabetes has been difficult to determine due to the lack of NK1.1 at the NKRP-1 locus in NOD mice ($NK1.1^{null}$), making identification of NK cells difficult. A promising strategy has been the congenic introgression of a diabetes resistant allele at *Idd6*, which includes genes important for NK function, including the NK1.1 allele at the NKRP-1 locus. NOD.*Idd6* mice have improved NK cell function and are modestly protected from diabetes [115].

B cells

NOD mice depleted of B cells were protected from the development of T1D, though the mechanisms by which B cells affect disease progression are unclear. It is possible that NOD B cells contribute to the development of autoimmunity through

production of autoantibodies, and in fact high titers of anti-insulin and anti-GAD autoantibodies are found in prediabetic NOD mice and diabetic humans. Paradoxically however, transfer of these autoantibodies failed to induce disease in NOD models [120]. In another study, an increased percentage of CD80+ and decreased percentage of CD86+ B cells were found in the spleens of NOD mice compared to the spleens of nonautoimmune C57BL/6 and BALB/c mice [121]. The skewed ratio of CD80 and CD86 expression was associated with the development of insulitis and CD4+CD25+ T reg deficiency, which suggests that NOD B cells may be associated with increased T cell costimulation and the development of insulitis.

Dendritic cells

In addition to B cell abnormalities, NOD mice harbor defects in DC maturation [122-127]. Recurrent to most of the dendritic cell studies done in NOD mice is the finding that both freshly isolated and bone marrow derived DC express lower basal levels of CD86, as seen above in the B cell compartment. Furthermore, following activation, CTLA-4 upregulation was reduced in NOD T cells as compared to levels seen in nonautoimmune control strains of mice. It has been hypothesized that lower levels of CD86 expression in the NOD mouse could contribute to defective regulation of autoreactive T cells by preventing the full activation of T cells and subsequent CTLA-4 upregulation [123].

Macrophages

Mø defects have also been reported in NOD mice [128-130]. One determination of Mø maturation is to quantify levels of secretion of IL-1β from bone marrow derived Mø stimulated with LPS [131], and NOD Mø secrete significantly less IL-1β than

similarly derived Mø from nonautoimmune C57BL/6 mice [132]. In addition, other groups have shown that Mø from NOD mice and patients with T1D display an increase in GM-CSF production and persistent STAT5 phosphorylation and are hypothesized to play a role in fostering a proinflammatory microenvironment within an immune system that is already conducive to the loss of tolerance regulation [129, 130].

T cells

Pathogenic CD4+ T cells restricted to the unique NOD I-A^{g7} MHC class II molecule can adoptively transfer disease when isolated from overtly diabetic NOD mice [133-136]. It has been hypothesized that while these T cells are probably not responsible for initiating pathogenesis, they develop at the time of disease onset [109].

Many believe that the inflammatory process of T1D in the NOD mouse is propagated by the secretion of Th1 cytokines (IFN-γ) and suppressed by Th2 anti-inflammatory cytokines (IL-4, IL-10). Several groups have shown that NOD mice have a generalized defect in the generation of an anti-inflammatory Th2 response [137-142]. In one study, thymic and peripheral T cell unresponsiveness after TCR cross-linking correlated with the time of insulitis and persisted until the onset of diabetes in NOD mice [143]. These T cells were shown to be deficient in the production of both IL-2 and IL-4 in response to TCR cross-linking compared to similarly activated T cells from non autoimmune BALB/c mice [139]. In addition, the administration of rIL-4 was shown to correct the thymic and peripheral T cell proliferation defect *in vitro* and protect against diabetes *in vivo*, suggesting that diabetes can be prevented in the NOD mouse by using Th2 secreted cytokines.

In additional support of the Th1/Th2 paradigm, the activation of NKT cells with α-galactosylceramide to induce the secretion of Th2 cytokines has been found to protect NOD mice from diabetes [144, 145]. While these studies suggest that activated NKT cells modulate the occurrence of TID in NOD mice by skewing cytokine production from a Th1 to a Th2 response, other groups have documented that α-galactosylceramide treatment also induces T1D resistance in NOD mice genetically deficient in the Th2 cytokines IL-4 and IL-10, which seems to suggest an alternative mechanism of protection [146].

The identification of regulatory T cells (Treg) as an important component of self-tolerance has lead to the hypothesis that impaired Treg function may be influencing the autoimmune phenotype seen in NOD mice. It has long been appreciated that a population of CD4+ T cells present in the periphery of pre-diabetic NOD mice is capable of suppressing diabetes [147]. Further support for this was shown in NOD mice deficient in CD28. These animals do not generate a CD4+CD25+ Treg population and develop T1D at an accelerated rate [148]. In addition, the depletion of CD4+CD25+ T cells from splenocytes of pre-diabetic mice rapidly induced diabetes after transfer into NOD.SCID recipients. Conversely, a population of islet antigen-specific Treg expanded from BDC2.5 TCR-transgenic mice has the ability to both prevent and reverse spontaneous diabetes in NOD mice [149, 150].

IL-2 has been shown to be an important component in the generation of Treg cells in the periphery [151]. Extending this observation to the NOD model, it has been shown that treatment with IL-2 *in vivo* inhibits diabetes development in NOD mice, with

protection associated with an improvement in Treg function. In addition, *in vitro* stimulated NOD thymocytes produce two-fold less IL-2 mRNA as compared to similarly treated thymocytes from non-autoimmune control mice [152]. This study also showed that the administration of exogenous IL-2 to NOD mice enhances the recruitment of regulatory T cells (Treg) to the inflammatory lesion in the islets, and the targeted genetic disruption of IL-2 accelerates the T1D phenotype. These data in their aggregate provide compelling evidence that reduced expression of IL-2 in the NOD leads to impaired Treg function and contributes to the T1D phenotype.

Tolerance induction in the NOD

An ongoing problem with using transplantation as a curative therapy for diabetes is in trying to induce tolerance to alloantigens in an autoagressive environment that has already lost tolerance to autoantigens. In addition to being targeted by alloreactive T cells, the transplanted tissue risks becoming the target of recurrent autoimmunity. The NOD mouse is the model of choice in developing therapies to cure type 1 diabetes, including islet transplantation. NOD mice have fundamental defects in T cell self-tolerance that appear to be related to breakdowns in both central and peripheral tolerance-inducing mechanisms.

Central tolerance

The establishment of central tolerance is dependent upon the deletion of potentially autoreactive T cells during development in the thymus and studies have shown that NOD mice have impaired thymic deletion of autoreactive T cells [153-155]. A possible factor that could be contributing to the breakdown in mechanisms responsible

for deleting or inactivating such pathogenic effectors is the expression in the NOD of a unique $H2^{g7}$ MHC. Multiple components of the $H2^{g7}$ haplotype contribute to T1D susceptibility. These include the $H2^{g7}$-encoded K^d and D^b class I molecules. Though K^d and D^b are common variants found in many non-autoimmune strains of mice, when expressed in the NOD they contribute to the development of diabetogenic CD8 T cell responses, most likely through interactions with *Idd* genes outside of the MHC [156]. The Class II genes contain the unusual H2-A^{g7} variant but are H2-E null [157, 158]. The first clue that the $H2^{g7}$ MHC was contributing to the breakdown in central tolerance was provided by studies using congenic strains of NOD mice that express MHC from non-autoimmune strains of mice [159-161]. In these studies, TID was dominantly inhibited through the heterozygous expression of protective MHC with the NOD $H2^{g7}$ MHC. It was also discovered that the transgenic or congenic expression of MHC from non-autoimmune strains of mice leads to restored function in hematopoietically-derived NOD APCs and triggered mechanisms which delete, anergize or regulate renegade autoreactive T cells [158, 161-165].

These studies suggest that hematopoietic chimerism in which APC express protective MHC molecules has the potential to prevent both autoimmune recurrence and the rejection of foreign tissues. To this end, there have been reports in which TID-protective allogeneic hematopoietic chimerism has been established in the NOD, however, the hematopoietic cells in the NOD recipient eventually converted almost exclusively to donor type [166, 167]. Though full hematopoietic chimerism in NOD mice prevents TID, it does so at the cost of a generalized immunosuppression [168]. This is

potentially a consequence of circulating peripheral T cells that were positively selected by thymic epithelial cells of non-hematopoietic origin. As a result, they are not specific for the MHC expressed on the bone marrow-derived APC circulating in the periphery that are able to activate their effector function. It was recently shown that permanently establishing a state of mixed allogeneic hematopoietic chimerism both prevents TID in NOD mice while avoiding the undesirable side effect of general immunosuppression [168].

Peripheral costimulation blockade based tolerance in the NOD

There are a number of tolerance induction protocols that establish peripheral transplantation tolerance in normal strains of mice [27]. Strategies usually include blockade of a) MHC/peptide-TCR interaction, b) costimulatory interaction, or c) CD80/86-CD28 interaction. Most of these protocols have failed in NOD mice, and we hypothesize that NOD mice have a generalized defect in their susceptibility to transplantation tolerance induction.

Early work demonstrated that chemically diabetic NOD mice resist tolerance induction to both syngeneic and allogeneic islets when treated with protocols that induce permanent allograft survival in non-autoimmune strains [169, 170]. This outcome could be due to the inability to induce allotolerance or an ongoing autoimmunity. The fact that prediabetic male NOD mice also reject allogeneic skin [169], which is not a target of the autoimmunity, supports our hypothesis that the NOD mouse has a generalized defect in peripheral tolerance induction to alloantigens. In addition, another group extended this

observation and showed a generalized defect in CD8+ peripheral tolerance induction using the targeted expression of a defined antigen on pancreatic β cells [171].

Many transplantation protocols target parts of the immune system that are abnormal in NOD mice and map to known *Idd* loci. Because of this, a common belief has been that the resistance seen in NOD mice to transplantation tolerance induction is a result of and/or controlled by the same genes that predispose it to autoimmunity [172]. The majority of *Idd* loci are NOD recessive susceptibility loci [173], and (NOD x C57BL/6)F1 mice, which are heterozygous at all *Idd* loci, are completely resistant to diabetes. Surprisingly, (NOD x C57BL/6)F1 mice remain resistant to costimulation blockade based tolerance induction to skin allografts, but not islet allografts [132]. These data suggest that the genetically dominant resistance of NOD mice to skin allograft tolerance induction is not a consequence of autoimmunity and that islet and skin allograft tolerance and the control of autoimmunity are not under identical genetic control [122, 172]. To investigate further the cellular and genetic control of costimulation blockade-induced transplantation tolerance, we have used NOD *Idd* congenic mice.

NOD Idd congenic mice

The contribution of multiple genes on many different chromosomes to disease susceptibility has been demonstrated in the NOD mouse [159, 174-177]. The first genome scan demonstrated the existence of multiple recessive loci, termed insulin-dependent diabetes (*Idd*) loci that were linked to diabetes resistance or susceptibility. Congenic inbred NOD strains bearing diabetes resistance alleles at one or multiple loci on a normal background, and reciprocal congenic strains in which NOD-derived *Idd*

susceptibility alleles have been introgressed into a normal background, have shed light on the contributions of individual loci to a polygenic, multifactoral disease process.

Idd9

Idd9 congenic mice carry at least three genes (*Idd9.1, 9.2* and *9.3*) derived from B10 mice within a 34-Mbp region. Candidate genes within this region include Lck and 4-1BB [178]. *Idd9* mice are profoundly protected from diabetes with the incidence of spontaneous autoimmunity reduced to less than 4%. In spite of this, they still develop significant insulitis and islet-specific autoantibodies [179].

Idd3

Idd3 maps within a 650-kb region on mouse chromosome 3 [180] and has a primary effect on the development of T1D in NOD mice. The *Idd*3 locus is known to play a role in the infiltration of autoreactive lymphocytes into the islets [181] and there is compelling evidence that *Idd3* is the IL-2 gene [152]. *In vitro* stimulated NOD thymocytes produce two-fold less IL-2 mRNA as compared to similarly treated thymocytes from NOD *Idd3* mice. Administration of exogenous IL-2 to NOD mice enhances the recruitment of Treg to the inflammatory lesion in the islets, and the targeted genetic disruption of IL-2 accelerates the T1D phenotype. In addition to reducing the frequency of spontaneous diabetes and insulitis in the NOD, *Idd3* also mediates protection from experimental autoimmune encephalomyelitis (EAE), which is highly sensitive to IL-2 [182].

Idd3/10/18

The *Idd3/10/18* congenic mouse contains *Idd3* described above with the addition of two other Idd loci termed *Idd10* and *Idd18*, also located on chromosome 3. Of interest within the *Idd10/18* region is the *Ptpn8* gene, which is orthologous to human *Ptpn22* [183]. Studies have shown that single-nucleotide polymorphisms (SNPs) in the lymphoid tyrosine phosphatase (LYP) encoded by the PTPN22 gene correlate with the development of type 1 diabetes in humans [184]. This observation has been extended to include an association between the same SNP in *Ptpn*22 and many other autoimmune diseases, including systemic lupus erythematosis, rheumatoid arthritis, and Grave's disease [185-191]. The disease-associated allele of *Ptpn*22 is a gain-of-function variant that *in vitro* has been shown to suppress TCR signaling in response to TCR/CD28 ligation more efficiently than the 'normal" allele [192]. Notably, the functional outcome of TCR signaling in the PTPN22 gain of function variant was reported to be reduced expression of IL-2.

Idd5

Idd5 contains four discreet loci termed *Idd5.1, 5.2, 5.3* and *5.4* located on mouse chromosome 1. Strong candidate genes within this region include CTLA-4 (*Idd5.1*), and NRAMP (*Idd5.2*) [193, 194]. *Idd5* alone causes only partial reduction in TID incidence. Thirty percent of NOD.B10*Idd5* females become spontaneously diabetic by 7 months of age as compared to 80% of NOD females [193]. In contrast, incidence of spontaneous diabetes development is reduced to less than 2% when *Idd5* is introgressed together with *Idd3* into the NOD background.

Tolerance induction in NOD.Idd mice

Costimulation blockade induced tolerance to skin allografts is prolonged on tolerized C57BL/6 mice and shortened on tolerized NOD mice. Surprisingly, NOD mice bearing very protective *Idd* loci remain resistant to tolerance induction and reject skin allografts rapidly [195]. Of note, NOD.B6*Idd3*B10*Idd5* and NOD.B10*Idd9* congenic stocks, which have a reduced frequency of spontaneously induced TID to 2% and 4%, respectively, still remain resistant to tolerance induction to skin allografts. These data, in addition to the data provided by the resistance to tolerance induction to skin allografts seen in the (NOD x C57BL/6)F1 mice , allow us to genetically separate the autoimmune phenotype from the resistance to tolerance induction phenotype.

In addition to rapidly rejecting skin allografts following costimulation blockade, NOD mice also fail to become tolerant to islet allografts. However, differences in the survival of skin versus islet allografts can be found in non-autoimmune mice [27, 196, 197]. In the case of the NOD mouse, destruction of the islet allograft could be due to underlying autoimmunity and not failure to induce allograft tolerance [170, 198]. While islet allograft tolerance cannot be induced in NOD.B10*Idd5* or NOD.B10*Idd9* mice that resist spontaneous autoimmunity, it can be achieved in non-autoimmune (NOD x C57BL/6)F1 and NOD.B6*Idd3* mice [199]. These data suggest that in order for islet allografts to survive in the setting of autoimmune diabetes, costimulation blockade must overcome both spontaneous diabetes and the genetic resistance of NOD mice to allotolerance induction.

Thesis goals and specific aims

A treatment for the cure of human type 1 diabetes (T1D) is the transplantation of insulin-producing islet cells. Excellent outcomes notwithstanding, contemporary immunosuppressive medications are toxic, are often not taken by patients, and pose long-term risks of infection and malignancy. An alternative to immunosuppressive therapy is to achieve donor allograft survival by inducing transplantation tolerance.

The NOD mouse is a model of type 1-like autoimmune diabetes mediated primarily by autoreactive T cells and is extensively used to study costimulation blockade-based transplantation tolerance within the context of autoimmunity. However, costimulation blockade-based transplantation tolerance protocols fail to prolong the survival of either islet or skin allografts in NOD mice. Interestingly, non-autoimmune (NOD x C57BL/6) F1 mice also resist the induction of transplantation tolerance to skin but not islet allografts. Furthermore, NOD mice bearing certain *Idd* loci that are potently protective of autoimmunity still fail to become tolerant to islet allografts. These data suggest that the genetically dominant resistance of NOD mice to skin allograft tolerance induction is not a consequence of autoimmunity and that islet and skin allograft tolerance and the control of autoimmunity are not under identical genetic control. To understand better the cellular and genetic contributions to both the susceptibility to autoimmunity and the resistance to transplantation tolerance in the NOD, three specific aims were addressed and we are hopeful that these studies will lead to a more thorough

understanding about the relationship between transplantation tolerance and autoimmunity.

Specific aim 1: We first wanted to test the hypothesis that dendritic cell maturation defects and resistance to tolerance induction in NOD mice are based in the hematopoietic cell compartment.

Specific aim 2: We hypothesized that *Idd* loci that are strongly protective against diabetes would enhance islet allograft survival in NOD mice treated with costimulation blockade. We chose to investigate *Idd* loci that contained promising candidate genes and confered varying degrees of protection from autoimmunity and compared their effects in alloimmunity.

Specific aim 3: To identify the cellular basis and mechanism for the resistance to transplantation tolerance in NOD mice. We hypothesized that in the NOD, failure to delete alloreactive CD8+ T cells is a major barrier to costimulation blockade induced transplantation tolerance. We further hypothesized that the exogenous administration of IL-2 can correct the tolerance defect by driving the deletion of the alloreactive CD8+ T cells.

METHODS

Animals

C3H/HeJ mice were obtained from the National Cancer Institute (Frederick, MD), The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY). NOD-*scid* mice were obtained from The Jackson Laboratory. C57BL/6J ($H2^b$), NOD/Mrk-TacfBR, NOD.B6 *Idd3*R450 (Line 1098), NOD.Czech *Idd3* (Line 1590), NOD.B6*Idd3*R450 + B10*Idd5*R444 (Line 1591 and re derived as Line 6109), NOD.B6*Idd3*R450 + B10*Idd5*R467 (Line 1573), NOD.B10*Idd5*R444 (Line 1094), NOD.B6*Idd3Idd10Idd18*R323 (Line 1538), NOD.B6*Idd10Idd18*R250 (Line 2410) NOD.B6*Idd9* (line 905) and NOD.B6*Idd10Idd18*R2 (Line 1101) were obtained from Taconic Farms. Because the experimental data using the NOD.B6 *Idd3*R450 (Line 1098) and NOD.Czech *Idd3* (Line 1590) congenic variants of *Idd3* were comparable, these groups have been combined for presentation of the experimental data and are referred to in the text as "*Idd3*" NOD congenic mice. A schematic of the congenic intervals on mouse chromosomes is shown in **Figure 8**. C57BL/6.NODc17 ($H2^{g7}$, hereafter termed C57BL/6.$H2^{g7}$) mice developed by Edward Wakeland, University of Texas Southwestern Medical Center, Dallas, TX were the gift of Dr. Edward Leiter (The Jackson Laboratory, Bar Harbor, ME). KB5 CBA x C57BL/6.$H2^{g7}$) F1 mice and (KB5 CBA x NOD) F1 mice were generated by a single intercross of the appropriate parental strains and were bred in our facility [200]. The KB5 breeding founders were the generous gift of Dr. John Iacomini (Harvard Medical School, Boston, MA) who obtained the mouse from the original developer, Dr. Andrew Mellor (Medical College of Georgia, Augusta, GA). The

TCR transgene is expressed in CBA ($H2^k$) mice by $CD8^+$ cells and the transgenic TCR has specificity for native H2-K^b [201].

