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The Genetic Basis of Resistance to Transplantation Tolerance Induced by Costimulation Blockade in NOD Mice: a Dissertation

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THE GENETIC BASIS OF RESISTANCE TO TRANSPLANTATION TOLERANCE INDUCED BY COSTIMULATION BLOCKADE IN NOD MICE

A Dissertation Presented

By

Todd Pearson

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

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ABSTRACT

The NOD mouse is a widely studied model of type 1 diabetes. The loss of self-tolerance leading to autoimmune diabetes in NOD mice involves at least 27 genetic loci. Curing type 1 diabetes in mice and humans by islet transplantation requires overcoming both allorejection and recurrent autoimmunity. This has been achieved with systemic immunosuppression, but tolerance induction would be preferable. In addition to their genetic defects in self-tolerance, NOD mice resist peripheral transplantation tolerance induced by costimulation blockade using donor-specific transfusion and anti-CD154 antibody. Failure has been attributed to the underlying autoimmunity, assuming that autoimmunity and resistance to transplantation tolerance have a common basis. Hypothesizing that these two abnormalities might be related, we investigated whether they had a common genetic basis. Diabetes-resistant NOD and C57BL/6 stocks congenic for various reciprocally introduced Idd loci were assessed for their ability to be tolerized. Surprisingly, in NOD congenic mice that are almost completely protected from diabetes, costimulation blockade failed to prolong skin allograft survival. In reciprocal C57BL/6 congenic mice with NOD-derived Idd loci, skin allograft survival was readily prolonged by costimulation blockade. Unexpectedly, we observed that (NOD x C57BL/6)F1 mice, which have no diabetes, nonetheless resist induction of tolerance to skin allografts. Further analyses revealed that the F1 mice shared the dendritic cell maturation defects and abnormal CD4+ T cell responses of the NOD but had lost its defects in macrophage maturation and NK cell activity. Finally, using a genome wide scan approach, we have
identified four suggestive markers in the mouse genome that control the survival of skin allografts following DST and anti-CD154 mAb therapy. We suggest that mechanisms controlling autoimmunity and transplantation tolerance in NOD mice are not completely overlapping and are potentially distinct, or that the genetic threshold for normalizing the transplantation tolerance defect is higher than that for preventing autoimmune diabetes. We conclude that resistance to allograft tolerance induction in the NOD mouse is not a direct consequence of overt autoimmunity and that autoimmunity and resistance to costimulation blockade-induced transplantation tolerance phenotypes in NOD mice are not under identical genetic control.
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ABBREVIATIONS

αGalCer, alpha-galactosylceramide; ADCC, antibody-dependent cellular cytotoxicity; AICD, activation-induced cell death; APC, antigen presenting cell; β2M, beta-two microglobulin; BCR, B cell receptor; B7.1, CD80; B7.2, CD86; CD152, CTLA4; CD154, CD40 ligand; CSF-1, macrophage colony stimulating factor/M-CSF; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DST, donor-specific transfusion; DTH, delayed-type hypersensitivity; FC, fetal clone (serum); GM-CSF, granulocyte/macrophage colony stimulating factor; GVHD, graft versus host disease; Idd, insulin-dependent diabetes (locus); IFNγ, interferon-gamma; Ig, immunoglobulin; IL-1β, interleukin-1-beta; IL-4, interleukin-4; IL-10, interleukin-10; LPS, lipopolysaccharide; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MST, median survival time; NK, natural killer; NKT, natural killer T; NOD, non-obese diabetic mouse; QTL, quantitative trait locus; TCR, T cell receptor; TGFβ, transforming growth factor-beta; Th1, T helper type 1 response; Th2, T helper type 2 response; TNFα, tumor necrosis factor-alpha.
INTRODUCTION