All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, lactate dehydrogenase–elevating virus, mouse poliovirus, Reo-3 virus, mouse adenovirus, lymphocytic choriomeningitis virus, polyoma, *Mycoplasma pulmonis*, and *Encephalitozoon cuniculi*. They were housed in a specific pathogen-free facility in microisolator cages and given autoclaved food and acidified water *ad libitum*. All animal use was in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Antibodies and Flow Cytometry

FITC-conjugated anti-Ly5.1 mAb (clone A20), PerCP-conjugated anti-Ly5.2 mAb (clone 104), PE-congugated anti-CD11c mAb (clone HL3), APC-congugated anti CD11b mAb (clone M1/70), FITC-conjugated anti-CD11a mAb (clone M17/4), PerCept®-conjugated anti CD8 mAb (clone 53-6.7), PerCept®-Cy5.5-conjugated anti-CD4 mAb (clone L3T4), Alexa Fluor 405-congugated anti-TNF-α mAb (clone MP6-XT22) and PE-Cy7-conjugated anti-IFNγ mAb (clone XMG1.2) were obtained from BD PharMingen (San Diego, CA). Alexa Fluor 700-conjugated anti CD8α mAb (clone 5H10) and Alexa Fluor 405-conjugated CD44 (clone IM7.8.1) were obtained from Caltag/Invitrogen (Carlsbad, CA). Isotype controls included FITC-conjugated rat $IgG_{2a,\kappa}$,

PerCept®-conjugated hamster IgG_1, PerCept®-Cy5.5-conjugated rat $IgG_{2a,\kappa}$, APC-conjugated rat IgG_1, PE-Cy7-conjugated rat IgG_1 and PE-conjugated rat IgG_{2b} and were obtained from BD PharMingen. Alexa Fluor 405-conjugated rat IgG_{2b} and Alexa Fluor 700-conjugated rat IgG_{2b} were obtained from Caltag/Invitrogen.

MR1 hamster anti-mouse CD154 mAb was produced as tissue culture supernatant and purified by affinity chromatography (National Cell Culture Center, Minneapolis, MN). Antibody concentration was determined by measurement of optical density and confirmed by ELISA. The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC) and was uniformly <10 units/mg of mAb.

Multiparameter flow cytometry analyses of freshly isolated spleen cells were performed as described [132, 195, 199]. Briefly, $1x10^6$ viable cells were incubated for 5 min at 4°C with anti-FcγRIII/II mAb (clone 2.4G2) to eliminate non-specific Fc binding of conjugated antibodies. Cells were then washed and reacted with a mixture of conjugated mAbs for 20 min. Stained cells were washed, suspended in 1% paraformaldehyde-PBS, and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Lymphoid cells were gated according their light-scattering properties, and approximately $2.5x10^4$ events were acquired for each analysis.

Generation of KB5 Synchimeras

KB5 synchimeric mice were generated using a procedure previously described with slight modifications (40). Briefly, (CBA/J x NOD)F1 and (CBA/J x C57BL/6.$H2^{g7}$)F1 non-transgenic mice were treated with 400 cGy of whole-body gamma

irradiation from a ^{137}Cs source (Gammacell 40; Atomic Energy of Canada, Ottawa, Ontario, Canada or Mark I-30 Series 2000 Ci; JL Shepherd & Associates, San Fernando, CA) and given a single i.v. injection of $5x10^6$ (KB5 CBA x NOD)F1 or (KB5 CBA x C57BL/6.$H2^{g7}$)F1 bone marrow cells, respectively. Mice were entered into experiments 8-12 weeks after bone marrow transplantation.

Preparation of LPS-treated splenocytes

Splenocytes were prepared from the indicated mouse strains ($2x10^6$ cells/ml in supplemented RPMI) and were treated with LPS (15 μg/ml) for 3 days *in vitro*. Following incubation, the cultures were washed 3 times with supplemented RPMI, γ-irradiated (20 Gy), and frozen at –70°C until used as previously described [202].

Intracellular Cytokine Staining

Cytokine-producing mouse T cells were detected using the Cytofix/Cytoperm Kit Plus™ (with GolgiPlug™, BD Biosciences, Boston, MA). Splenocytes ($2x10^6$ cells) from mice were incubated with 250 ng/ml of anti-mouse CD3ε mAb (145-2C11, BD Biosciences) or with the indicated LPS-treated splenocytes populations ($1x10^6$ stimulator cells per sample) in the presence of 1 U/ml of human recombinant IL-2 (BD Biosciences) and 1 μl/ml GolgiPlugTM at 37°C in an atmosphere of 5% CO_2 for 5 hr. Following incubation, splenocytes were stained with mAb specific for CD8 (53-6.7), CD4 (RM4-5) and CD44 (RM5726). Samples were fixed and permeabilized with Cytofix/Cytoperm™ solution and stained with mAb specific for IFN-γ , TNF-a or IL-2 (XMG1.2 and MP6-XT22, respectively, BD Biosciences), or with an IgG1-isotype control (R3-34, BD Biosciences).

Generation of Hematopoietic Chimeras

Bone marrow donor NOD or C57BL/6^{g7} mice were killed in 100% CO_2. Bone marrow was obtained by flushing the femurs and tibias of donor mice with RPMI using a 24-gauge needle. Recovered cells were filtered through sterile nylon mesh (70 μm, Becton Dickinson, Franklin Lakes, NJ), counted by hemocytometer, and re-suspended in RPMI. 6-8 week old NOD or C57BL/6^{g7} recipient mice received 700 cGy of whole-body gamma irradiation from a ^{137}Cs source (Gammacell 40; Atomic Energy of Canada, Ottawa, Ontario, Canada or Mark I-30 Series 2000 Ci; JL Shepherd & Associates, San Fernando, CA) two times with a 4 hour interval between. 10 x 10^6 prepared NOD cells or 10 x 10^6 prepared C57BL/6^{g7}cells, (or 5 x 10^6 NOD donor cells plus 5 x 10^6 C57BL/6^{g7} donor cells in the case of the mixed chimeras) were injected in a volume of 0.5-1.0 ml via the lateral tail vein within 1-5 hrs of irradiation. Blood samples were obtained from all bone marrow recipients 4-6 weeks after transplantation for determination of the percentages of donor and host cells by flow microfluorometry.

Dendritic Cell Maturation Assay

Bone marrow was flushed from the femurs and tibias of euthanized donors into RPMI-10% FC serum. Erythrocytes were lysed with a 0.85% hypotonic NH_4Cl lysis buffer and the mononuclear cells were washed twice more in RPMI-10% FC. Viable mononuclear cells were counted and suspended at 2 x 10^6 cells/ml in RPMI-10% FC supplemented with 500 U/ml recombinant mouse GM-CSF and 1000 U/ml recombinant mouse IL-4 (R & D Systems). Bone marrow cells (3 x 10^6) were cultured in a six well tissue culture plate in a total volume of 3 ml/well. Typically, the cultures for each strain

consisted of pooled bone marrow from two mice. Cultures were incubated at 37 ° C in an atmosphere of 95% air 5% CO_2. On day two, non-adherent cells were removed by gentle swirling and half the medium was replaced with fresh medium supplemented with 500 U/ml GM-CSF and 1000 U/ml recombinant mouse IL-4. On day four, non-adherent cells were removed and fresh medium supplemented with cytokines was added as on day two and 5 ug/ml of an agonist anti-CD40 mAb (clone HM40-3, BD PharMingen) was added to the appropriate wells. Cultures were incubated an additional 48 hours at which point all cells (adherent and non-adherent) were harvested. Adherent cells were removed by gently scraping and pooled with the non-adherent cells. Each population was counted and then analyzed by flow cytometry. In all cases, cultured cells were washed twice in PBS-1% FC in the presence of 0.1% sodium azide and prepared for flow cytometry.

Tolerance Induction and Allograft Transplantation

Diabetes was induced in male C57BL/6, NOD, and NOD *Idd* congenic mice by a single intraperitoneal injection of streptozotocin (150 mg/kg). Animals were tested for glycosuria (test strips, Glucosin, Bayer, Elkhart, IN) twice weekly. Diabetes was confirmed by documenting plasma glucose concentration >250 mg/dl (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN.). Mice hyperglycemic for at least one week were used in the experiments. Chemically diabetic mice were treated with our standard costimulation blockade protocol consisting of a single C3H/HeJ donor-specific transfusion (DST) and anti-CD154 mAb and transplanted with a C3H/HeJ islet allograft as described [199]. Briefly, $1x10^7$ spleen cells obtained from 5-10 week old female C3H/HeJ mice were injected intravenously in a volume of 0.5 ml. DST was given on day

–7 relative to transplantation. Islet allograft recipients were also injected intraperitoneally with anti-CD154 mAb (0.5 mg/dose) on days –7, –4, 0, and +4 relative to islet transplantation on day 0.

Islets for transplantation were isolated from C3H/HeJ donors by collagenase digestion followed by density gradient separation as described (46,47). Handpicked islets (20 islets/gram body weight) were transplanted into the renal subcapsular space of chemically diabetic recipients. Plasma glucose concentrations were measured twice weekly and allograft rejection was defined as recurrent hyperglycemia (>250 mg/dL) on at least 2 consecutive days.

(KB5 CBA x NOD)F1 or (KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeric mice were treated with a single C57BL/6 donor-specific transfusion (DST) and anti-CD154 mAb and transplanted with a C57BL/6 skin allograft as described (40). Full-thickness skin grafts 1-2 cm in diameter were transplanted onto the dorsal flanks of recipients (48). Skin graft survival was evaluated three times a week, and graft rejection was defined as the first day on which the entire graft was necrotic.

Histology

Kidneys into which islet grafts had been transplanted were fixed in Boin's solution overnight, washed, and stored in 10% buffered formalin. Paraffin- embedded sections were prepared and stained with hematoxylin and eosin. Additional sections were stained immunohistochemically for the presence of insulin and glucagon.

Injection of IL-2 During Costimulation Blockade

In some experiments, mice were treated during costimulation blockade with exogenous IL-2. For these experiments, 0.8μg recombinant murine IL-2 (R & D systems, Minneapolis, MN) was injected intraperitoneally on days −7, −6, −5, −4 and −3 relative to analysis of KB5 DES^+ $CD8^+$ T cells or transplantation on day 0. Concurrently, costimulation blockade consisting of a single DST on day −7, and 4 injections of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0.

Statistics

Average duration of allograft survival is presented as the median. Graft survival among groups was compared using the method of Kaplan and Meier. The equality of allograft survival distributions for animals in different treatment groups was tested using the log rank statistic. P values <0.05 were considered statistically significant. Data is presented as the mean ± 1 s.d. Comparisons of two means used Student's t-test with separate variance estimates. Comparisons of three or more means used one-way analysis of variance and the LSD procedure for *a posteriori* contrasts.

Chapter 1: Resistance to transplantation tolerance and defects in dendritic cell maturation is intrinsic to the hematopoietic system

Introduction

The ability to induce transplantation tolerance to skin allografts by costimulation blockade in NOD mice is not a consequence of their expression of autoimmunity [132]. (NOD x C57BL/6)F1 mice are heterozygous at all *Idd* loci and are completely protected from autoimmune diabetes, yet they exhibit a genetically dominant resistance to the prolongation of skin allograft survival following treatment with a costimulation blockade protocol consisting of a donor-specific transfusion (DST) and anti-CD153 mAb [132]. It has also been shown that diabetes can be prevented by the adoptive transfer of bone marrow from diabetes-resistant donors [166, 167]. For example, TID was dominantly inhibited by the heterozygous expression of a protective MHC together with the diabetes prone NOD $H2^{g7}$ MHC. Furthermore, the transgenic or congenic expression of MHC from non-autoimmune strains of mice led to restored function in NOD-derived APCs and triggered mechanisms that delete, anergize or regulate renegade autoreactive T cells [158, 161-165].

These data suggest that the susceptibility to diabetes resides in the hematopoietic compartment. It follows that non-autoimmune-prone donor stem cells developing into APCs that express diabetes-resistant MHC acquire the ability to "reset the immune system" through mechanisms that limit the development and function of autoreactive T cells. In NOD bone marrow chimeras in which the $H2^{nb1}$ diabetes resistant MHC was

expressed on DC, macrophages and B cells, complete protection from diabetes was observed [203]. In this system, the $H2^{nb1}$ expressing DC and macrophages from the bone marrow donor mediated virtually complete deletion of the autoagressive CD8+ T cell population. We would like to extend these observations to determine if hematopoietic chimerism can likewise correct the tolerance induction defect of NOD mice. Because the expression of diabetes and the resistance to transplantation tolerance can be separated genetically, it is not known whether the tolerance induction defects in NOD mice reside in the hematopoietic cell compartment or the stromal environment in which they mature and function.

In this chapter, we first tested the hypothesis that the resistance of NOD mice to the induction of transplantation tolerance was dependent on the hematopoietic cell population, not the environment in which the hematopoietic cells mature and function. To investigate this, we created full hematopoietic chimeras by performing reciprocal NOD and C57BL/6^{g7} bone marrow transfers. We chose to use C57BL/6^{g7} based on their 1) lack of autoimmunity, 2) normal dendritic cell maturation, 3) ability to exhibit prolonged skin allograft survival following costimulation blockade, and 4) histocompatibility with NOD mice (both express the $H2^{g7}$ MHC). This minimized the potential for development of graft-versus-host disease in the hematopoietic chimeras.

The cellular basis for the resistance of NOD mice to transplantation tolerance is not known, but NOD mice do exhibit a broad spectrum of cellular and humoral immune abnormalities [116, 123, 131, 148, 204-208]. These include abnormal deletion of activated CD4+ and CD8+ T cells, decreased Treg function, defective cytokine secretion

by NK and NKT cells, B cell production of autoantibodies, and abnormal maturation of both DC and macrophages. Many of these cellular defects are genetically recessive and are not expressed in (NOD x C57BL/6)F1 mice. However, not all cellular defects are corrected, and CD8+ function and DC maturation in (NOD x C57BL/6)F1 mice continue to be abnormal. Based on these observations, we hypothesized that because of their critical role in modulating immune responses, DC maturation defects were important in the resistance of NOD mice to the induction of transplantation tolerance. To begin to test this hypothesis, we first determined if the dendritic cell maturation defect was intrinsic to the hematopoietic cells or the result of the non-hematopoietic environment.

Our overall hypothesis in this chapter is that dendritic cell maturation defects and resistance to tolerance induction are both based in the hematopoietic cell compartment. In this chapter we demonstrate that skin allograft survival in NOD or C57BL/6^{g7} hematopoietic chimeras is dependent on the hematopoietic cell donor, not the host. Furthermore, in full chimeras, dendritic cell maturation defects tracked with the genotype of the bone marrow donor. Together, these data demonstrate that the dendritic cell defects and resistance to tolerance induction of NOD mice are dependent on the source of the hematopoietic cell system.

Results

1. *Skin allograft survival in hematopoietic chimeras treated with costimulation blockade is dependent on the hematopoietic donor*

To begin to understand the basis for the dominant genetic resistance to tolerance induction seen in (NOD x C57BL/6) F1 mice, we first determined whether the resistance is dependent on the hematopoietic (irradiation-sensitive) or the non-hematopoietic (irradiation-resistant) cell compartment in which they mature and function. To begin to investigate this, we generated hematopoietic chimeras in which the entire hematopoietic system is of donor-origin. As shown in figure 2, the phenotype of skin allograft survival in mice treated with costimulation blockade tracked with the donor bone marrow. In control C57BL/6^{g7} into C57BL/6^{g7} hematopoietic chimeras, fully allogeneic C3H/HeJ *($H2^k$)* skin allograft survival was prolonged (Figure 2, median survival time MST=37.5 days, N=10). As expected, the NOD into NOD hematopoietic chimeras had skin allograft survival that was relatively short (Figure 2, MST=12 days, N=15 $p<0.0001$ versus C57BL/6^{g7}). Not shown is the NOD bone marrow into C57BL/6^{g7} recipient hematopoietic chimera group. Though this is an important experimental cohort, technical difficulties prevented this group from being included in the skin allograft experiments. There are plans to include this group in future allograft survival experiments. In the C57BL/6^{g7} into NOD hematopoietic chimeras, skin allograft survival was significantly prolonged (Figure 2, MST= 42 days, N=34 $p<0.0001$ versus NOD) relative to that achieved in the NOD into NOD hematopoietic chimera. These data demonstrate that skin allograft survival in NOD or C57BL/6^{g7} hematopoietic chimeras is dependent on the

hematopoietic cell donor, not the host, and support our hypothesis that tolerance induction defects present in the NOD are based in the hematopoietic cell compartment. Likewise, skin allograft survival in the NOD bone marrow into C57BL/6^{g7} recipient hematopoietic chimera group that is significantly shortened compared to the control C57BL/6^{g7} into C57BL/6^{g7} group will further support our hypothesis

Figure 2: Skin allograft survival in hematopoietic chimeras tracks with the hematopoietic system

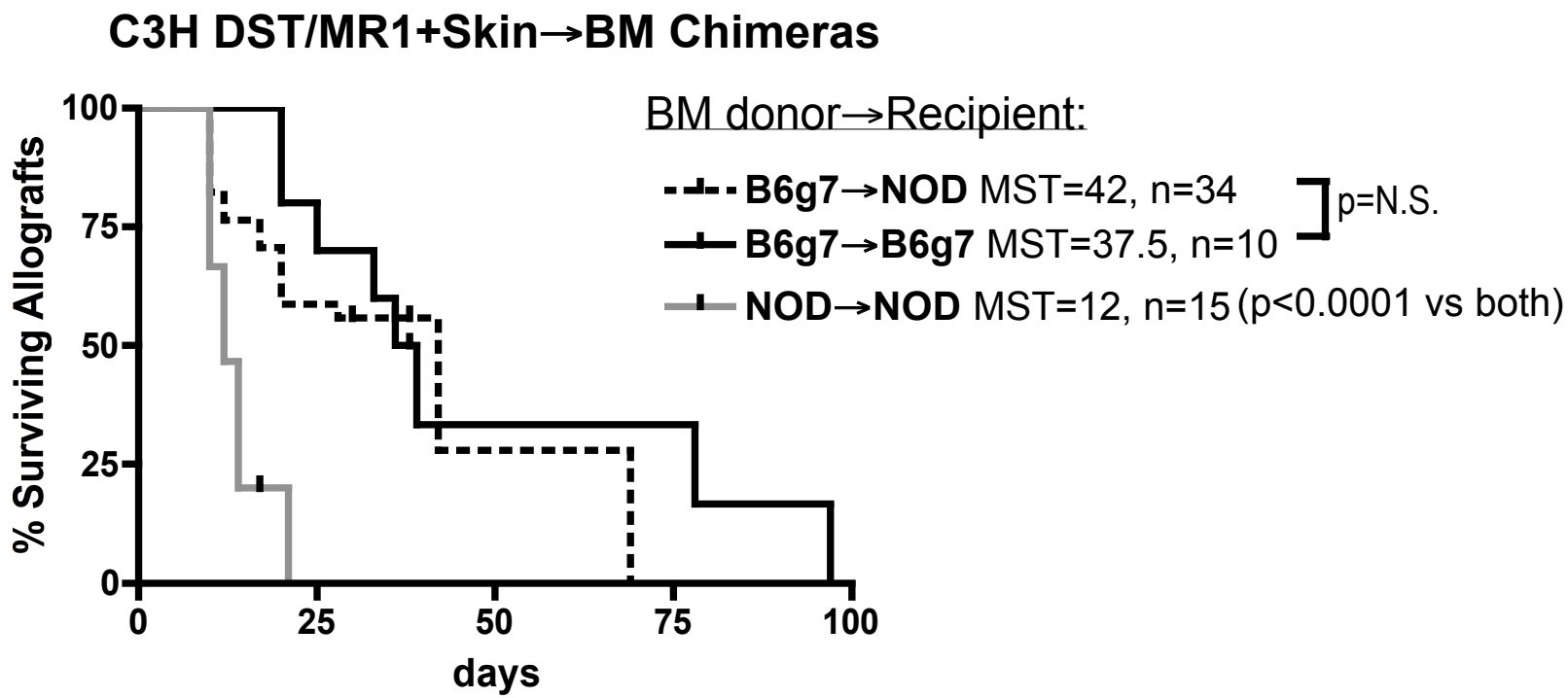


Figure 2 Legend: Skin allograft survival on hematopoietic chimeric mice. Irradiated (1400G) recipient NOD and C57BL/6^{g7} mice were injected with 5 x 10^6 bone marrow cells from the indicated donor. Eight weeks later all mice were given a single C3H/HeJ DST consisting of 1 x 10^7 spleen cells intravenously on day -7, four injections of 0.5 mg of anti-CD154 mAb intraperitoneally on days -7, -4, 0 and +4 and a C3H/HeJ skin graft on day 0. In control C57BL/6^{g7} into C57BL/6^{g7} hematopoietic chimeras, fully allogeneic C3H/HeJ *(H2^k)* skin allograft survival was prolonged (MST=37.5 days, N=10). As expected, in NOD into NOD hematopoietic chimeras, skin allograft survival was relatively short (MST=12 days, N=15 $p<0.0001$ versus C57BL/6^{g7}). In C57BL/6^{g7} into NOD hematopoietic chimeras, skin allograft survival was significantly prolonged (MST= 42 days, N=34 $p<0.0001$ versus NOD) relative to that achieved in the NOD into NOD hematopoietic chimeras and was not significantly different from that achieved in C57BL/6^{g7} into C57BL/6^{g7} hematopoietic chimeras.

2. Dendritic cell maturation phenotype in hematopoietic chimeras

We next tested the hypothesis that dendritic cell defects, similar to resistance to tolerance induction, also depended on the hematopoietic cell donor, not the environment in which they matured and functioned. First we developed a flow cytometry strategy that would permit us to determine whether the dendritic cells were NOD or C57BL/6^{g7} origin. To do this we used an allele specific mAb recognizing the Ly5 antigen. NOD cells express Ly5.1 while C57BL/6^{g7} express Ly5.2. As shown in figure 3, use of the Ly5.1 and Ly5.2 antibodies allowed us to distinguish the two different genotypes of DC, even in the mixed hematopoietic chimeras that circulated equal numbers of donor and host DC.

As shown in figure 3, we can easily discriminate NOD (Ly5.1+) and C57BL/6^{g7} (Ly5.2+) dendritic cells ($CD11b^+CD11c^+$) and their expression of CD86 in mixed hematopoietic chimeras. CD86 is a maturation marker; dendritic cells in NOD mice express low levels of CD86 as compared to expression of CD86 in C57BL/6^{g7} dendritic cells [132]. Figure 4 is a representative histogram from mixed hematopoietic chimeras showing the determination of % CD86 expression on $CD11b^+$ $CD11c^+$ bone marrow derived DC cultures treated with anti-CD40. Figure 5 shows the pooled data described in Figure 4. As expected, a significantly higher percentage of bone marrow derived dendritic cells from control C57BL/6^{g7} hematopoietic chimeras expressed CD86 in response to anti-CD40 stimulation than dendritic cells from similarly treated NOD hematopoietic chimeras ($p < 0.0001$). This was also seen in dendritic cells from the mixed hematopoietic chimeras. The percentage of dendritic cells of the C57BL/6^{g7} phenotype expressing CD86 upon stimulation in culture was significantly higher than the NOD

dendritic cells, regardless of whether the recipient was of C57BL/6^{g7} or NOD origin. In addition to CD86 expression, we also looked at the parameters of CD80 and CD25 expression as markers of DC maturation and activation. As seen with CD86 expression, there were significant differences between NOD and C57BL/6^{g7} dendritic cells in their ability to upregulate CD80 and CD25 in response to anti-CD40 (data not shown), though the percentages of $CD11b^+$ $CD11c^+$ $CD80^+$ and $CD11b^+$ $CD11c^+$ $CD25^+$ were much lower and more inconsistent than that seen with CD86 expression.

We were also interested in determining whether differences in DC maturation could be found *in vivo* in the mixed chimeric mice at the time of allogeneic skin graft rejection. In some experimental groups, freshly isolated splenic DC were briefly stimulated *in vitro* with anti-CD40 and analyzed for their expression of CD80, CD86, CD25 and IFNγ (data not shown). In these experiments, the percent of recovered $CD11b^+$ $CD11c^+$ splenic DC was extremely low and made determination of percent expression differences difficult.

Surprisingly, a significantly higher percentage of NOD dendritic cells from the mixed hematopoietic chimeras expressed CD86 upon stimulation in culture as compared to NOD controls. These data suggest that the maturation defect seen in NOD bone marrow derived dendritic cells in response to stimulation with CD40 can be improved in mixed hematopoietic chimeras by the presence of C57BL/6^{g7} bone marrow, suggesting that the C57BL/6^{g7} dendritic cells are able to provide a critical factor that is missing in NOD mice.

Figure 3: Use of allele specific monoclonal antibodies to differentiate between NOD and C57BL/6 dendritic cells

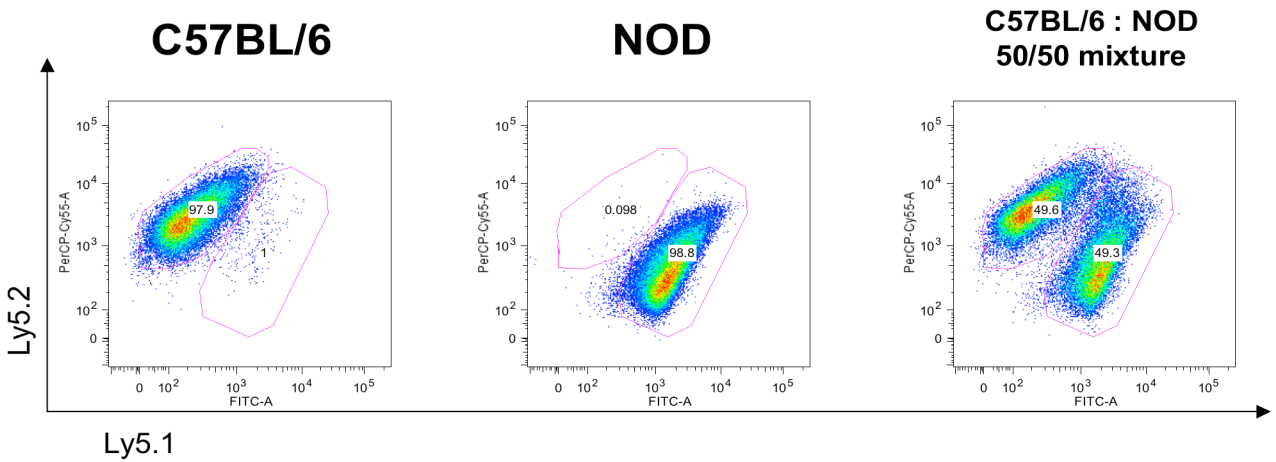


Figure 3 Legend: We cultured bone marrow from C57BL/6^{g7}, NOD and C57BL/6^{g7}:NOD experimental groups in the presence of 500 U/mGM-CSF and 1000 U/ml IL-4 for 6 days as described in Methods and added 5 μg/ml of an agonist anti-CD40 mAb for the last 2 days. The total cultured cell population (adherent and non-adherent) was recovered on day 6, and labeled with antibodies to Ly5.1 and Ly5.2. Figure 3 shows the allele specific differentiation of both NOD and C57BL/6 populations of dendritic cells. The last panel is representative of a mixed hematopoietic chimera.

Figure 4: Dendritic cell maturation phenotype in hematopoietic chimeras

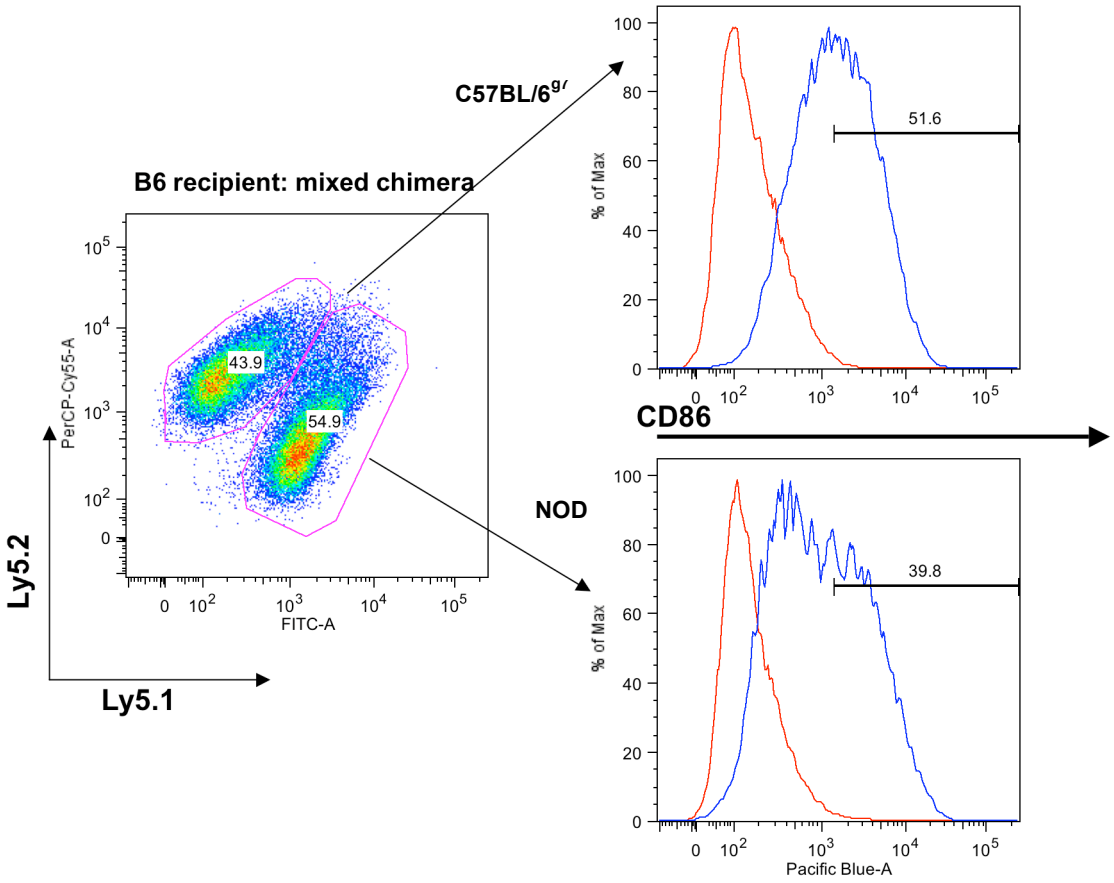


Figure 4 Legend: Expression of CD86 on Ly5.1+ or Ly5.2+ dendritic cells from a mixed hematopoietic chimera. We cultured bone marrow in the presence of 500 U/mGM-CSF and 1000 U/ml IL-4 for 6 days and added 5 μg/ml of an agonist anti-CD40 mAb for the last 2 days. The total cultured cell population (adherent and non-adherent) was recovered on day 6, and labeled with antibodies to Ly5.1, Ly5.2, CD11b, CD11c, and CD86. Figure 4 shows Ly5.1+ and Ly5.2+ populations that were further gated on the coexpression of the DC markers $CD11b^{+}CD11c^{+}$ cells (this intermediate gate is not shown). The histogram shows the percent of CD86 positive DC in the Ly5.1/5.2 $CD11b^{+}CD11c^{+}$ populations. The blue line represents CD86 expression on cells incubated in the presence of anti-CD40 mAb (activated), the red line is the isotype control. Representative plot from multiple experiments. Aggregate data shown in Figure 5.

Figure 5: Dendritic cell maturation phenotype

	Full chimera NOD (NOD)	Full chimera B6 (B6)
Full chimera B6 (NOD)	P<0.001	P=0.02
Partial chimera B6 (NOD)	P=0.0002	P=0.47
Partial chimera NOD (NOD)	P=0.0092	P=0.02
Partial chimera B6 (B6)	P=0.0002	P=0.005
Partial chimera NOD (B6)	P=0.0003	P<0.001

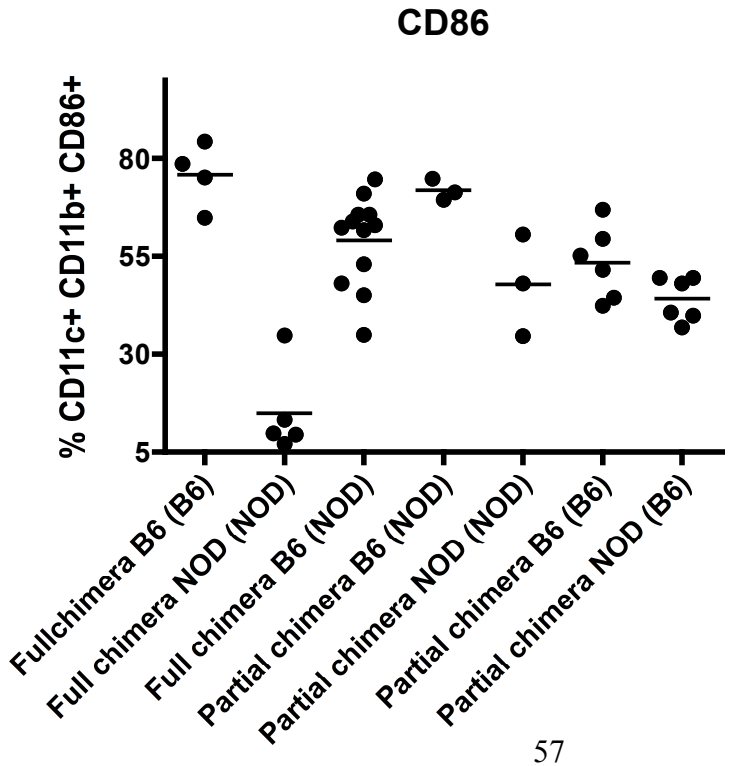


Figure 5 Legend: Mixed and full bone marrow chimeras were established using 14 Gy total body irradiation (7 Gy + 7 Gy 4 hr later) and an i.v. injection of a) 2.5 x 10^6 NOD and 2.5 x 10^6 C57BL/6^{g7} bone marrow cells for the mixed and b) 5 x 10^6 either NOD or C57BL/6^{g7} bone marrow cells for the full chimeras into the recipients indicated. Before injection, the bone marrow cells from each donor were counted, mixed (for the mixed chimeras) and T cell depleted (anti-CD4 plus anti-CD8 mAb). Recipients were tested for % chimerism 6-8 weeks later using Ly5.1 and Ly5.2 mAb. Recipients circulating 50% NOD and 50% C57BL/6^{g7} PBL were determined "mixed hematopoietic chimeras". Animals circulating 100% PBL of bone marrow donor origin were considered "full hematopoietic chimeras". Figure 5 shows the expression of CD86 on Ly5.1+ or Ly5.2+ CD11b+ CD11c+ dendritic cells from hematopoietic chimeras. We cultured bone marrow in the presence of 500 U/mGM-CSF and 1000 U/ml IL-4 for 6 days and added 5 μg/ml of an agonist anti-CD40 mAb for the last 2 days. The total cultured cell population (adherent and non-adherent) was recovered on day 6, and labeled with antibodies to Ly5.1, Ly5.2, CD11b, CD11c, and CD86. Parentheses indicate the recipient animal. The genotype preceding the parenthesis is the genotype of the bone marrow derived dendritic cell graphically represented for that line. Comparitive P values for % CD86 expression on CD11b+ CD11c+ double positive dendritic cells shown in table.

Summary

We show that resistance to transplantation tolerance in response to costimulation blockade in the NOD resides in the hematopoietic compartment and can be reversed with bone marrow from a transplantation tolerance susceptible donor. In addition, maturation defects in dendritic cells reside in the hematopoietic cell compartment of the donor. The maturation defect seen in NOD dendritic cells can be improved with the addition of C57BL/6^{g7} bone marrow, suggesting that the C57BL/6^{g7} dendritic cells are able to provide a critical factor that is missing in NOD mice.

Chapter 2

Idd Loci Synergize to Prolong Islet Allograft Survival Induced by Costimulation Blockade in NOD Mice

Introduction

Costimulation blockade-based tolerance protocols that induce prolonged skin and permanent islet allograft survival in non-autoimmune mice have failed in NOD mice. To investigate the underlying mechanisms, we studied congenic NOD mice bearing insulin dependent diabetes (*Idd*) loci that reduce diabetes incidence. Our underlying assumption was that understanding the genetic basis for these phenotypes would lead to a more thorough understanding of the mechanisms that control transplantation tolerance and autoimmunity.

We hypothesized that *Idd* loci that are strongly protective against diabetes would enhance islet allograft survival in NOD mice treated with costimulation blockade (Figure 6). In order to obtain proof of principle we first documented that islet graft rejection seen in the NOD mouse was in fact due to the recognition of alloantigen and not a consequence of an underlying autoimmune process. Several NOD congenic lines were then studied based upon the candidate genes found within their defined intervals. We also determined that there was a synergistic effect between various *Idd* loci as graphically depicted in Figure 8, and investigated whether a stepwise improvement in diabetes protection seen in multi-congenic lines would also translate into an increased ability to induce costimulation blockade-based tolerance to islet allografts.

Figure 6: Islet allograft tolerance induction protocol

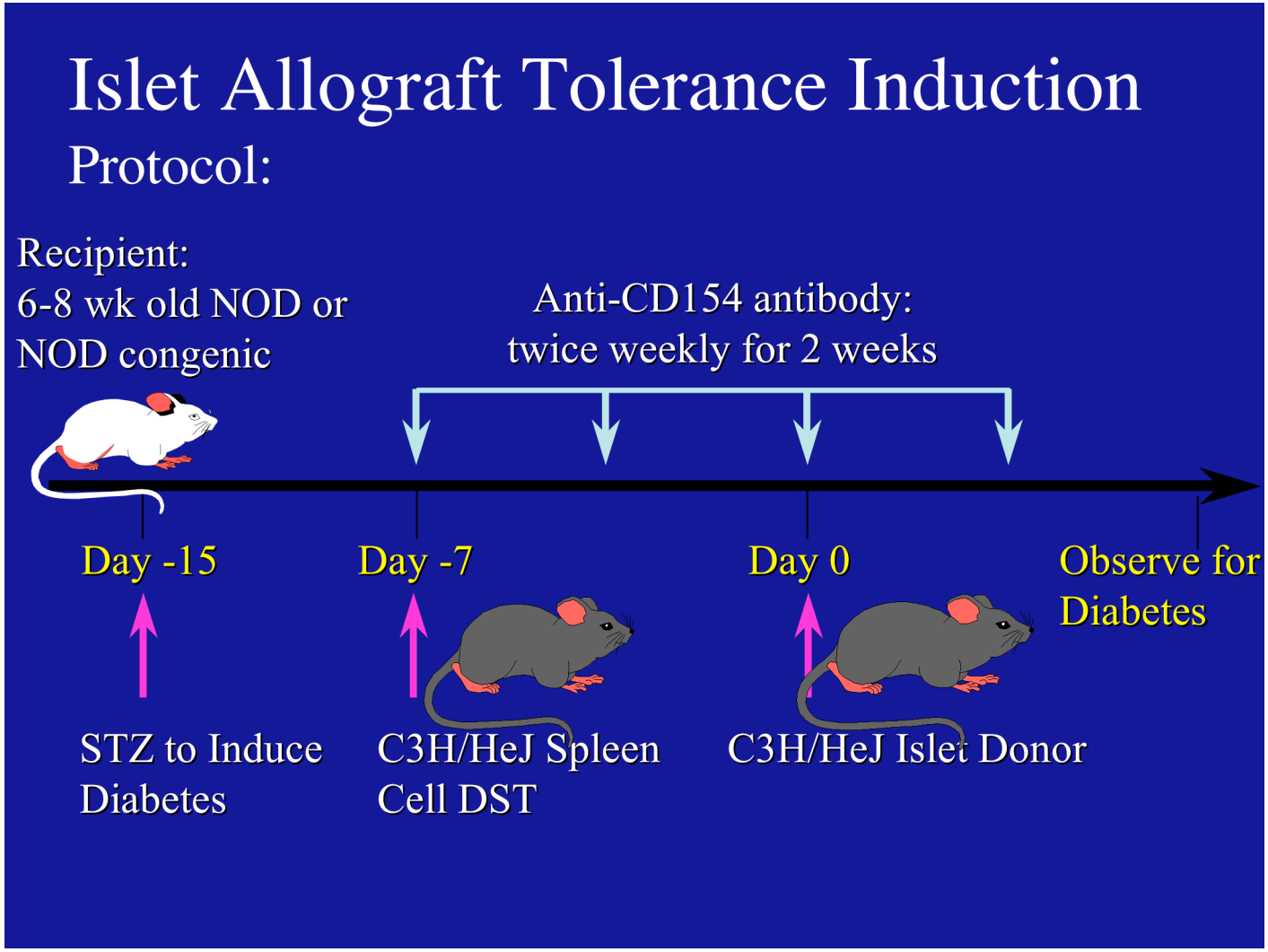


Figure 6 Legend: Diabetes was induced in male C57BL/6, NOD, and NOD *Idd* congenic mice by a single intraperitoneal injection of streptozotocin (150 mg/kg). Diabetes was confirmed by documenting plasma glucose concentration >250 mg/dl. Mice hyperglycemic for at least one week were used in the experiments. Chemically diabetic mice were treated with our standard costimulation blockade protocol consisting of a single C3H/HeJ donor-specific transfusion (DST) and anti-CD154 mAb and transplanted with a C3H/HeJ islet allograft as described in materials and methods. Islets for transplantation were isolated from C3H/HeJ donors and were transplanted into the renal subcapsular space of the chemically diabetic recipients. Plasma glucose concentrations were measured twice weekly and allograft rejection was defined as recurrent hyperglycemia (>250 mg/dL) on at least 2 consecutive days.