Overview of diabetes mellitus

Diabetes mellitus comprises a group of disorders characterized by hyperglycemia and affects 14 million Americans, with 800,000 diagnosed new cases each year (1). Most cases are classified as type 1 or type 2 (2). Both types can cause debilitating symptoms, predispose to infection, complicate pregnancy, and lead to many secondary complications. Indeed, diabetes is a leading cause of blindness and amputation in the United States. Type 2 diabetes is the more common syndrome, accounting for approximately 85% of cases. It is associated with obesity and generally occurs in adults, but can often be controlled by diet and exercise. Individuals with type 2 diabetes suffer from insulin resistance or a relative deficiency in insulin, meaning that insulin secretion is altered, but not totally lost. Type 1 diabetes is less common, accounting for about 10% of cases. It is an autoimmune disease caused by T cell mediated destruction of pancreatic beta cells, resulting in an absolute insulin deficiency. In contrast to type 2 diabetes, type 1 occurs most commonly during childhood. Patients with type 1 diabetes require exogenous insulin for survival, but insulin therapy never achieves perfect glucose regulation, and secondary complications of diabetes inevitably develop. The only currently available alternative is transplantation of replacement beta cells, which would be curative.

Segmental pancreas transplantation can achieve normal glucose regulation and insulin independence, but requires major surgery and immunosuppression and has a substantial rate of post-surgical complications (3). Transplantation of islets isolated from the pancreas would clearly be preferable because it is much less invasive. Successful islet
transplantation for type 1 diabetes was first reported in 1990 (4), but until recently success rates were very low.

In 2000, however, researchers in Edmonton, Canada demonstrated that transplantation of islets using a new regimen of islet preparation and immunosuppression could cure type 1 diabetes with a success rate that appeared to rival that of segmental pancreas transplantation (5, 6). Promising results for more than 30 patients were reported at the 2002 meeting of the American Diabetes Association, and two patients have received transplants using this protocol at the University of Massachusetts Medical School.

These successes have been encouraging, but islet transplantation to cure type 1 diabetes continues to require immunosuppressive drugs. These are medications with both detrimental side effects and potential long-term health risks. Although allograft recipients comprise a uniquely motivated group of patients, overall medication non-compliance rates reportedly vary from 20 to 50% (7). In addition, many of these drugs have deleterious effects on the function of beta cells (8). New formulations and treatment schedules have made immunosuppression safer and more effective than in the past, but diabetic patients considering pancreas or islet transplantation are still forced to choose between the risks of hyperglycemia and its complications on the one hand, and the risks and side effects of lifelong systemic immunosuppression on the other. Because of the risks, transplantation to ameliorate diabetes is primarily reserved for patients who have extremely labile glycemic control and severe recurrent hypoglycemia, or who have renal failure and need a kidney transplant. To make islet transplantation safe and more
acceptable, research in transplantation is focused on developing alternatives to immunosuppression, most notably the induction of transplantation tolerance (9).

**Rationale for small animal models**

The pathology and genetic basis of type 1 diabetes in humans have been studied extensively, but understanding of type 1 diabetes remains incomplete. The disease is under polygenic control and penetrance is modified by environmental factors; identifying at risk individuals is difficult (10). In addition, studying disease in humans in general and children in particular poses serious ethical limitations, especially when considering a therapeutic intervention like an experimental transplantation procedure. Fortunately, reliable animal model systems are available that allow us to analyze the pathogenesis of autoimmune diabetes (11), the induction of transplantation tolerance (9), and the special problem of tolerance induction in the setting of autoimmunity (12).

Islet transplantation into autoimmune diabetics represents a unique situation because two obstacles must be overcome: the induction of tolerance to a foreign tissue and the prevention of autoimmune recurrence that is the cause for the transplant in the first place. Autoimmune recurrence has been elegantly demonstrated by syngeneic segmental pancreas transplantation between monozygotic twins discordant for diabetes, a situation where immunosuppressive drugs are not required. The graft fails in the absence of an alloimmune response because of a preexisting autoimmune condition in the recipient (13-15). Successes that have been seen with current pancreas and islet transplantation protocols are likely due to the fact that the immunosuppressive regimens that block the alloimmune response also inhibit the autoimmune process (9).
Central and peripheral transplantation tolerance

Tolerance in the immune system can be generically described as unresponsiveness to antigen. T cell tolerance is accomplished by many mechanisms, but is most broadly divided into two categories: central tolerance and peripheral tolerance (16). In the field of transplantation, there are various definitions of what it means to be "tolerant." "Immunological" transplantation tolerance refers to the complete absence of an immune response to the foreign tissue in the absence of immunosuppression. "Functional" transplantation tolerance, a more clinically relevant definition, refers to the survival of a foreign tissue in the absence of immunosuppression (9). Throughout this dissertation, we will use the "functional" definition of transplantation tolerance.

Central tolerance refers to tolerance that develops in the thymus during T cell development (17). The main process that leads to central tolerance is clonal deletion. Deletion removes immature thymocytes based on the strength of their TCR interaction with a cognate MHC/peptide complex. Self-reactive cells that have a high avidity for self MHC/peptide complexes are deleted intrathymically and don't enter the periphery (18). There is also a non-deletional central tolerance mechanism, called clonal inactivation that allows self-reactive cells to escape the thymus, but they are antigen unresponsive or clonally anergic. Central tolerance mechanisms have been exploited to induce transplantation tolerance by introducing alloantigens into the thymus to eliminate alloreactive thymocytes as they develop. This can be accomplished with intrathymic injection of antigen, or as discussed later, the induction of hematopoietic chimerism.
In spite of central tolerance mechanisms, some self-reactive cells do escape into the peripheral immune system (19, 20). Peripheral tolerance mechanisms are in place to prevent these cells from becoming inappropriately activated. There are numerous mechanisms believed to be involved, including anergy, suppression, clonal exhaustion and immune deviation (21). Transplantation researchers use peripheral tolerance mechanisms to induce the long term survival of foreign tissues. It is this type of transplantation tolerance that is the focus of this dissertation.

**Modeling costimulation blockade-based transplantation tolerance**

The transplantation of pancreata (3) and islets (22) using immunosuppression are clinical procedures that were prototyped in animals. Development of transplantation strategies that do not require immunosuppression is similarly based on animal experimentation. Findings that translate reproducibly from animals to humans are based on a detailed understanding of the underlying mechanisms responsible for immune responses (23).

Several transplantation tolerance protocols prolong allograft survival in normal inbred strains of mice (9). The strategies typically involve blockade of 1) TCR-MHC/peptide interaction, 2) costimulatory CD40-CD154 interaction, or 3) CD80/86-CD28 interaction. These protocols can be used either to prevent activation of alloreactive peripheral T cells (peripheral tolerance) or to establish hematopoietic chimerism (central tolerance) (9).

In our laboratory we have developed a two-element protocol that consists of a single transfusion of donor spleen cells (a donor-specific transfusion or DST) and a brief
course of anti-CD154 mAb to induce peripheral transplantation tolerance (9). Its design is based on interference of normal T cell activation (Figure 1A). We hypothesize that this protocol operates through the following mechanism: 1) Donor-origin resting APCs first engage the TCR of alloantigen-specific T cells. 2) Anti-CD154 mAb then prevents ligand engagement of CD40 on the resting APC, 3) thereby preventing the up-regulation of B7 costimulatory molecules, MHC class II, and cytokine production (Figure 1B). This protocol results in permanent islet and prolonged skin allograft survival in normal mouse strains (9) and non-human primates (24). Additionally, this protocol can prolong rat skin and islet xenograft survival in normal mouse strains (25). We have determined that the mechanism of prolonged allograft survival requires the elimination of alloreactive CD8+ T cells from the circulation in a CTLA-4 dependent manner, the presence of CD4+ T cells, and the secretion of IFNγ but not IL-4 or IL-10 (26, 27).
Figure 1