Results

1. *Islet allograft rejection in chemically diabetic male NOD mice is not due to islet autoimmunity*

We have previously shown that islet allograft survival in chemically diabetic male NOD mice treated with our costimulation blockade protocol is relatively short [199]. However, in those experiments, islet graft rejection could have resulted from either the development of islet autoimmunity or from the failure of costimulation blockade to induce allograft tolerance. To address this question, 6-8 week old male NOD mice were rendered hyperglycemic by treatment with streptozotocin and transplanted with syngeneic NOD-*scid* islets. Through 150 days after islet transplantation, all mice (5/5) remained normoglycemic. Histopathological analysis of the islet-bearing kidney revealed an insulin-producing islet graft present at the time of necropsy (Figure 7B). Of note, only a small amount of leukocytic infiltrate was observed and it was localized to margins of the islet graft, akin to "peri-insulitis" in the pancreas (Figure 7A). These data suggest that autoimmunity is substantially impaired in chemically diabetic NOD mice, and that islet graft rejection in our model system is due to the failure to induce tolerance to islet allografts and not due to the development of islet autoimmunity.

Figure 7: Islet allograft rejection in chemically diabetic male NOD mice is not due to islet autoimmunity

A. **B.**

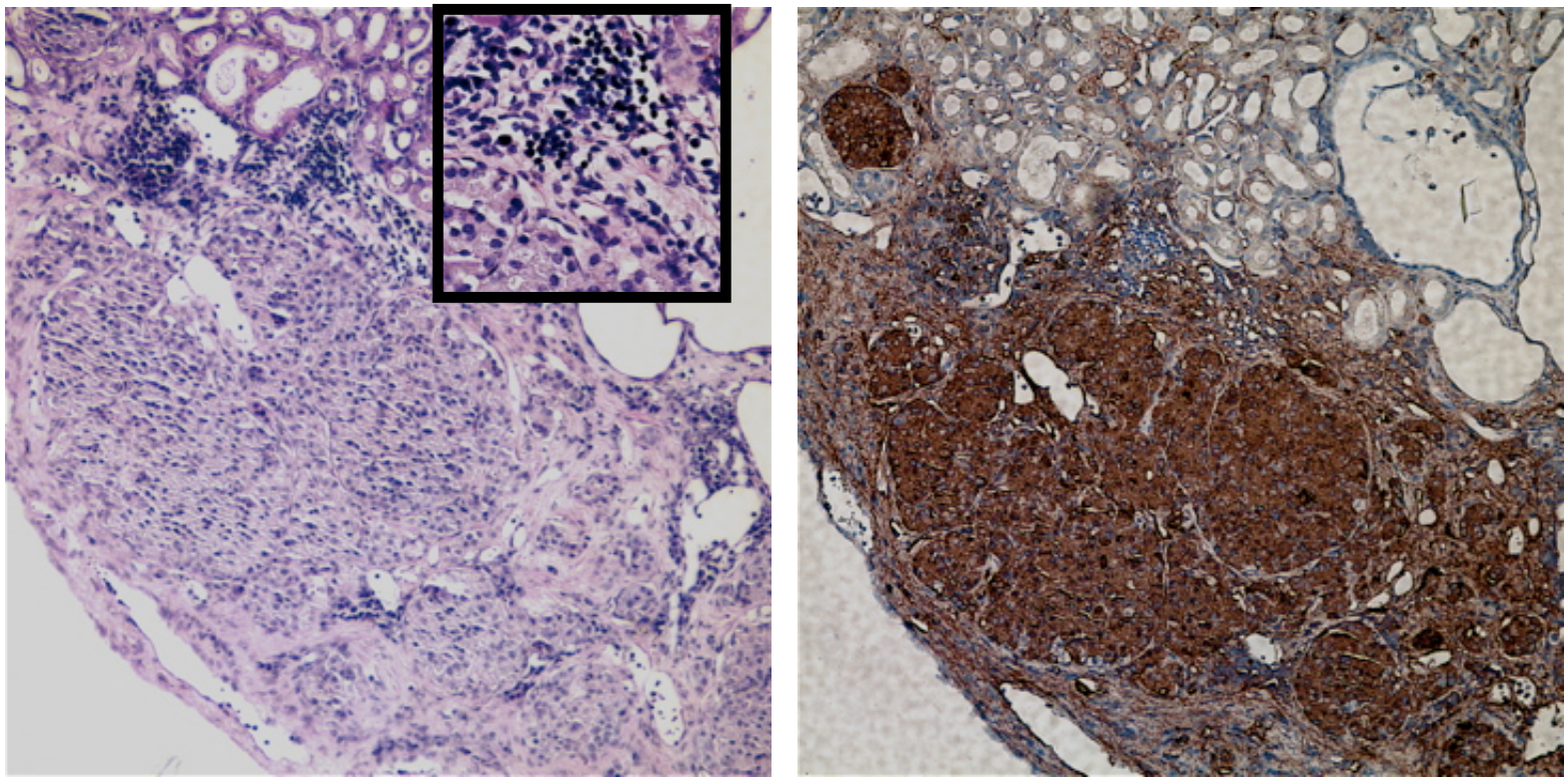


Figure 7 Legend: Histopathological analysis of the islet-bearing kidney revealed an insulin-producing islet graft present at the time of necropsy. The specimen was recovered 150 days post transplant from a recipient who was euglycemic and had not rejected the islet graft. A) A small amount of leukocytic infiltrate was observed and localized to the margins of the islet graft. B) Immunohistochemical staining for insulin reveals the presence of insulin producing β-cells within the graft. Hematoxylin and eosin (original magnification x 160).

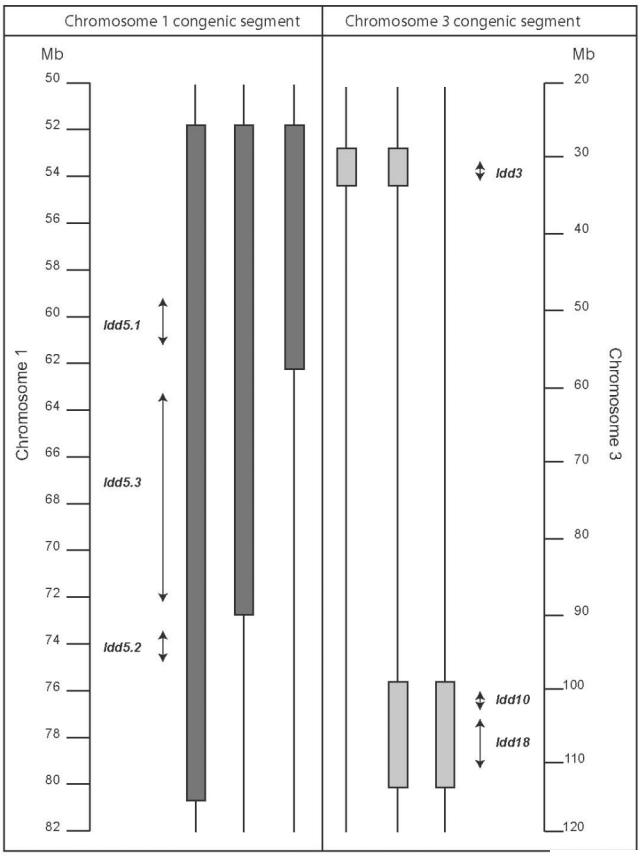
2. Islet allograft survival in NOD.B6 *Idd10Idd18 or* NOD.B6 *Idd3Idd10Idd18 congenic mice following treatment with DST and anti-CD154 mAb*

Although islet allograft survival in NOD mice treated with costimulation blockade is relatively short, we have also shown that it can be prolonged in NOD mice bearing the diabetes resistant *Idd3* congenic interval from C57BL/6 mice [199]. However, *Idd3* is only partially protective against diabetes, but when combined with certain other *Idd* loci, can almost completely protect NOD mice from diabetes expression (Figure 8). We therefore hypothesized that the combinations of *Idd* loci that are strongly protective against diabetes would enhance islet allograft survival in NOD mice treated with costimulation blockade. NOD mice congenic for the *Idd10 Idd18* intervals have reduced incidence of diabetes and when combined with *Idd3*, have a very low frequency of diabetes [181, 209, 210]. These congenic NOD mice were rendered chemically-diabetic, treated with donor-specific transfusion (DST) and anti-CD154 mAb, and transplanted with C3H/HeJ ($H2^k$) islet allografts.

Confirming our previous reports [199], islet allograft survival in NOD mice treated with costimulation blockade is short (median survival time, MST=74 days) whereas permanent islet allograft survival (MST>240 days) is observed in the majority of similarly treated C57BL/6 mice (Figure 9). We also confirmed our previous report that NOD mice bearing the *Idd3* congenic interval exhibit prolonged islet allograft survival (MST=140 days) as compared with NOD mice treated with costimulation blockade, but that graft survival remains significantly shorter than that achieved in similarly treated C57BL/6 mice (Figure 10). Surprisingly, we observed that NOD *Idd10 Idd18* congenic

mice exhibited statistically shorter islet allograft survival (MST=63 days) than that achieved in NOD mice treated with costimulation blockade (Figure 9). However, combination of the detrimental effects of the *Idd10 Idd18* genetic intervals with the beneficial effects of *Idd3* did not decrease islet allograft survival to less than that achieved in NOD.B6 *Idd3* congenic mice (p=N.S., Figures 9 and 10).

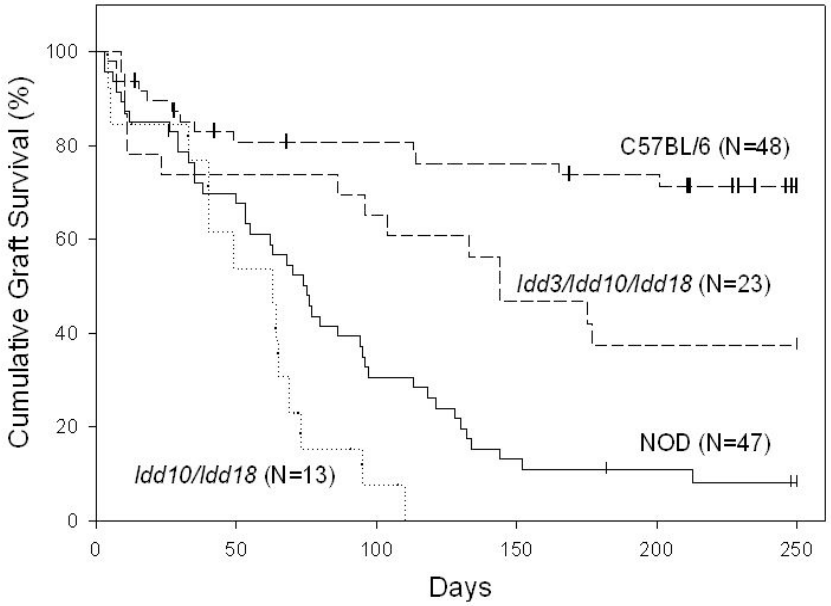
Figure 8: Schematic representation of candidate gene interval and chromosomal location



STRAIN	*Idd* interval(s) present in each strain						Diabetes
Idd5.1/5.3/5.2	+						10-35%
Idd3/Idd5.1/5.3/5.2	+			+			0-2%
Idd3/Idd5.1/5.3		+		+			2%
Idd3/Idd5.1			+	+			13%
Idd3				+			10-30%
Idd3/Idd10/Idd18					+		1-9%
Idd10/Idd18						+	45-55%

Figure 8 Legend: The filled bars represent B10-derived or B6-derived congenic segments on chromosomes 1 and 3, respectively. The arrows represent the size of each *Idd* interval as previously defined using additional congenic strains of mice: *Idd3* (650 kb) [152], *Idd10* (950 kb) [211] ,*Idd18* (4.0 Mb) [210], *Idd5.1* (2.1 Mb) [193], *Idd5.2* (1.52 Mb) [193],and *Idd5.3* (9.1 Mb)[in press] . The "Diabetes" column indicates the percentage of females developing diabetes by 7 months of age. Where a range is indicated, this summarizes the results of a number of frequency studies performed over many years.

Figure 9: Life table analysis of islet allograft survival in chemically diabetic Idd3 Idd10 Idd18 congenic NOD mice



	B6	*Idd10/18*	*Idd3/10/18*
NOD	<0.0001	0.0279	0.0032
B6		<0.0001	0.0096
Idd10/18			0.0003

Figure 9 Legend: Groups of 6-8 week old chemically diabetic mice were treated with a donor-specific transfusion (DST) plus anti-CD154 mAb. DST ($1x10^7$ C3H/HeJ spleen cells) was given on day −7, and anti-CD154 mAb (0.5 mg/dose) was given on days −7, −4, 0, and +4 relative to transplantation with C3H/HeJ islets on day 0. Vertical bars indicate mice removed from the study with intact grafts or alive with intact grafts at the conclusion of the period of observation. Comparative p values of islet allograft survival in the various groups is shown in the table.

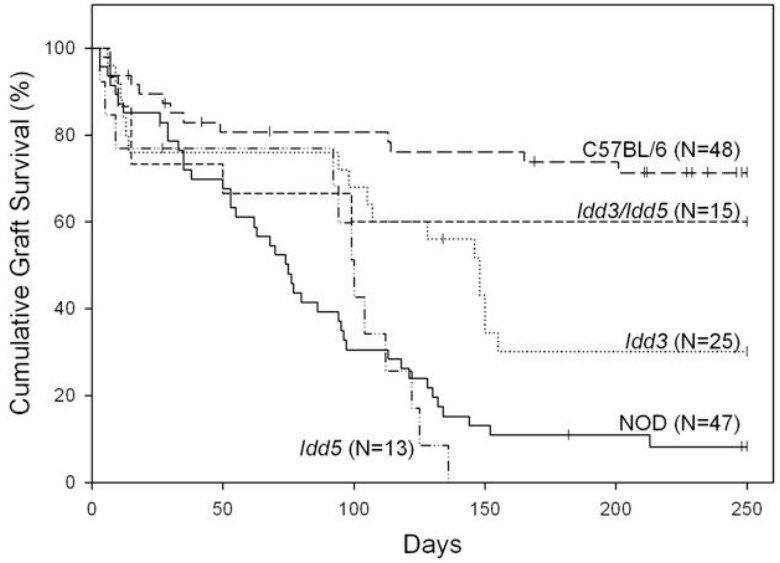
3. Idd5 synergizes with Idd3 to prolong islet allograft survival in chemically diabetic NOD mice treated with DST and anti-CD154 mAb

We next tested the hypothesis that other *Idd* congenic loci that reduce diabetes incidence in NOD mice would prolong islet allograft survival following treatment with costimulation blockade. We studied the effects of *Idd5* alone or in combination with *Idd3* on islet allograft survival in NOD mice. NOD mice bearing the *Idd5* loci are partially protected from diabetes [193], and the addition of *Idd3* results in nearly complete disease suppression [in press][212] . NOD.B6 *Idd5* and NOD.B6 *Idd3Idd5* congenic mice were rendered chemically diabetic, treated with DST and anti-CD154 mAb, and transplanted with fully MHC-mismatched C3H/HeJ ($H2^k$) islet allografts.

Islet allograft survival in NOD.B6 *Idd5* congenic mice (MST=96 days) was similar to that achieved in NOD mice (p=N.S., Figure 10). Islet allograft survival in the *Idd5* congenic NOD mice was significantly shorter than that achieved in NOD mice bearing the *Idd3* congenic region ($p<0.005$, Figure 10). These data, combined with the NOD.B6 *Idd10Idd18* results show that enhancement of islet allograft survival by *Idd* loci does not strictly correlate with the extent they suppress diabetes expression.

Strikingly, NOD mice bearing both the partially diabetes-protective *Idd3* and *Idd5* congenic intervals exhibited prolonged islet allograft survival (MST>250 days) that was similar to that achieved in C57BL/6 mice (p=N.S.) and significantly greater than that achieved in NOD ($p<0.005$) or NOD. B6 *Idd5* mice ($p<0.01$, Figure 10).

Figure 10: Life table analysis of islet allograft survival in chemically diabetic *Idd3*, *Idd5,* and *Idd3/Idd5* congenic NOD mice



	B6	*Idd3*	*Idd5*	*Idd3/5*
NOD	<0.0001	0.0044	0.8869	0.0023
B6		0.0013	<0.0001	0.3409
Idd3			0.0027	0.1828
Idd5				0.0100

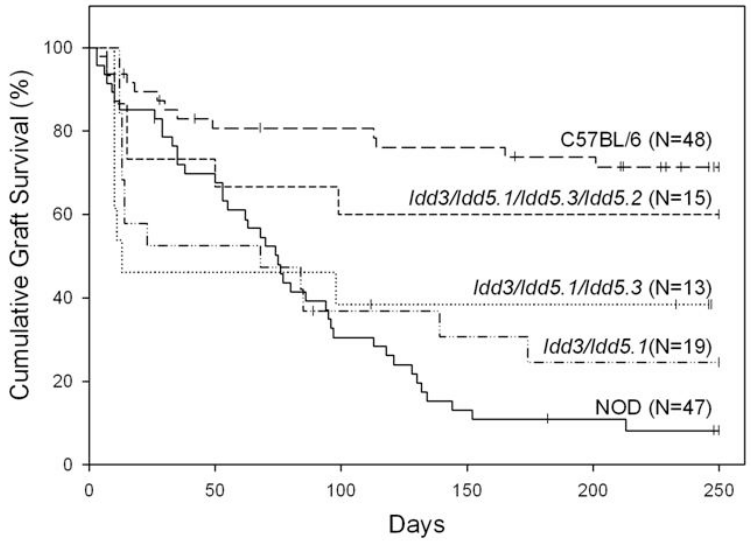
Figure 10 Legend: Groups of 6-8 week old chemically diabetic mice were treated with a donor-specific transfusion (DST) plus anti-CD154 mAb. DST ($1x10^7$ C3H/HeJ spleen cells) was given on day −7, and anti-CD154 mAb (0.5 mg/dose) was given on days −7, −4, 0, and +4 relative to transplantation with C3H/HeJ islets on day 0. Vertical bars indicate mice removed from the study with intact grafts or alive with intact grafts at the conclusion of the period of observation. Islet allograft survival in C57BL/6 and NOD mice shown in Figure 9 is reproduced here for ease of comparison with other strains. Comparative p values of islet allograft survival in the various groups is shown in the table.

4. Islet allograft survival in Idd3 congenic NOD mice bearing different Idd5 congenic intervals treated with costimulation blockade

It is now known that the *Idd5* interval contains at least 3 diabetes resistant loci termed *Idd5.1*, *Idd5.2*, and *Idd5.3* [193][in press]. Strong candidate genes within this region include CTLA-4 (*Idd5.1*) and NRAMP (*Idd5.2*) [193, 194]. To begin to identify the congenic *Idd5* interval that synergizes with *Idd3* to prolong islet allograft survival in NOD mice treated with costimulation blockade, we tested two newly developed NOD congenic lines. The new congenic lines carry the *Idd3* congenic interval as well as *Idd5.1* or *Idd5.1* plus *Idd5.3* (Figure 8). These congenic NOD mice were rendered chemically-diabetic, treated with DST and anti-CD154 mAb, and transplanted with C3H/HeJ ($H2^k$) islet allografts.

NOD.B6 *Idd3Idd5.1* congenic mice treated with costimulation blockade exhibited islet allograft survival that was shorter (MST=69 days) than that achieved in C57BL/6 mice ($p<0.0001$) and not different from that achieved in NOD mice (p=N.S., Figure 11). Adding back the *Idd5.3* region to the NOD.B6 *Idd3Idd5.1* congenic line did not significantly improve islet allograft survival (MST=13 days) over that achieved in NOD.B6 *Idd3Idd5.1* congenic mice (p=N.S., Figure 11).