A

Recognition
MHC $\rightarrow$ TCR
Step 1

Activation
CD40 $\rightarrow$ CD80/86
Step 2

Co-stimulation
CD154 $\rightarrow$ CD28
Step 3

Activated APC

Activated Reactive T Lymphocyte

B

Step 1
MHC $\rightarrow$ TCR

Step 2
CD154 $\rightarrow$ Anti-CD154

Donor APC

Recipient T Lymphocyte

No Expression of CD80/86

Deletion
**Legend to Figure 1:** The three-step model of T cell activation and the proposed function of anti-CD154 mAb in preventing full T cell activation to establish tolerance. **Panel A:** Summary of T cell activation. The TCR on a T cell engages the MHC/peptide complex of an APC (Step 1). This interaction induces the upregulation of CD154 on the T cell and CD40-CD154 interactions result in activation of the APC (Step 2). When the APC is activated, it upregulates the B7-family molecules CD80 and CD86, which provide the costimulatory signal to the T cell needed for its full activation (Step 3). **Panel B:** Prevention of T cell activation by anti-CD154 mAb. TCR-MHC interaction still takes place (Step 1), but the anti-CD154 mAb blocks CD154-CD40 interaction, preventing full activation of the APC (i.e. blocks Step 2). As a consequence of failure to achieve full activation, the APC does not upregulate B7 molecules, preventing the delivery of costimulation to the T cell (no Step 3). Signaling through the TCR in the absence of costimulation renders the T cell tolerant.
Modeling type 1 diabetes in rodents

The two most common small animal models of type 1 diabetes are the BioBreeding (BB) rat and the Non-obese Diabetic (NOD) mouse (11). Both strains develop an autoimmune, type 1 diabetes-like syndrome. The pathophysiology of autoimmune diabetes in both strains has been well studied and has similarities and differences to each other and to human type 1 diabetes (11). These comparisons have been well documented in the literature and have prompted investigators to exercise caution when applying conclusions from animal studies to the human syndrome (23, 28).

We have chosen the NOD mouse to study the special case of transplantation tolerance in the context of autoimmunity. First, the mouse genome in general and the immunogenetics of the NOD mouse in particular are very well characterized. Second, the availability of knockout, transgenic and congenic strains of mice allows for in depth analysis. Third, reagents such as recombinant cytokines and monoclonal antibodies are more abundant for mice. Finally, our transplantation tolerance induction protocol has been extensively characterized in mice.

The NOD mouse model of type 1 diabetes

The NOD mouse was derived from outbred Swiss mice in Japan during the 1970's (29). Swiss mice are also the progenitors of other common inbred strains, including SJL and SWR (30). Researchers were originally screening brother x sister matings of Swiss mice for lines that developed cataracts, and developed two sublines, both free of cataracts: one had high fasting blood glucose levels and a second "control" strain with normal fasting blood glucose levels. These sublines were inbred, and
amazingly, a female in the "control" strain spontaneously developed an autoimmune diabetes-like syndrome. The offspring of this female gave rise to the NOD inbred strain of mice. The other subline, originally designed to be the diabetic line, gave rise to the nonobese nondiabetic (NON) strain (29, 30).

Type 1 diabetes in the NOD mouse is characterized by the T cell mediated destruction of insulin producing beta cells in the pancreas (11, 31, 32). The onset of hyperglycemia is predated by infiltration of mononuclear cells at 4 to 5 weeks of age; at first peripheral to the islet (termed "peri-insulitis") and followed by more invasive infiltration directly into the islet ("insulitis"). Prevalence of type 1 diabetes in NOD/Lt mice is >90% in females and approximately 60% in males by 20 weeks of age (11, 31, 32). In addition to the presence of pathogenic autoreactive T cells (33), NOD mice harbor additional immunological defects.

These defects include abnormal maturation of bone marrow-derived antigen presenting cells (34), reduced natural killer (NK) cell (35, 36) and natural killer T (NKT) cell numbers and function (37-40), a pathogenic role for B cells (41-43), the absence of hemolytic complement (44), and defects in regulatory CD4+CD25+ T cells (45, 46). Some of these immunologic abnormalities, such as a defect in NKT cells are believed to be important in the pathogenesis of human type 1 diabetes (47), although this has recently been questioned (48), while a role for others, such as the absence of hemolytic complement, isn’t even associated with diabetes in the mouse (44). Of particular interest is the observation that many of the cell defects associated with the pathogenesis of
diabetes are in the same cell subsets that are important for the induction of transplantation tolerance (Table 1).