Figure 11: Life table analysis of islet allograft survival in chemically diabetic *Idd3* congenic NOD mice bearing different *Idd5* congenic intervals



	B6	*Idd3/5*	*Idd3/5.1/5.3*	*Idd3/5.1*
NOD	<0.0001	0.0023	0.2634	0.4153
B6		0.3409	0.0055	0.0001
Idd3/5			0.2005	0.0677
Idd3/5.1/5.3				0.9574

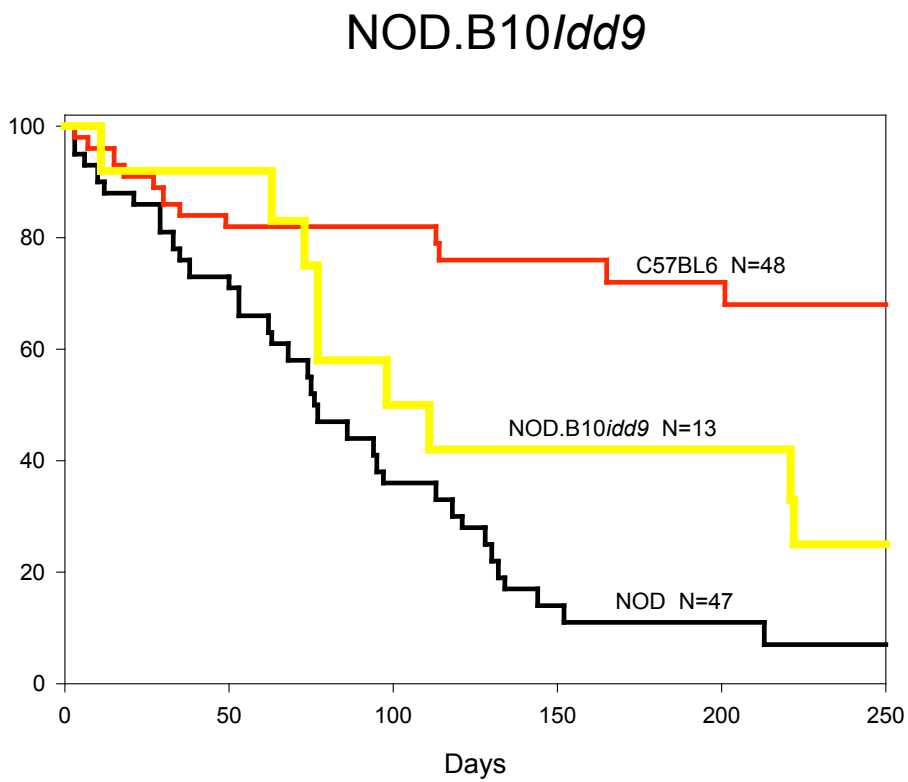
Figure 11 Legend: Groups of 6-8 week old chemically diabetic mice were treated with a donor-specific transfusion (DST) plus anti-CD154 mAb. DST ($1x10^7$ C3H/HeJ spleen cells) was given on day -7, and anti-CD154 mAb (0.5 mg/dose) was given on days −7, −4, 0, and +4 relative to transplantation with C3H/HeJ islets on day 0. Vertical bars indicate mice removed from the study with intact grafts or alive with intact grafts at the conclusion of the period of observation. Islet allograft survival in C57BL/6, NOD, and NOD *Idd3 Idd5* congenic mice shown in Figures 9 and 10 are reproduced here for ease of comparison with other strains. Comparative p values of islet allograft survival in various groups is shown in the table.

5. Islet allograft survival in NOD.B10 *Idd9 congenic mice following treatment with DST and anti-CD154 mAb*

Having shown that *Idd5* synergizes with *Idd3* to achieve prolonged islet allograft survival in NOD mice that is similar to that achieved in C57BL/6 mice, we next wanted to test whether *Idd9* would also result in improved islet allograft survival. *Idd9* mice carry three intervals (*Idd9.1, 9.2* and *9.3*) derived from B10 mice that include the candidate genes Lck and 4-1BB [178]. Like the *Idd3/5* mice, *Idd9* mice are extremely resistant to autoimmune diabetes [178], however, they develop insulitis and islet specific autoantibodies [179] that are absent in the *Idd3/5* mice. Because *Idd3/5* and *Idd9* mice represent different checkpoints at which loss of self-tolerance and disease progression can be halted, we next compared islet allograft survival in *Idd9* mice with that achieved the *Idd3/5* mice treated with costimulation blockade.

NOD, C57BL/6 and NOD.B10*Idd9* congenic mice were rendered chemically-diabetic, treated with DST and anti-CD154 mAb, and transplanted with C3H/HeJ ($H2^k$) islet allografts. NOD.B10 *Idd9* congenic mice treated with costimulation blockade exhibited islet allograft survival that was shorter (MST=69 days) than that achieved in C57BL/6 mice ($p<0.0001$) and not different from that achieved in NOD mice (p=N.S., Figure 12).

Figure 12: Life table analysis of islet allograft survival in chemically diabetic *Idd9* congenic NOD mice



	B6	NOD.B10*Idd9*
NOD	<0.0001	0.96
B6		0.0001

Figure 12 Legend: Groups of 6-8 week old chemically diabetic mice were treated with a donor-specific transfusion (DST) plus anti-CD154 mAb. DST ($1x10^7$ C3H/HeJ spleen cells) was given on day -7, and anti-CD154 mAb (0.5 mg/dose) was given on days −7, −4, 0, and +4 relative to transplantation with C3H/HeJ islets on day 0. Islet allograft survival in C57BL/6 and NOD mice shown in Figure 9 is reproduced here for ease of comparison with *Idd9.* Comparative p values of islet allograft survival in various groups is shown in the table.

Summary

We examined the contributions various *Idd* loci have in prolonging islet allograft survival in NOD mice. We were able to demonstrate that some *Idd* loci that are strongly protective against autoimmunity do not correct the tolerance induction defect of NOD mice. Furthermore, we identified a protective effect of *Idd3* (IL-2) in transplantation tolerance that when combined with *Idd5* is a potent determinant in susceptibility to tolerance induction to islet allografts.

Chapter 3: Failure of costimulation blockade to delete alloreactive CD8$^+$ T cells in (NOD x KB5)F1 synchimeric mice can be reversed by administration of exogenous IL-2

Introduction

The *Idd3* interval, alone or in combination with *Idd5*, mediates strong islet allograft survival prolonging effects in NOD mice treated with costimulation blockade. The *Idd3* loci, located on mouse chromosome 3, has been shown to contain the encoding region for IL-2 [213]. Variation of the N-terminal amino acid sequence of IL-2 correlates with *Idd3* allelic variation, and electrophoretic migration of NOD and C57BL/6 IL-2 allotypes were consistent with differences in IL-2 glycosylation patterns and correlated with diabetes-resistance. Extending this observation, the *Idd3* effect on diabetes expression in NOD mice appears to result from an IL-2 allele that is transcribed at lower levels than variants contributing to disease resistance [152]. In an elegant series of experiments, Wicker *et al*. were able to demonstrate a stepwise increase in IL-2 mRNA transcription from similarly treated NOD, (NOD x NOD.B6*Idd3*)F1, NOD.B6*Idd3* and C57BL/6 splenocytes. The homozygous expression of NOD *Idd3* alleles in the NOD splenocytes resulted in the lowest level of IL-2 mRNA expression upon stimulation. Heterozygous (NOD x NOD.B6*Idd3*)F1 splenocytes, carrying one copy of the B6 *Idd3* allele, produced significantly more IL-2 than NOD splenocytes and half the amount seen produced in the homozygous NOD.B6*Idd3* and C567BL/6 splenocytes. Functionally, the enhanced production of IL-2 from the B6 *Idd3* allele had a drastic effect on the ability of the diabetogenic clone 8.3 CD8+ T cells to differentiate into cytotoxic effectors both *in*

vitro and *in vivo*. Clone 8.3 CD8+ T cells are transgenic NOD CD8+ T cells that target islet specific antigens and are highly pathogenic [214]. When compared to similarly treated NOD.*Idd3*.8.3+ T cells, the autoantigen-activated autoreactive 8.3 CD8 T cells from NOD.B6*Idd3*.8.3 mice produced more IL-2 and efficiently recruited more CD25+CD4+ Treg cells into the pancreatic draining lymph nodes [152]. What was most striking was that in NOD.B6*Idd3* mice, increased IL-2 production and CD4+CD25+ recruitment and function were shown to be associated with decreased cross presentation of β-cell autoantigens to autoreactive CD8+ T cells by mature dendritic cells in the peripheral lymph nodes. It was previously shown that CD4+CD25+ Treg cells inhibit 8.3+-like CD8+ T cell responses by suppressing the maturation of dendritic cells and their ability to cross-prime T cells [215]. The receptor for IL-2 (IL-2R), also known as CD25, is highly expressed on Treg cells (CD4+CD25+ T cells), and the differentiation, activation and homeostasis of CD4+CD25+ Treg cells is critically dependent on CD25 function and IL-2 signaling [216-221].

Several groups have shown that IL-2 is important for costimulation blockade based tolerance induction by promoting AICD of alloreactive T cell clones [60, 222-224]. In addition, we [22, 28] and others [60, 224, 225] have shown that skin allograft survival, a robust tissue for analysis of tolerance, is strongly dependent on the ability of costimulation blockade to delete host alloreactive $CD8^+$ T cells. Based on these observations, we hypothesized that a deficiency in IL-2 production in NOD mice would impair host alloreactive $CD8^+$ T cell deletion, leading to shortened allograft survival.

Results

1. *Alloreactive CD8$^+$ T cell deletion in synchimeric mice treated with costimulatory blockade and exogenous IL-2*

To test the hypothesis that the failure to delete alloreactive CD8+ T cells is a major barrier to tolerance induction in the NOD mouse, we used a modified synchimera model system that is based on KB5 TCR transgenic alloreactive CD8$^+$ T cells [22]. This synchimeric system permits a direct analysis of the fate of host alloreactive CD8$^+$ T cells in mice that have a partially NOD genetic background. As illustrated in figure 13, KB5 CBA mice were mated with NOD mice, or with C57BL/6.*H2*^{g7} mice as normal controls, and were used to generate F1 synchimeric mice for these studies as described [22]. Synchimeric mice were treated with a C57BL/6 DST on day −7 and anti-CD154 mAb on days −7 and −4 relative to analysis of their circulating levels of KB5 transgenic alloreactive CD8$^+$ T cells on day 0, the day islets would be transplanted. One cohort of synchimeric mice was also given 5 daily injections of recombinant mouse IL-2 beginning on the day of DST.

A representative FACS plot showing the detection of circulating KB5+ transgenic T cells in a (KB5 CBA x C57BL/6.*H2*^{g7})F1 synchimeric mouse prior to (left panel) or 7 days after DST and anti-CD154 mAb administration (right panel, Figure 14A**).** (KB5 CBA x C57BL/6.*H2*^{g7})F1 synchimeric mice treated with costimulation blockade exhibited marked deletion of their alloreactive CD8$^+$ T cells, with an average deletion of 63±30% (Figure 14B). In contrast, (KB5 CBA x NOD)F1 synchimeric mice exhibited

significantly less deletion of their alloreactive $CD8^+$ T cell population (34±24%). To determine if the reduced deletion was due to insufficient levels of IL-2, exogenous IL-2 was administered to (KB5 CBA x NOD)F1 synchimeric mice during the peri-transplant period when deletion occurs [226]. (KB5 CBA x NOD)F1 synchimeric mice treated with IL-2 and costimulation blockade exhibited enhanced alloreactive $CD8^+$ T cell deletion (63±21%) that was significantly improved over that of (KB5 CBA x NOD)F1 synchimeric mice treated with costimulation blockade alone and was not significantly different than that observed in (KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeric mice treated with costimulation blockade (Figure 14B). Alloreactive $CD8^+$ T cell deletion in control(KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeric mice treated with IL-2 and costimulation blockade was not significantly different (69.7±5.6%, N=3) than that observed in both the (KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeric mice treated with costimulation blockade and (KB5 CBA x NOD)F1 synchimeric mice treated with IL-2 and costimulation blockade (data not shown).

Figure 13: Generation of a KB5 Synchimera

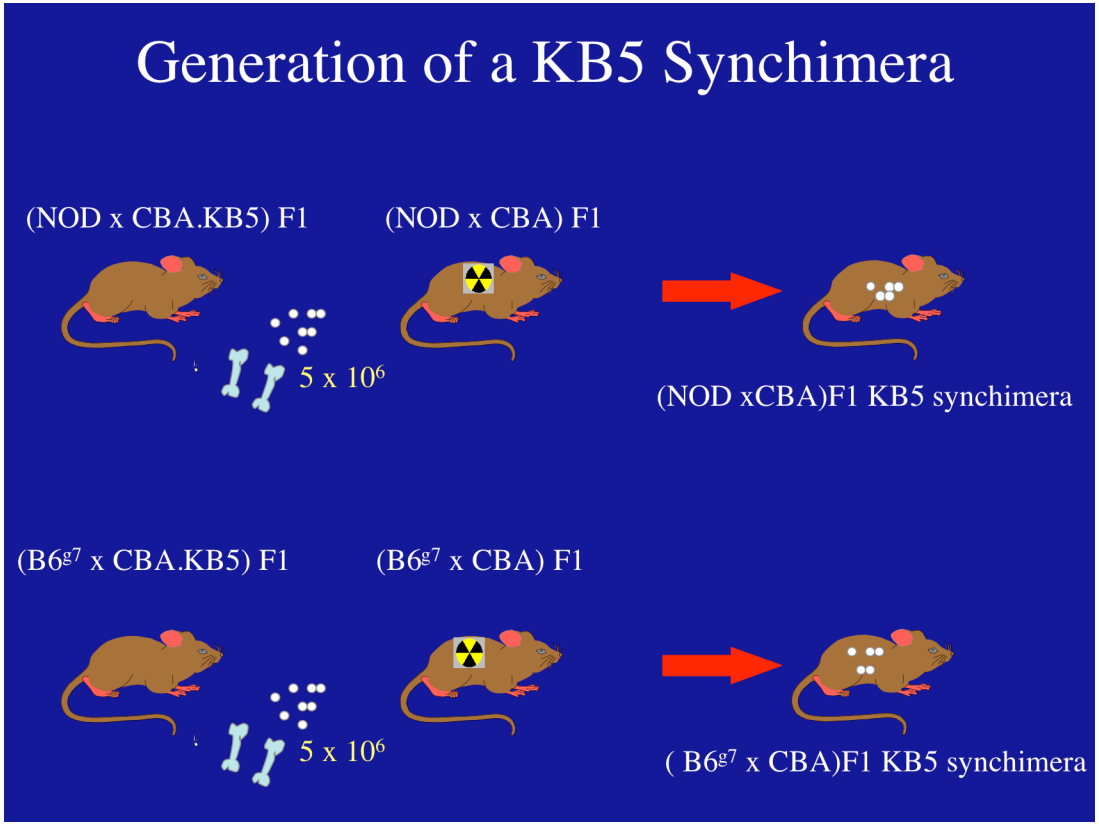


Figure 13 Legend: KB5 synchimeric mice were generated using a procedure previously described with slight modifications [22]. Briefly, (CBA/J x NOD)F1 and (CBA/J x C57BL/6.$H2^{g7}$)F1 non-transgenic mice were treated with 400 cGy of whole-body gamma irradiation from a ^{137}Cs source and given a single i.v. injection of $5x10^6$ (KB5 CBA x NOD)F1 or (KB5 CBA x C57BL/6.$H2^{g7}$)F1 bone marrow cells, respectively. Mice were entered into experiments 8-12 weeks after bone marrow transplantation.

Figure 14 Panel A: Representative FACS plot of (CBA x C57BL/6H2g7)F1 KB5 synchimeric mouse treated with costimulation blockade and exogenous IL-2

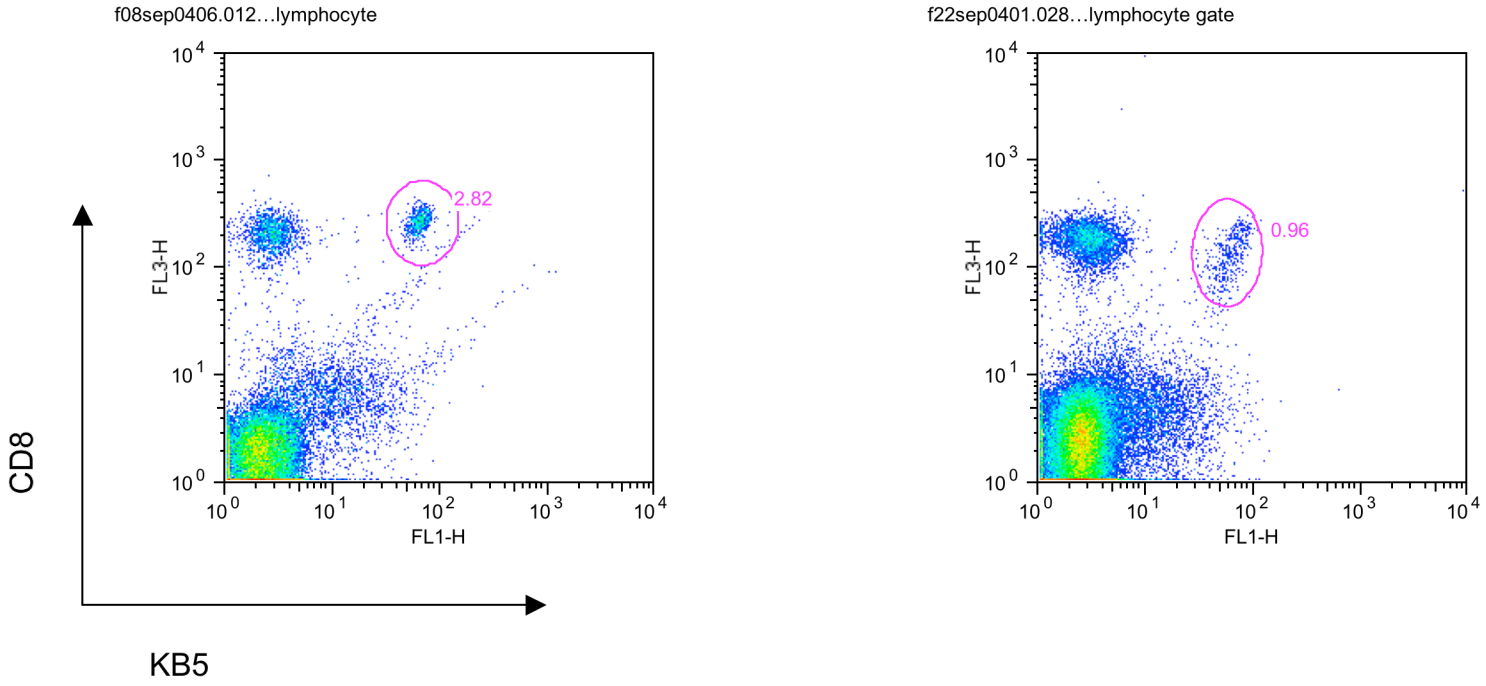


Figure 14 Panel B: Scatter plot of alloreactive CD8+ T cell deletion in synchimeric mice treated with costimulatory blockade

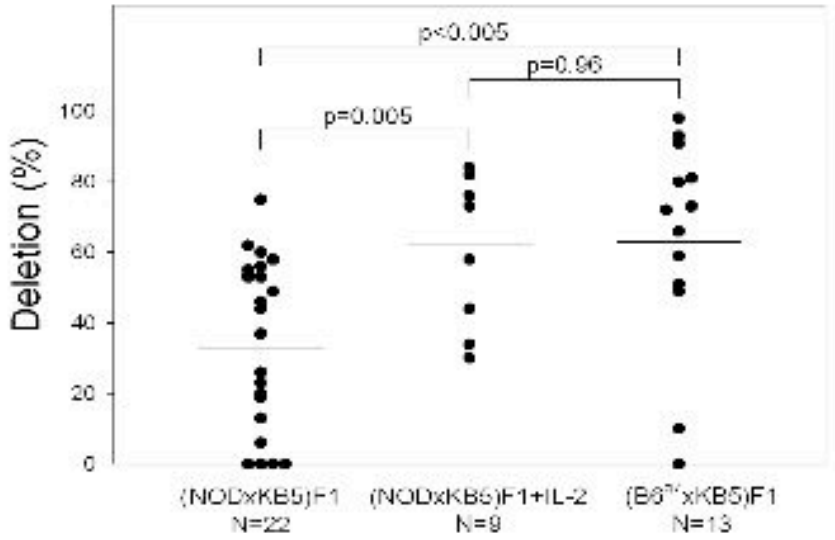


Figure 14 Legend: (KB5 CBA x C57BL/6.*H2*^{g7})F1 mice and (KB5 CBA x NOD)F1 synchimeric mice were treated with a C57BL/6 DST on day −7 and anti-CD154 mAb on days −7 and −4 relative to analysis of their circulating levels of KB5 transgenic CD8^{+} T cells on day 0 as described in Methods. Indicated groups also received 5 consecutive intravenous doses of IL-2 on days -7, -6, -5, -4 and -3 concomitant with the initiation of the DST. Panel A shows a representative FACS plot of circulating KB5 transgenic CD8+ T cells from a (KB5 CBA x C57BL/6.*H2*^{g7})F1 synchimeric mouse on day -7 (before DST and costimulation blockade) and again in the same mouse on day 0 (after KB5 transgenic CD8+ T cell deletion). Panel B graphically depicts the FACS data from several experiments. Each dot in Panel B represents an individual mouse. P values are indicated by horizontal bars. % deletion was calculated as the percent of KB5+ CD8+ transgenic T cells that were deleted in each mouse 7 days after initiating costimulation blockade, DST and IL-2 as compared to the day -7 pre-bleed levels.

2. Failure of costimulation blockade to prolong skin allograft survival in (KB5 CBA x NOD)F1 synchimeric mice

Having shown that costimulation blockade failed to efficiently delete alloreactive T cells, we next determined if skin allograft survival was impaired in the (KB5 CBA x NOD)F1 synchimeric mice. (KB5 CBA x NOD)F1 synchimeric mice treated with costimulation blockade and transplanted with C57BL/6 skin allografts exhibited statistically significant shorter skin allograft survival (MST=12 days) than that achieved in similarly treated (KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeric mice (MST>59 days, Figure 15). These data document that, as predicted, (non-autoimmune prone x NOD)F1 mice, *i.e.*, (KB5 CBA x NOD)F1 synchimeric mice, are resistant to costimulation blockade induced skin allograft tolerance induction.

Figure 15: Life table analysis of skin allograft survival in (CBA x C57BL/6^{g7})F1 and (CBA x NOD)F1 KB5 synchimeric mice treated with costimulation blockade

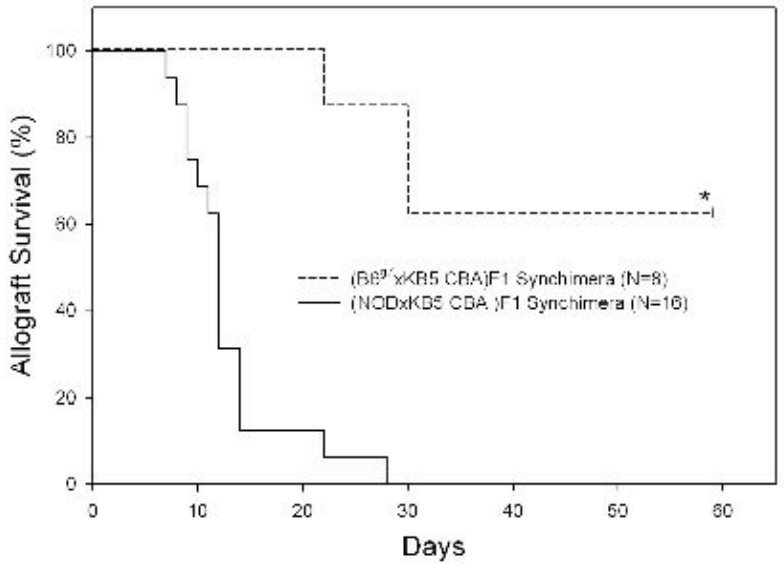


Figure 15 Legend: Groups of 6-8 week old synchimeric mice of the indicated strains were treated with a donor-specific transfusion (DST) plus anti-CD154 mAb. DST ($1x10^7$ C57BL/6 spleen cells) on day −7, and anti-CD154 mAb (0.5 mg/dose) was given on days −7, −4, 0, and +4 relative to transplantation with C57BL/6 skin on day 0. Vertical bars indicate mice removed from the study with intact grafts or alive with intact grafts at the conclusion of the period of observation. *$p<0.001$ *vs*. (KB5 CBA x NOD)F1 synchimeric mice.

3. Exogenous IL-2 improves islet allograft survival in NOD mice treated with costimulation blockade

We next hypothesized that increased deletion of functional alloreactive $CD8^+$ effector T cells in (KB5 CBA x NOD)F1 synchimeric mice treated with costimulation blockade plus IL-2 would lead to a difference in islet allograft survival. To test this, chemically diabetic NOD mice were treated with costimulation blockade and transplanted with C3H/HeJ islets with or without injection of exogenous IL-2 administration during the peri-transplant period as described in Methods.

As expected, islet allograft survival in NOD mice treated with costimulation blockade was relatively short (MST=46 days, Figure 16). In contrast, NOD mice treated with costimulation blockade and exogenous IL-2 during the peri-transplant period exhibited slightly but significantly prolonged islet allograft survival (MST=83 days), although all islet allografts were eventually rejected (Figure 16). The addition of IL-2 had no effect on costimulation blockade based tolerance induction in B6 mice, as B6 mice treated with exogenous IL-2 exhibited islet allograft survival that was significantly no different than that achieved in other studies with B6 mice treated with costimulation blockade alone (data not shown).

Figure 16: Life table analysis of islet allograft survival in NOD mice treated with IL-2

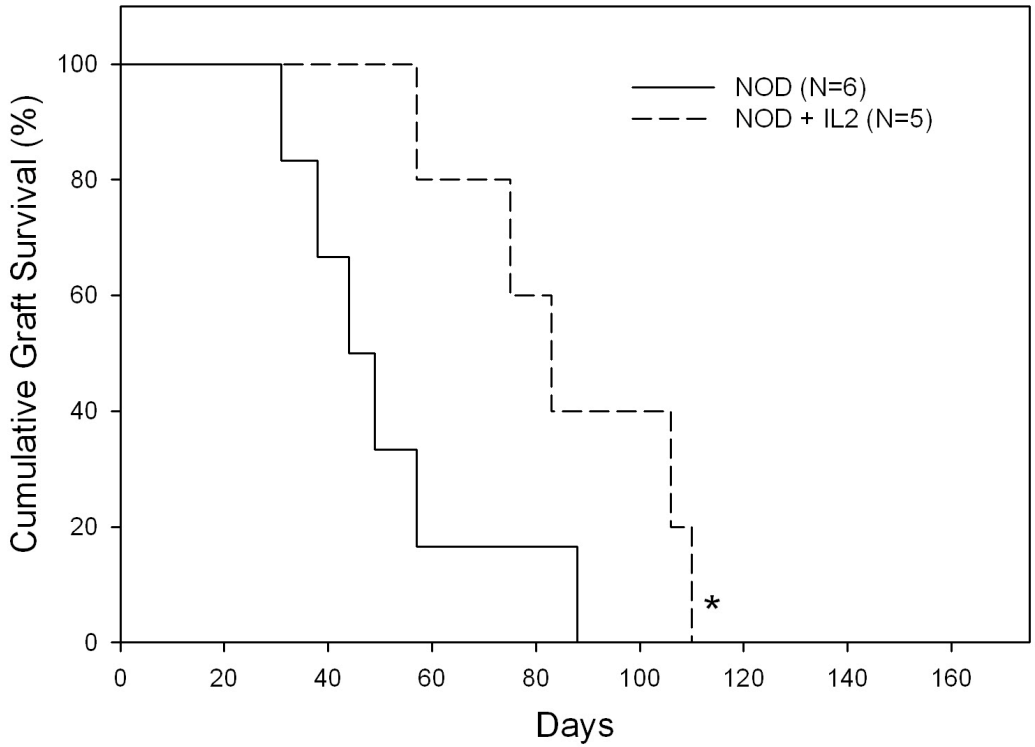


Figure 16 Legend*:* Groups of 6-8 week old chemically diabetic NOD mice were treated with a C3H/HeJ DST on day −7 and anti-CD154 mAb (0.5 mg/dose) was given on days −7, −4, 0, and +4 relative to transplantation with C3H/HeJ islets on day 0. One group of mice also received 0.8μg recombinant murine IL-2 (R & D systems, Minneapolis, MN) intraperitoneally on days −7, −6, −5, −4 and −3 relative to islet transplantation on day 0 . *$p<0.05$ *vs.* NOD.

Summary

We demonstrated that (KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeric mice treated with costimulation blockade showed a marked deletion of alloreactive $CD8^+$ T cell that was significantly greater than that achieved in the (KB5 CBA x NOD)F1 synchimeric mice. These (KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeric mice also showed significantly prolonged skin allograft survival compared to the (KB5 CBA x NOD)F1 synchimeric mice. We interpret this to mean that the failure to delete alloreactive CD8 T cells in response to costimulation blockade is a major barrier to tolerance induction in the NOD F1 mice.

When (KB5 CBA x NOD)F1 synchimeric mice were treated with exogenous IL-2 during the peri-transplant period, the alloreactive $CD8^+$ T cell deletion was significantly improved and comparable to that achieved in (KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeras. From these experiments we conclude that defects in IL-2 production in NOD mice lead to inefficient deletion of alloreactive $CD8^+$ T cells in response to costimulation blockade. This failure to delete can in part be rescued by administration of exogenous IL-2 during the peri transplant period. To extend this finding, we showed that islet allograft survival in NOD mice treated with costimulation blockade plus IL-2 was slightly, but significantly longer than in NOD mice treated with costimulation blockade alone.

Chapter 4

In vitro identification of allospecific CD8$^+$ T cells in mice treated with costimulation blockade

Introduction

In Chapter 3 we showed that NOD mice fail to delete alloreactive CD8+ T cells in response to costimulation blockade. We also showed that this failure to delete was a major barrier to tolerance induction, and that the addition of exogenous IL-2 could both improve deletion of alloreactive CD8+ T cells and prolong islet allograft survival in NOD mice. We next wanted to extend this observation to determine if the alloreactive CD8 T cells that escaped deletion in the NOD developed into functional effector cells and were responsible for shortened allograft survival. The ability to identify the presence of alloreactive T cells has important implications clinically. Quantification of effector allospecific T cells before the onset of clinical signs of rejection could lead to better therapies for the maintenance of allograft survival. A hallmark characteristic of naïve CD8+ T cells is that they rapidly produce TNF-α but not IFN-γ following TCR engagement [227]. This differential expression of TNF-α and IFN-γ can be used to distinguish between naïve and effector/memory alloreactive T cells [202, 228]. To detect the presence of alloreactive effector CD8+ T cells that escape deletion in NOD mice we used an intracellular cytokine assay for detection of IFNγ-producing cells. We observed

that the CD8 T cells that were not deleted in NOD mice treated with costimulation blockade exhibited effector function when stimulated donor-specific antigen.

We also wanted to determine whether NOD *Idd* congenic mice bearing two copies of the C57BL/10 *Idd3* locus would show improved CD8+ T cell deletion and reduced numbers of functional effector cells in response to costimulation blockade compared to the NOD. Recently, we showed that the frequency of host alloreactive CD8 T cells as measured by $CD44^{hi}$ expression and IFN-γ production is a powerful predictor of allograft survival [227]. Since allograft survival in response to costimulation blockade and DST in NOD.B6*Idd3* mice is intermediate to that seen in similarly treated NOD and C57BL/6 mice and slightly shorter than that observed in NOD.B6*Idd3*B10*Idd5* double congenic mice, we reasoned that the presence of $CD44^{hi}$ IFN-γ^{+} effector/memory cells would likewise show a stepwise increase and correlate with islet allograft survival.

1. Intracellular cytokine analysis of stimulated T cells from NOD and C57BL/6 mice

In order to determine whether alloreactive CD8 T cells that escaped deletion in the NOD developed into functional effector cells, we first had to ensure that in our hands we could detect differences in cytokine production between both NOD and C57BL/6 CD4 and CD8 T cells. Using the conditions described in Methods, we cultured freshly isolated NOD and C57BL/6 splenocytes in the presence or absence of anti-CD3 stimulation. As shown in Figure 17, significant differences in IL-2 production were seen between NOD and C57BL/6 stimulated CD4 and CD8 splenocytes. These differences were apparent in both the $CD44^{lo}$ and $CD44^{hi}$ compartments. CD4 and CD8 C57BL/6 splenocytes produce significantly more IL-2 in response to anti-CD3 stimulation than similarly treated NOD splenocytes. The mean fluorescent intensity (MFI) for IL-2 was not different (p=ns) between the NOD unstimulated and stimulated $CD8+CD44^{hi}$ populations. In contrast, significant differences were seen in the MFI of the unstimulated versus stimulated C57BL/6 $CD8+CD44^{hi}$ groups (MFI=717±55 and 1470±64 respectively, $p<0.0001$) indicating higher IL-2 expression per cell in the C57BL/6 population in response to stimulation. Significant differences were also seen within the parameters of percent of cells producing TNFα and IFN-γ in response to stimulation (Figure 18 and Figure 19, respectively). While a greater percentage of C57BL/6 $CD44^{hi}$ and $CD44^{lo}$ CD4 and CD8 T cells produced TNFα when stimulated compared to similarly treated NOD cells, there were no differences in the MFI of these populations. In contrast, when IFNγ was assessed, there were no differences in the MFI of NOD unstimulated and stimulated splenocytes, while the differences between C57BL/6

unstimulated and stimulated splenocytes was significant (MFI= 528±20 and 660±16, respectively, p=0.0004). From these pilot studies we concluded that significant differences in cytokine production in response to stimulation were detectable between NOD and C57BL/6 mice.

Figure 17: Intracellular cytokine analysis of stimulated and unstimulated NOD and C57BL/6 splenocytes

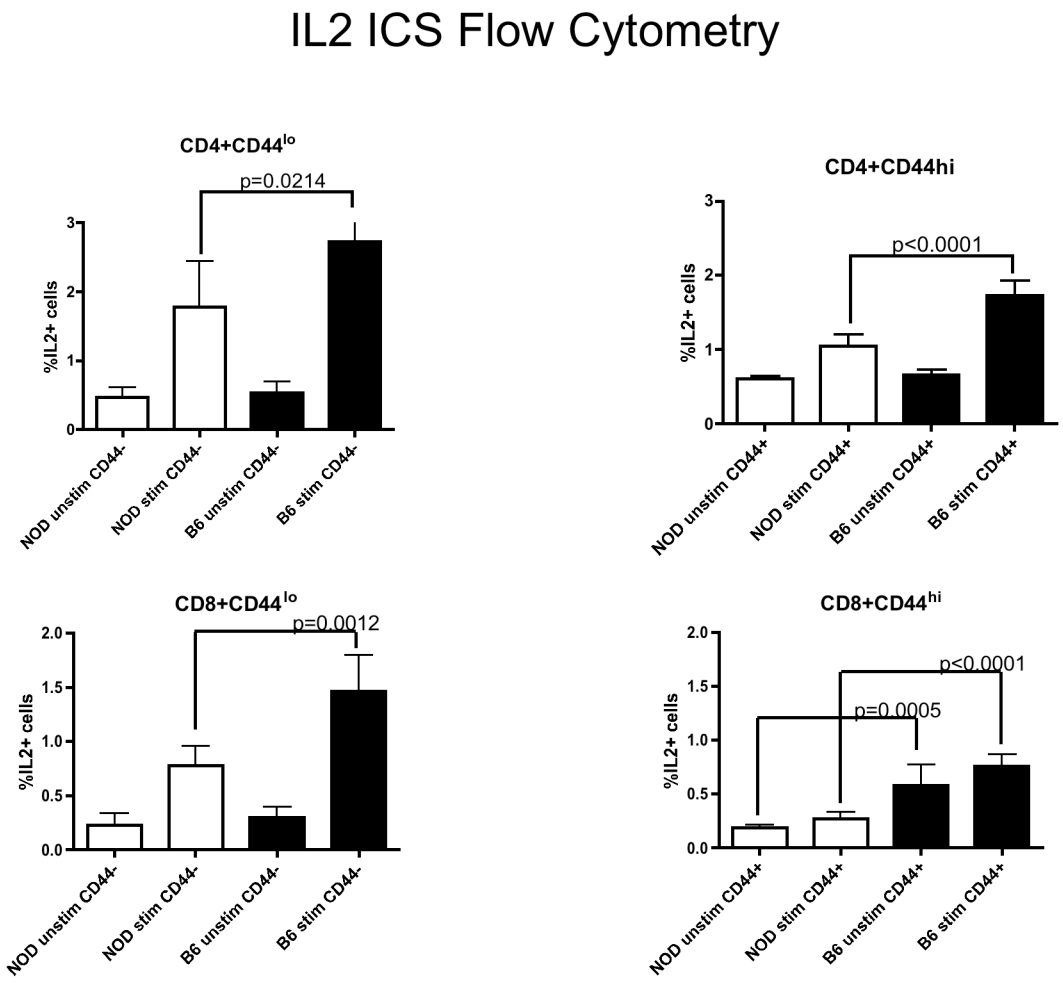


Figure 17 Legend: Freshly isolated NOD and C57BL/6 splenocytes were cultured either in the presence or absence of anti-CD3 as described in methods. Five hours later the cultured cells were harvested and labeled with fluorescent antibodies to detect CD4, CD8, CD44 and the intracellular cytokine IL-2. Cells were gated on CD4+ $CD44^{hi}$, CD4+ $CD44^{lo}$, CD8+ $CD44^{hi}$ and CD8+ $CD44^{lo}$ and the percent of IL-2 positive cells from each group was determined. Each bar represents an N of 6.

Figure 18: Intracellular cytokine analysis of stimulated and unstimulated NOD and C57BL/6 splenocytes

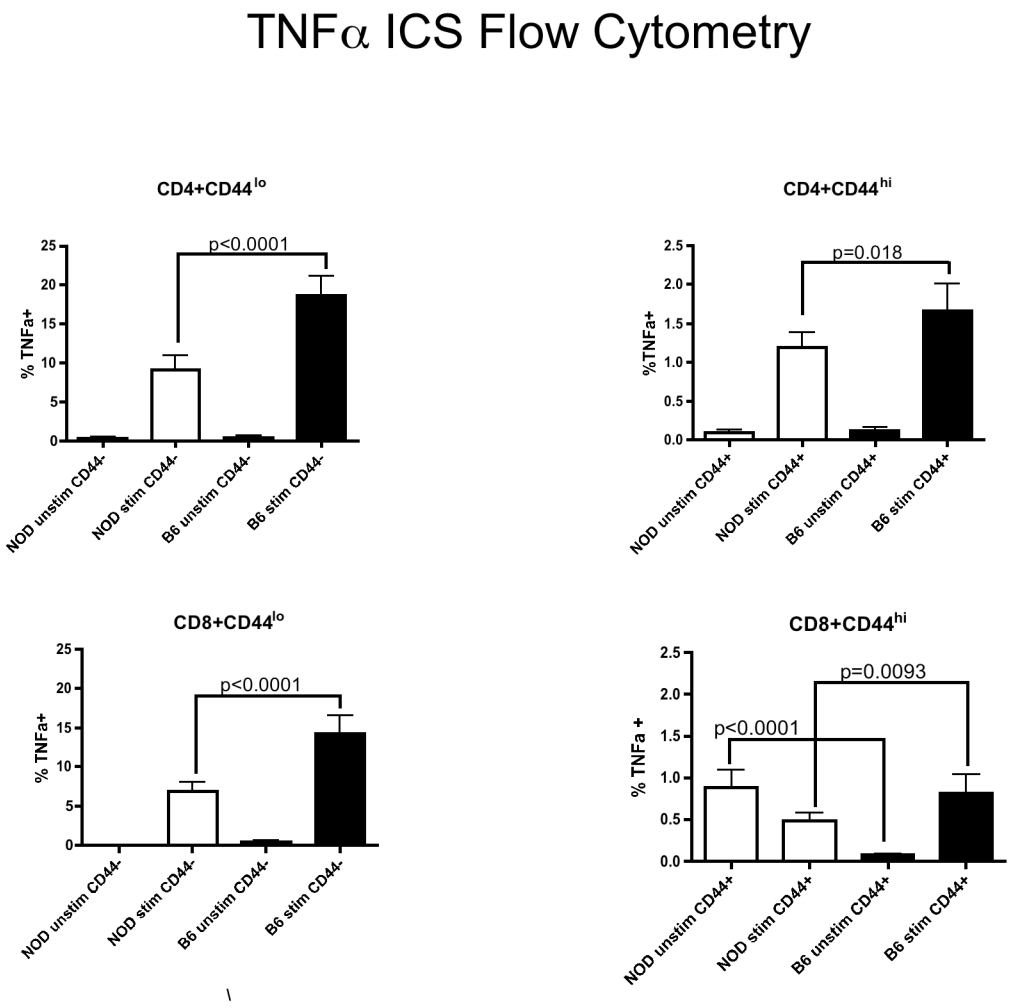


Figure 18 Legend: Freshly isolated NOD and C57BL/6 splenocytes were cultured either in the presence or absence of anti-CD3 as described in methods. Five hours later the cultured cells were harvested and labeled with fluorescent antibodies to detect CD4, CD8, CD44 and the intracellular cytokine TNF-α. Cells were gated on CD4+ $CD44^{hi}$, CD4+ $CD44^{lo}$, CD8+ $CD44^{hi}$ and CD8+ $CD44^{lo}$ and the percent of TNF-α positive cells from each group was determined. Each bar represents an N of 6.