**Preventing and curing type 1 diabetes in NOD mice**

Fundamentally, NOD mice have a defect in T cell self-tolerance that appears to be a failure of both central and peripheral tolerance mechanisms. The failure to delete autoreactive thymocytes during development in the thymus has been demonstrated in a number of studies (49-51). Similarly, defects in peripheral T cells have been observed. Activation induced cell death (AICD) induced by *in vivo* administration of superantigen is a classic demonstration of peripheral CD4⁺ T cell tolerance (52, 53). NOD CD4⁺ T cells initially expand, but fail to delete following superantigen treatment (42). Using a model self-antigen and TCR transgenic mice, Sherman and co-workers have demonstrated that NOD CD8⁺ T cells are relatively resistant to the induction of tolerance (54). T cell self-tolerance defects are likely to be controlled by both T cell intrinsic effects (49) as well as defects in other immunological compartments (41, 50). Therefore, preventing type 1 diabetes will require protocols that result in improved central and/or peripheral tolerance, and will need to target T cells both directly and indirectly. An impressive number of strategies have been attempted.

**Prevention of diabetes in NOD mice**

There are numerous (>100) immunosuppressive and immunoregulatory protocols that prevent the spontaneous development of diabetes in NOD mice (11, 28, 31). These include non-specific modulations such as elevated temperature or overcrowded housing, or more specific interventions such as administration of exogenous
cytokines. Caution must be taken with studies documenting protection from diabetes in NOD mice, as the intervention may not translate into an appropriate therapy in humans (23). In many cases, the mechanisms by which these protocols prevent diabetes are unknown, but various hypotheses have been put forward. These include the skewing of cytokine polarization from a Th1 to a Th2 phenotype (55), the induction of regulatory T cells (T_{reg}) (56) and the maturation of antigen-presenting cells (50, 57-59).

**Prevention of autoimmune recurrence in NOD mice**

Remarkably, the prevention of autoimmune recurrence in NOD mice has been quite poor in spite of the numerous treatments that prevent the spontaneous onset of diabetes. To date, only chronic administration of immunosuppressive drugs (60, 61), treatment with anti-CD3 antibody (62), or the establishment of hematopoietic chimerism (63) prevent both autoimmune recurrence and the rejection of foreign tissues.

**Induction of central tolerance by establishment of hematopoietic chimerism**

The establishment of hematopoietic chimerism has the potential to both prevent autoimmune diabetes and to establish tolerance to tissue allografts of the same donor type as the bone marrow graft (9, 64). The mechanism of action is thought to involve the elimination of autoreactive and/or alloreactive thymocytes during development (65, 66). Work by Serreze (32, 67) and others (68) has demonstrated the powerful ability of a protective MHC haplotype on bone marrow-derived antigen-presenting cells in the thymus to mediate the elimination of self-reactive T cells and to prevent the spontaneous onset of type 1 diabetes in NOD mice. Similar results have been observed when
employing chimerism to establish transplantation tolerance. Seung et al. were able to induce donor-derived chimerism with sublethal irradiation, a brief course of anti-CD154 mAb and a single hematopoietic graft (63). This protocol in combination with an allogeneic islet graft of the same donor strain can permanently reverse diabetes in both chemically induced diabetic non-autoimmune prone strains and in spontaneously autoimmune diabetic NOD mice. In related studies, Sykes and co-workers have established allogeneic hematopoietic chimerism in autoimmune diabetic NOD mice with depleting antibodies against CD4, CD8, Thy1.2 plus a blocking anti-CD154 mAb followed by sublethal conditioning (69). To date, the establishment of allogeneic hematopoietic chimerism is the most potent form of transplantation tolerance that can be achieved.