Figure 19: Intracellular cytokine analysis of stimulated and unstimulated NOD andC57BL/6 splenocytes

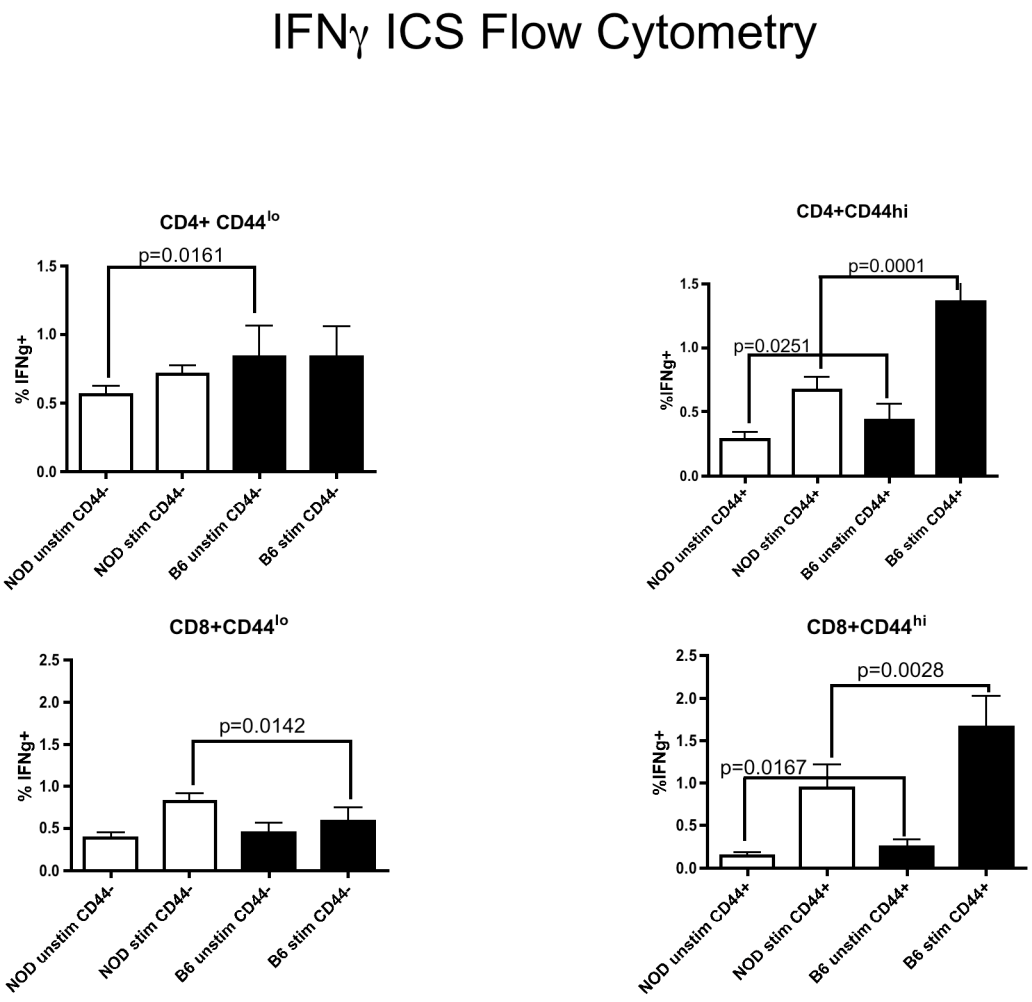


Figure 19 Legend: Freshly isolated NOD and C57BL/6 splenocytes were cultured either in the presence or absence of anti-CD3 as described in methods. Five hours later the cultured cells were harvested and labeled with fluorescent antibodies to detect CD4, CD8, CD44 and the intracellular cytokine IFN-γ. Cells were gated on CD4+ $CD44^{hi}$, CD4+ $CD44^{lo}$, CD8+ $CD44^{hi}$ and CD8+ $CD44^{lo}$ and the percent of IFN-γ positive cells from each group was determined. Each bar represents an N of 6.

2. Intracellular cytokine analysis of the frequency of effector/memory alloreactive T cells in congenic NOD mice treated with costimulation blockade

To determine if the alloreactive CD8 T cells that escaped deletion developed into functional effector cells, we used an intracellular cytokine assay for detection of IFNγ-producing cells that we have shown is a sensitive measure of the presence of effector/memory alloreactive $CD8^+$ T cells [202, 228]. As shown in Figure 20, treatment of C57BL/6 mice with DST on day 0 and anti-CD154 mAb on days 0 and +4 resulted in low levels of $CD8^+CD44^{hi}IFN\gamma^+$T cells on day +7 for all stimulating conditions indicated. It is interesting to note that the percent of $CD8^+CD44^{hi}$splenocytes producing IFNγ in response to *in vitro* anti-CD3 stimulation from the tolerized C57BL/6 mice treated with K^k DST and anti-CD40 is so low (0.23 %) compared to the response seen in similar populations of splenocytes from C57BL/6 mice that were primed with K^k DST in the absence of anti-CD40 (9.02%) (Figure 20). Since splenocytes from both naïve and DST primed mice respond so robustly to anti-CD3 stimulation, this data seems to suggest that a large population of alloreactive cells has been eliminated in the tolerized C57BL/6 mice. Alternatively, several members of our lab have seen a general immunosuppressive effect of anti- CD40 treatment (unpublished observations).

In contrast to the results seen in the tolerized C57BL/6 mice , priming C57BL/6 mice with DST on day 0 in the absence of costimulation blockade lead to high levels of $CD8^+CD44^{hi}IFN\gamma^+$T cells on day +7 in response to anti-CD3 and alloantigen (k^k), but not in the unstimulated or syngeneic controls. The data in Figure 20 are a representative FACS plot of experiments depicted graphically in Figure 21.

Priming C57BL/6, NOD, NOD.B6 *Idd3*, and NOD.B6*Idd3* B10*Idd5* with an allogeneic DST led to readily detectable levels of splenic alloreactive $CD8^{+}CD44^{hi}IFN\gamma^{+}$ T cells 7 days later in each of these lines of mice (Figure 21 A). As expected [195, 202], treatment of C57BL/6 mice with DST on day 0 and anti-CD154 mAb on days 0 and +4 resulted in low levels of $CD8^{+}CD44^{hi}IFN\gamma^{+}$T cells on day +7 (0.10±0.03%, Figures 20 and 21 B). In contrast, high levels of $CD8^{+}CD44^{hi}IFN\gamma^{+}$T cells were detected in similarly treated NOD mice (0.28±0.18%), correlating with their short islet allograft survival (Figure 9 and Figure 21 B). Levels of $CD8^{+}CD44^{hi}IFN\gamma^{+}$ T cells in similarly treated NOD.*Idd3* mice on day +7 (0.20±0.04%) were reduced, but not significantly lower than that observed in NOD mice. These levels were decreased further in NOD.B6 *Idd3Idd5* congenic mice (0.07±0.04%, Figure 20B) as compared to NOD mice ($p<0.05$) and were not significantly different than levels observed in C57BL/6 mice (p=N.S.), again correlating with improved islet allograft survival in NOD.B6 *Idd3Idd5* congenic NOD mice that was similar to that achieved in C57BL/6 mice (Figure 10).

Figure 20: Detection of effector/memory $CD8^{+}CD44^{high}$ IFNγ-producing cells

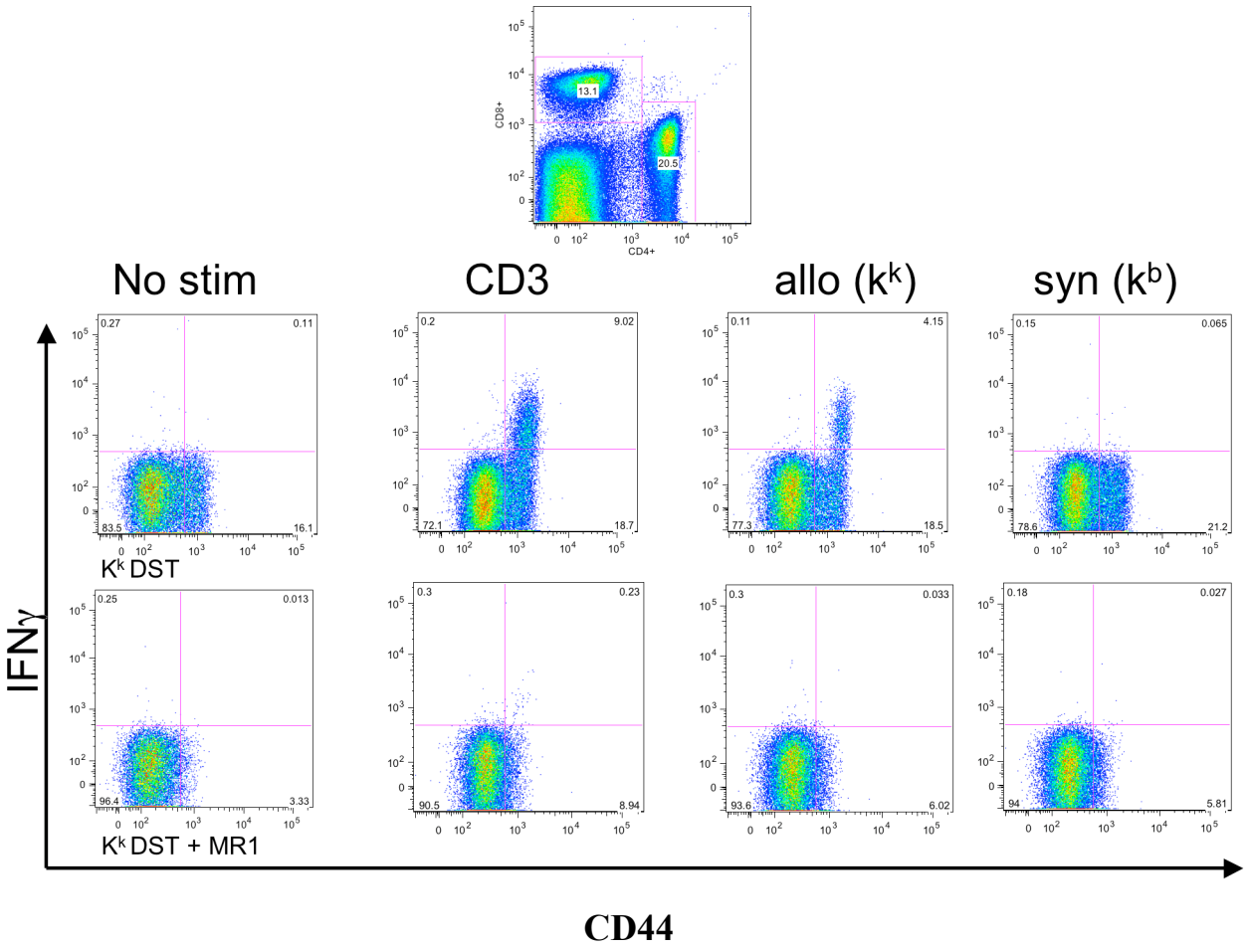


Figure 20 Legend: Naïve C57BL/6 mice were treated with C3H/HeJ DST on day -7 with (bottom panel) or without (top panel) 0.5 mg anti-CD154 mAb on days –7 and -4 relative to analysis on day 0. Splenocytes were recovered and stimulated for 5 hr *in vitro* with the culture conditions described at the top of the panel (unstimulated, anti-CD3, allogeneic K^k and syngeneic K^b) and stained for cell surface CD8 and CD44 and for intracellular IFNγ (representative example shown in Figure 20). A graphic representation of aggregate data is shown in Figure 21.

Figure 21: Detection of effector/memory $CD8^{+}CD44^{high}$ IFNγ-producing alloreactive T cells in mice treated with costimulatory blockade

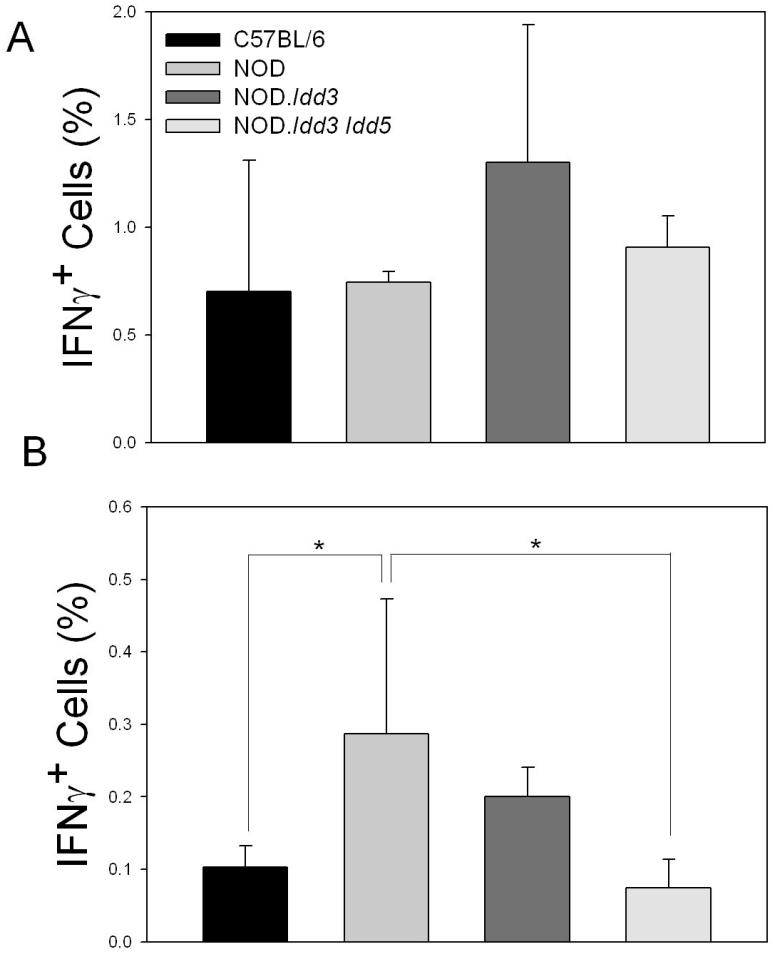


Figure 21 Legend: C57BL/6, NOD and NOD *Idd* congenic mice were treated with a C3H/HeJ DST on day –7 and 0.5 mg anti-CD154 mAb on days –7 and -4 relative to analysis on day 0 (Panel B). Splenocytes were recovered and stimulated for 5 hr *in vitro* with allogeneic $H2^k$ splenocytes and stained for cell surface CD8 and CD44 and for intracellular IFNg. As controls, naïve C57BL/6, NOD, and congenic mice were treated with an injection of C3H/HeJ DST seven days earlier to prime alloreactive T cells (Panel A). Samples were gated on $CD8^+$ T cells and the percentage of $CD44^{high}$ cells staining positive for IFNg were quantified by flow cytometry. Shown is the mean percent positive cytokine producing cells (mean ± s.d.). Student's t test was used to compare groups (*$p<0.05$).

Summary

We demonstrate that NOD mice treated with costimulation blockade and DST fail to delete alloreactive CD8 T cells as compared with C57BL/6 mice, and these cells develop cytotoxic effector/memory function when re-challenged with alloantigen. The stepwise improvement in islet allograft survival seen in the *Idd3* and *Idd3/5* congenic mice was also associated with a similar decrease in the levels of functional alloreactive cytotoxic $CD8^+$ T cells detected after costimulation blockade. Priming C57BL/6, NOD, NOD.B6 *Idd3* and NOD.B6 *Idd3*B10*Idd5* mice with allogeneic DST led to a reduction in the numbers of functional alloreactive $CD8^+$ T cells, and that reduction corresponded with the presence of *Idd3Idd5* or *Idd3* alone.

DISCUSSION

The aims described within this thesis were based upon the overall hypothesis that the susceptibility to autoimmunity and resistance to transplantation tolerance are genetically separate phenotypes in the NOD mouse. The results in Chapter 1 show that the resistance to transplantation tolerance in response to costimulation blockade in the NOD resides in the hematopoietic compartment and can be reversed with bone marrow from a tolerance susceptible donor. In addition, the maturation defects seen in NOD dendritic cells were partially corrected in NOD mixed hematopoietic chimeras, suggesting that NOD dendritic cells lack a critical component required for maturation that is provided by the B6 bone marrow. Next, in Chapter 2 we examined the contributions various *Idd* loci have in prolonging islet allograft survival in NOD mice. Because autoimmunity and resistance to transplantation tolerance are genetically separate in the NOD mouse, we hypothesized that tolerance induction to islet alloantigens, as well as tolerance to self, are polygeneically controlled mechanisms that may involve both distinct and overlapping genes. We were able to demonstrate that some *Idd* loci that are strongly protective against autoimmunity do not correct the tolerance induction defect of NOD mice. Furthermore, we identified a protective effect of *Idd3* (IL-2) in transplantation tolerance that when combined with *Idd5* is a potent determinant in susceptibility to tolerance induction to islet allografts. Finally, in Chapters 3 and 4 we present data that demonstrates a role for IL-2 in costimulation blockade based tolerance induction in the NOD. This effect was dependent on the ability of IL-2 to correct a defect in alloreactive CD8+ T cell deletion seen in the NOD following treatment with DST and costimulation

blockade. The remaining alloreactive CD8+ T cells in the NOD respond with cytotoxic effector/memory function when re-challenged with alloantigen. The stepwise improvement in islet allograft survival seen in the *Idd3* and *Idd3/5* congenic mice was also associated with a similar decrease in the levels of functional alloreactive cytotoxic $CD8^+$ T cells detected after costimulation blockade. Treatment of C57BL/6, NOD, NOD.B6 *Idd3* and NOD.B6 *Idd3*B10*Idd5* mice with allogeneic DST and anti-CD154 mAb led to a reduction in the numbers of functional alloreactive $CD8^+$ T cells that corresponded with the presence of *Idd3Idd5* or *Idd3* alone.

Hematopoietic chimerism and tolerance induction in the NOD

Our overall hypothesis is that dendritic cell maturation defects and resistance to transplantation tolerance induction are both based in the hematopoietic cell compartment. To investigate this, we created hematopoietic chimeras by performing reciprocal NOD and C57BL/6^{g7} bone marrow transfers.

This hypothesis predicts that NOD mice fully reconstituted with C57BL/6^{g7} bone marrow will exhibit prolonged skin allograft survival in response to costimulation blockade. It also predicts the reciprocal will be true, that is, C57BL/6^{g7} mice with a hematopoietic compartment of NOD origin will exhibit short skin allograft survival. Our results support this hypothesis, and the skin allograft survival seen in NOD mice with C57BL/6^{g7} hematopoietic origin was significantly prolonged over that achieved in similarly treated NOD recipients of NOD bone marrow.

It has been shown that diabetes can be prevented by the adoptive transfer of bone marrow from diabetes-resistant donors [166, 167]. We wanted to extend this observation

and determine if hematopoietic chimerism can likewise correct the tolerance induction defect to alloantigens seen in the NOD. Because the expression of diabetes and the resistance to transplantation tolerance are phenotypes that can be separated genetically, it is not known if the tolerance induction defects in NOD mice reside in the hematopoietic cell compartment, as is the case for TID, or the stromal environment in which they mature and function. Our data are consistent with the tolerance induction defect residing in the hematopoietic compartment. We conclude that the generation of bone marrow chimerism in NOD mice, that is, the provision of non-autoimmune prone stem cells in a genetically autoimmune prone mouse, can prevent resistance to tolerance induction and has an important role in the mechanisms regulating both autoimmunity and alloimmunity.

We wanted to extend this finding and try to determine if defects in dendritic cell maturation likewise track with the donor hematopoietic compartment and susceptibility to tolerance induction. Dendritic cells are regulators of immunity and self-tolerance [229, 230] and have an important role in transplantation tolerance [231-233]. Maturation of dendritic cells is dependent on CD40-CD154 interaction [234] and NOD mice have abnormalities in dendritic cell maturation [123, 126, 127, 235, 236] and costimulation blockade-based tolerance induction [132]. Importantly, while many of the immunologic cellular defects demonstrated in the NOD are corrected in the tolerance-resistant (NOD x C57BL6^{g7})F1, the dendritic cell maturation defect is still expressed.

We hypothesized that dendritic cell maturation defects in the NOD are based in the hematopoietic cell compartment. In the case of NOD recipients fully reconstituted with C57BL/6^{g7} bone marrow, this would predict that the bone marrow derived dendritic

cells from these animals would be of C57BL/6^{g7} origin and would not have the maturation defect normally seen in the non-chimeric NOD recipient strain.

We also wanted to determine whether the dendritic cell defect seen in the NOD was intrinsic or environmental. The relative contribution of NOD and C57BL/6^{g7} dendritic cells in NOD:C57BL/6^{g7} mixed chimeric animals is 50%. Bone marrow derived NOD dendritic cells from these animals showed a partial but significant improvement in maturation in response to CD40 stimulation when evaluated by CD86 expression. In both combinations of mixed chimeras, whether it was the NOD recipient or the of C57BL/6^{g7} recipient, the NOD derived dendritic cells had a significantly higher percentage of CD86 expression than the dendritic cells derived from control NOD:NOD chimeras. These results suggest that a critical component required for dendritic cell maturation is absent in NOD mice but can be provided by the presence of C57BL/6^{g7} bone marrow.

The role of specific *Idd* loci in transplantation tolerance

Our first goal was to determine whether the shortened islet allograft survival seen in the NOD results from the development of islet autoimmunity or from the failure of costimulation blockade to induce allograft tolerance. To address this question we rendered prediabetic male NOD mice hyperglycemic by treatment with streptozotocin and transplanted them with syngeneic NOD-*scid* islets. All mice remained normoglycemic through 150 days after islet transplantation, and histopathological analysis of the islet-bearing kidney revealed an insulin-producing islet graft present at the time of necropsy. Studies have shown that islets transplanted beneath the kidney capsule can in fact be rejected by an ongoing autoimmune process, as syngeneic islets

transplanted into diabetic NOD males were rapidly rejected. These data suggest that autoimmunity is substantially impaired in chemically diabetic NOD mice.

It would be interesting to examine the immunomodulatory effects of streptozotocin more closely in the NOD model system. Some have suggested that there is actually a temporal window of opportunity by which β cell death and the concomitant display of β autoantigens by DC in the pancreatic draining lymph node leads to the establishment of β cell autoimmunity. [237]. According to this model, physiological priming of islet reactive T cells by pancreatic DC must occur during a discrete timeframe for the establishment of type 1 diabetes. The autoimmune suppressive effect of streptozotocin treatment could be mediated by the death, thus removal, of β cells during this important window of antigenic priming. This seems unlikely, however, because treatment of NOD mice with alloxan, a similar chemical used to target the destruction of β cells, does not have the same subsequent autoimmune suppressing effects [238].

A closer study revealed that the streptozotocin effect is due to the apoptosis of islet β cells and skews the development of Treg [239], though another study revealed a bigger role for CD8+ T cells [238]. It is clear that the mechanisms leading to the immunomodulatory effects of streptozotocin in NOD mice are complex and may involve a balance between autoimmune effector cells and the generation of a Treg population to control them. Because streptozotocin treatment is one of so few strategies that exist to reverse autoimmune diabetes in NOD mice,

understanding streptozotocin mediated immune modulation could provide new strategies for the treatment of patients with type 1 diabetes.

Having removed the autoimmune component from the equation with streptozotocin treatment, we were confident that loss of euglycemia in our NOD islet allograft recipients would be indicative of a true allograft rejection process. This allowed us to take a close look at allotolerance in the NOD compared to C57 mice in which tolerance to alloantigens is readily induced via costimulation blockade. We were able to demonstrate that some *Idd* loci that are strongly protective against autoimmunity do not correct the tolerance induction defect of NOD mice. This led us to hypothesize that tolerance induction to islet alloantigens, as well as tolerance to self, are polygeneically controlled mechanisms that may involve both distinct and overlapping genes. Our goal was to understand the genetic relationship between transplantation tolerance and autoimmunity. The previously held dogma that autoimmunity and transplantation tolerance in the NOD are under the same genetic control may not be correct, and a further understanding of the mechanisms underlying these two processes may lead to insights into the mechanisms that control transplantation tolerance and may lead to protocols for islet transplantation in type 1 diabetes.

To begin to test the hypothesis that tolerance to self, as well as tolerance to islet alloantigens, involves both distinct and overlapping genes, we investigated the role of *Idd9* in transplantation tolerance induction. NOD *Idd9* congenic mice carry at least three genes (*Idd9.1, 9.2* and *9.3*). Candidate genes within this region include Lck and 4-1BB, both of which have immunomodulatory functions [178]. *Idd9* mice are profoundly

protected from diabetes with the incidence of spontaneous autoimmunity reduced to less than 4%. In spite of this, they still develop significant insulitis and islet-specific autoantibodies [179]. We chose to look at *Idd9* because previous reports have shown that the presence of *Idd9* genes in the NOD background leads to restored CD8 T cell tolerance to islet specific antigens [240]. We hypothesized that the protective *Idd9* genes would also result in restored tolerance to islet alloantigens in response to costimulation blockade. Surprisingly this was not the case. The NOD.B10*Idd9* mice resisted tolerance induction and rejected allogeneic islets with the same kinetics as the control NOD mice. These results clearly demonstrate that certain *Idd* loci that are profoundly protective for autoimmune diabetes may play little or no role in tolerance induction to alloantigen.

We wanted to determine whether other profoundly diabetes-resistant *Idd* loci could confer susceptibility to allotolerance induction. Together, the *Idd 3/10/18* regions confer almost complete protection from diabetes when homozygous B6/B10 alleles are introgressed into the NOD background [209]. This protection from diabetes is dependent upon the presence of both the *Idd10* and *Idd18* loci being combined with *Idd3*, as the protection seen when each is individually combined with *Idd3* is indistinguishable than that achieved with *Idd3* alone [183]. Because the *Idd3/10/18* loci lead to almost complete protection from diabetes [181, 209, 210] and the *Idd10/18* alleles provide moderate protection (45-55%) [183, 209, 241], we hypothesized that NOD.B6 *Idd10/18* mice and NOD.B6 *Idd3/10/18* mice would demonstrate a stepwise improvement in costimulation blockade induced tolerance to islet allografts. Surprisingly, this was not the case. The islet allograft survival we observed in the NOD.B6 *Idd10/18* mice was significantly

shorter than what we observed in NOD mice. In addition, the detrimental effects of the *Idd10/18* loci did not decrease islet allograft survival when combined with the beneficial effect of *Idd3*, as the MST between the NOD.B6 *Idd3/10/18* mice and the NOD.B6 *Idd3* mice was not statistically different.

Of interest within the *Idd10/18* region is the *Ptpn8* gene, which is orthologous to human *Ptpn22* [183]. Studies have shown that single-nucleotide polymorphisms (SNPs) in the lymphoid tyrosine phosphatase (LYP) encoded by the PTPN22 gene correlate with the development of type 1 diabetes in humans [184]. This observation has been extended to include an association between the same SNP in *Ptpn*22 and many other autoimmune diseases, including systemic lupus erythematosis, rheumatoid arthritis, and Grave's disease [185-191]. The disease-associated allele of *Ptpn*22 is a gain-of-function variant that *in vitro* has been shown to suppress TCR signaling in response to TCR/CD28 ligation more efficiently than the 'normal" allele [192]. Notably, the functional outcome of TCR signaling in the PTPN22 gain of function variant was reported to be reduced expression of IL-2. It is tantalizing to speculate that the aggressive rejection demonstrated in the *Idd10/18* mice could likewise be due to the gain of function variant within that interval resulting in decreased IL-2 production. It is possible that the homozygous presence of the B6 *Idd3* (IL-2) allele, either with or without the B6 *Idd10/18* region, is enough to effect a modest increase in IL-2R signaling and protect against islet cell autoimmunity and permit costimulation blockade induced tolerance induction. However, in the absence of *Idd3*, the amount of IL-2 produced by the B6 copy of *Idd10/18* is not enough to participate in the induction of transplantation tolerance. Additional studies are

underway to determine the role(s) *Ptpn8* may have in transplantation and autoimmunity in the NOD.

Given the surprising results seen with the NOD.B6 *Idd3/10/18* islet allograft studies, we next wanted to determine if a synergistic effect could be found between *Idd3* and other *Idd* loci such as *Idd5*. NOD.B10 *Idd5* mice are much less susceptible to spontaneous diabetes than NOD mice, and when combined with *Idd3* the incidence of spontaneous diabetes is reduced to less than two percent [193, 212]. Surprisingly, NOD.B6*Idd3*B10*Idd5* double congenic mice achieved islet allograft survival that was statistically no different than similarly treated C57BL/6 controls. Islet allograft survival in mice bearing the *Idd5* resistance region was not significantly different than in NOD mice but was significantly shorter than the islet allograft survival achieved in the NOD.B6 *Idd3* mice. Interestingly, the tempo of islet allograft rejection seen in the NOD.B10 *Idd5* mice seems to be biphasic. It is tantalizing to speculate that there is a differential requirement for *Idd5* in the induction of central versus peripheral tolerance. Mechanisms involved in peripheral tolerance induction would translate into allograft survival at earlier timepoints, while those involved in the establishment of central tolerance would have more of a role at later timepoints.

The islet allograft survival data from NOD.B6 *Idd10/18* and NOD.B10 *Idd5* congenic regions, either alone or with *Idd3*, show that not all *Idd* loci that confer diabetes resistance lead to improved islet allograft survival.

We next wanted to identify the *Idd5* sub-region interval that synergizes with *Idd3* to improve islet allograft survival in NOD mice treated with costimulation blockade. *Idd5*

contains at least 4 different regions termed *Idd5.1, Idd5.2, Idd5.3,* and *Idd5.4* [193, 212]. The *Idd5.1* region is most likely to be variants of *Ctla4*, with the diabetes prone NOD allele producing less of the ligand-independent CTLA-4 (liCTLA-4) molecule than the resistant B10 allele [242]. CTLA-4 has been shown to be important for the induction of tolerance using costimulation blockade [23].

Nramp1 is a candidate gene within the *Idd5.2* region [193]. We became interested in interrogating the role of *Idd5.2* in both allo and autoimmunity because of the plietropic effects the NRAMP1 protein has in immune processes. *Nramp1* codes for a metal ion transport protein that is important in macrophage function. It is expressed in macrophage lysosomes and recruited to the membranes of phagosomes that have ingested either live bacteria or inert particles. There is evidence that NRAMP1 exerts its antimicrobial effect through its ability to deplete phagosomal divalent cations, which are essential for bacterial replication and important components of microbial metabolic activity [243]. In addition, other groups showed that *Nramp1* expression leads to qualitative differences in macrophage MHC class II expression and in processing of antigen for presentation to T cells [244-249]. Recently, another group was able to demonstrate *Nramp1* expression in CD11c+ bone marrow-derived dendritic cells [250]. By utilizing *Nramp1* congenic mouse strains this group was able to identify a role for NRAMP1 in modulating cytokine transcription, class II expression and antigen presenting function in DC. Notably, several human and mouse studies have implicated a role for NRAMP1 variation and function in autoimmunity, including type 1 diabetes, rheumatoid arthritis, and the inducible experimental autoimmune encephalomyelitis (EAE) [182, 251-253]. RNAi experiments

in which Nramp1 silencing leads to protection from both EAE and T1D in mice further supports a role for NRAMP in autoimmunity [182, 194].

Interestingly, the B10 diabetes resistant NRAMP1 allele is nonfunctional [243]. The loss of function *Nramp1* mutation derived from B10 mice confers protection from the development of spontaneous T1D while concomitantly resulting in susceptibility to intracellular pathogen infection, demonstrating a clear role for the NRAMP1 protein in diverse immune processes [248, 254, 255]. These data in their aggregate seem to suggest that NRAMP1 may be an important modulator of allo and autoimmunity. When diabetes is assessed, *Idd5.2/Nramp1* is not required for the interaction with *Idd3*. In addition, the *Idd5.1/Ctla4* and *Idd3* resistance alleles did not increase protection from diabetes as compared to *Idd3* alone. These results are consistent with our islet allograft survival data and seem to suggest that the polymorphisms seen in both the *Ctla4* and *Nramp1* diabetes susceptibility alleles do not lead to a functional difference in tolerance induction when allograft survival is assessed. That being said, the synergy observed between the *Idd5* and *Idd3* loci that results in nearly complete diabetes protection was shown to be dependent on the presence of the *Idd5.3* locus [256]. To extend this observation, it would be interesting to test *Idd3/5.3* in the absence of the *Idd5.1* interval mice to determine the contribution *Idd5.3* has in peripheral tolerance induction.

Idd3, which is partially protective of diabetes, significantly improves islet allograft survival in the NOD mouse, with the strongest effect seen in the *Idd3/5* bicongenic. The *Idd3* region of the NOD has approximately 100 SNPs per 10 kb when compared to the syntenic B6/B10 region [213]. Recent work has shown the *Idd3* effect

likely results from differential expression of IL-2 and results in diminished function of $CD4^+CD25^+$ Treg cells in NOD mice [152].

Cellular basis for the resistance to transplantation tolerance induction in the NOD

Based upon the islet allograft survival achieved in the mice containing the *Idd3* interval, we became interested in interrogating the role of IL-2 in the resistance to transplantation tolerance induction seen in the NOD mouse. IL-2 has been shown to be required for the development of self-tolerance and for costimulation blockade induced allograft tolerance [223, 257]. It is essential for activation induced cell death via the CD95 pathway, and administration of IL-2 *in vivo* can either enhance or downregulate a CTL response [258, 259]. Because *Idd3* significantly improves costimulation blockade induced tolerance and is likely the *Il2* gene, we decided to interrogate the role of IL-2 in transplantation tolerance in the NOD.

We have previously shown that deletion of recipient alloreactive $CD8^+$ T cells is required for prolonged skin allograft survival [22, 23, 28]. We hypothesized that the failure to induce tolerance in NOD mice is due to a failure to delete host alloreactive $CD8^+$ T cells because of insufficient levels of IL-2, and that administration of exogenous IL-2 would correct this defect. As expected, (KB5 CBA x C57BL/6.$H2^{g7}$)F1 synchimeric mice treated with costimulation blockade showed a marked deletion of alloreactive $CD8^+$ T cell that was significantly greater than that achieved in the (KB5 CBA x NOD)F1 synchimeric mice. When (KB5 CBA x NOD)F1 synchimeric mice were treated with exogenous IL-2 during the peri-transplant period, the alloreactive $CD8^+$ T cell deletion was significantly improved and comparable to that achieved in (KB5 CBA x

C57BL/6.*H2*^{g7})F1 synchimeras. From these experiments we conclude that NOD mice fail to efficiently delete alloreactive CD8^{+} T cells in response to costimulation blockade. This failure to delete can in part be rescued by administration of exogenous IL-2 during the peri-transplant period.

To extend this finding we determined if the increased deletion of alloreactive CD8^{+} T cells we observed in (KB5 CBA x NOD)F1 synchimeric mice treated with costimulation blockade plus IL-2 would also enhance islet allograft survival. We hypothesized that the failure to induce tolerance to islet allografts in the NOD is partly due to dysregulated IL-2 production from the NOD *Idd3* locus, and that the exogenous administration of IL-2 during the peri-transplant period would drive the deletion of the alloreactive recipient CD8^{+} T cells leading to prolonged islet allograft survival. Islet allograft survival in NOD mice treated with costimulation blockade plus IL-2 was slightly, but significantly longer than in NOD mice treated with costimulation blockade alone. The fact that the survival doesn't appear to be as long as that achieved in NOD.B6 *Idd3* mice may be a due to the fact that the exogenous IL-2 used to correct this defect was only transient in its administration whereas the increased IL-2 achieved in NOD.B6 *Idd3* mice is presumably at the proper physiologic tolerogenic level throughout the animal's life.

It would be interesting to use the NOD.*Idd3* congenics to determine the source of IL-2 provision responsible for correcting the tolerogenic defect in the NOD. Wicker *et al* have shown that in the NOD model, IL-2 has a remarkable effect on the ability of CD8+ T cells to differentiate into cytotoxic effectors both in vitro and in vivo [152]. The

increased production of IL-2 by *Idd3* CD8+ T cells results in more efficient CD25+CD4+ Treg recruitment , which functionally translates into inhibition of the autoreactive CD8+ T cells. IL-2 production and signaling is crucial for both CD8+ mediated Treg recruitment and for the subsequent Treg differentiation, activation and homeostasis . A possible model that could be developed to tease apart the source of IL-2 responsible for correcting the tolerogenic defect in the NOD could involve the seperation and transfer of discreet populations of *Idd3* CD4+ T cells or *Idd3* CD8+ T cells into an NOD background.

The stepwise improvement in islet allograft survival seen in the *Idd3* and *Idd3/5* congenic mice was also associated with a similar decrease in the levels of functional alloreactive cytotoxic $CD8^+$ T cells detected after costimulation blockade. The presence of a $CD8^+CD44^+IFN\gamma^{hi}$ population is a sensitive measure for effector/memory alloreactive $CD8^+$ T cells [202]. Treatment of C57BL/6, NOD, NOD.B6 *Idd3* and NOD.B6 *Idd3*B10*Idd5* mice with allogeneic DST and anti-CD154 mAb led to a reduction in the numbers of functional alloreactive $CD8^+$ T cells that corresponded with the presence of *Idd3Idd5* or *Idd3* alone.

These data suggest that impaired production of IL-2 in NOD mice is a barrier to costimulation blockade induced tolerance to skin and islet allografts. It is interesting that (NOD x C57BL/6)F1 mice carrying one copy of an *Idd3* resistance variant are resistant to tolerance induction for skin but not islet allografts [132, 199]. It has long been appreciated that tolerance induction to distinct tissues is differentially regulated [27]. For example, costimulation blockade induced tolerance to skin has an absolute requirement

for the presence of CD4+CD25+ Treg in the host, whereas the same protocol induces tolerance to islet allografts in the absence of Treg activity. IL-2 is indispensable for supporting the *in vivo* growth, survival and function of naturally occurring Tregs [219, 221, 260-262], and because of their corrected *Idd3* haplotype, NOD.B6 *Idd3* mice have $CD4^{+}CD25^{+}$ Tregs with enhanced regulatory activity [152].

Conclusions

A confounding variable in using islet transplantation as a curative therapy for diabetes is in trying to induce tolerance to alloantigens in an autoagressive environment that has already lost tolerance to autoantigens. In addition to being targeted by alloreactive T cells, the transplanted tissue risks becoming the target of recurrent autoimmunity. The NOD mouse is the model of choice in developing therapies to cure type 1 diabetes, including islet transplantation. These mice have fundamental defects in T cell self-tolerance that appear to be related to breakdowns in both central and peripheral tolerance-inducing mechanisms. Understanding these fundamental defects in tolerance, and the relationship between transplantation tolerance and autoimmunity, represent an important area of research that can lead to more effective curative therapies for type 1 diabetes.

Because the expression of diabetes and the resistance to transplantation tolerance can be separated genetically, it was not known if the tolerance induction defects in NOD mice reside in the hematopoietic cell compartment or the stromal environment in which they mature and function. Preliminary results of Chapter 1 show that the defect lies in the hematopoietic compartment. We further hypothesized that dendritic cell maturation defects and resistance to tolerance induction are linked phenotypes in NOD mice and that both are based in the hematopoietic cell compartment. Bone marrow derived dendritic cell cultures from NOD and C57BL/6^{g7} reciprocal chimeras revealed that the dendritic cell defects track with the NOD bone marrow. Together, these data demonstrate that the

dendritic cell defects and resistance to tolerance induction of NOD mice are dependent on the source of the hematopoietic cell system.

We have also shown in Chapter 3 that the cellular basis of NOD mouse resistance to costimulation blockade induced transplantation tolerance is in part due to the failure to efficiently delete alloreactive CD8 T cells. Genetically, this can be overcome by the introgression of a normal *Idd3* (i.e. Il2) gene that synergizes with some but not all *Idd* protective loci to promote allograft tolerance, as we demonstrated in Chapter 2.

The effects of *Idd3* can be recapitulated by the administration of exogenous IL-2 suggesting that the tolerance-enhancing effect of *Idd3* is mediated through improved production of IL-2. This effect is mediated through enhanced deletion of alloreactive CD8 T cells (Chapter 3) as well as enhanced Treg activity [152]. The "normal" dendritic cells circulating in NOD mixed hematopoietic chimeras have been shown to drive the deletion of autoreactive CD8 T cells [168], and other groups have shown that dendritic cells are important mediators of Treg maintenance in the periphery [263, 264]. Notably, these studies showed that the ability of dendritic cells to support Treg populations is linked to a mature phenotype dendritic cell and requires IL-2 production by Treg. NOD Treg cells are deficient in IL-2 production, which can be rescued by the expression of *Idd3* [152].

Taken together, these data suggest a model in which alloreactive CD8 T cell deletion in response to costimulation blockade is controlled by a collaboration between regulatory T cells and dendritic cells. Mature dendritic cells acquire the ability to delete/control alloreactive CD8 T cells. They also participate in the maintenance of Treg

populations. This maintenance is dependent on both the mature dendritic cell and the ability of the Treg to produce IL-2. Treg can also drive the deletion of alloreactive CD8 T cells with the provision of IL-2.

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