A major goal for translating hematopoietic chimerism to the clinic is to develop protocols that reduce or eliminate the need for host myeloablative conditioning. In animal models, the most promising approaches to achieve this goal are based on the use of costimulation blockade. Chronic administration of anti-CD154 mAb and repeated hematopoietic cell injections is sufficient to establish mixed chimerism in non-autoimmune prone strains (70). Although low levels of chimerism were obtained, recipients were able to maintain the long-term survival of matched allogeneic cardiac grafts. More recently, researchers have developed a protocol for establishing mixed allogeneic chimerism using a short course of anti-CD154 mAb in combination with CTLA-4 Ig (to block the B7:CD28 costimulatory pathway) with a conditioning regimen of busulfan (71). Another non-myeloablative protocol based on costimulation blockade
uses a very high dose of bone marrow (65). However, to date, translation of these protocols to the NOD mouse have not been reported. We speculate that this is the result of the resistance of NOD mice to the induction of peripheral transplantation tolerance (12, 72), which we hypothesize is critical for the establishment of hematopoietic chimerism using costimulation blockade. Based on the work in NOD mice, future studies using hematopoietic chimerism to prevent autoimmune recurrence and to induce transplantation tolerance should take into account the MHC haplotype of the stem cell donor. This will be important in both the induction of donor-specific transplantation tolerance as well as the engraftment of cells expressing protective class I and class II molecules that eliminate autoreactive T cells and prevent diabetes recurrence (32, 67, 68).

**Induction of peripheral tolerance in NOD mice**

There are a number of approaches for the establishment of peripheral transplantation tolerance that are successful in normal, non-autoimmune mice (9). However, most of these approaches have failed when translated to the NOD mouse. We have hypothesized that NOD mice have a generalized defect in their response to transplantation tolerance induction. NOD mice reject both allogeneic islet and skin grafts when treated with therapies that induce prolonged or even permanent survival in non-autoimmune strains (12). The cellular basis for the resistance of NOD mice to transplantation tolerance induction is thought to be a consequence of their autoimmunity. As discussed below, there are multiple immune defects that may be involved in each of these two phenotypes; autoimmunity and resistance to transplantation tolerance.
Cellular mechanisms that mediate resistance to transplantation tolerance and expression of autoimmunity in NOD mice

The current understanding of the resistance to transplantation tolerance induction in NOD mice is that they are: 1) controlled by the same genetic pathways; 2) mediated by the same cellular abnormalities, and/or; 3) a function of the ongoing autoimmune condition (Figure 2). Unfortunately, there has been little evidence that supports or refutes these assumptions because the resources necessary to resolve these issues have not been available. These hypotheses seem attractive, because as highlighted in Table 1 and Table 2, a number of genetic and cellular defects that could be important in the induction of transplantation tolerance and susceptibility to type 1 diabetes appear to be shared.
CURRENT VIEW

Idd Loci

↓

Cellular Abnormalities

↓

Autoimmune Diabetes

↓

Transplantation Tolerance Defect
Legend to Figure 2: Schematic diagram depicting the contribution of genetic and cellular defects to autoimmunity and resistance to transplantation tolerance in NOD mice. Represented is the conventional view that the Idd loci are responsible for a set of cellular immunological defects that cause both autoimmune diabetes and resistance to transplantation tolerance. These cellular abnormalities with a single genetic origin could cause transplantation tolerance resistance either directly or as a secondary consequence of the expression of autoimmune diabetes.
The role of CD4+ T cells in autoimmune diabetes and transplantation tolerance

MHC class II restricted CD4+ T cells, also known as "helper" T cells (Th) are central to the generation of both humoral and cell-mediated adaptive immune responses (73). This function is achieved through the expression of secreted cytokines as well as cell surface co-stimulatory molecules. CD4+ T cells are the primary cell type that expresses CD154, the ligand for CD40, an important B cell and APC activation receptor (74, 75). Cytokines secreted by CD4+ T cells control the type of immune response by activating specific cell types. Classically, this response has been divided into two broad categories: Th1 and Th2 (76). A Th1 cytokine profile is characterized by secretion of IFNγ, IL-2 and TNF-β and primarily activates cell-mediated responses such as DTH and CTL. A Th2 profile mediates humoral responses and activates eosinophils by secreting IL-4, IL-5 and IL-10 cytokines. The CD4+ T cell cytokine profile has been hypothesized to be important in the immunopathogenesis of a number of diseases, including type 1 diabetes (55).

Role in type I diabetes in NOD mice

Pathogenic CD4+ T cells, restricted to the unique I-Aβ7 MHC class II molecule, have been shown to be important in disease progression in NOD mice. Indeed, CD4+ T cells isolated from overtly diabetic NOD mice can adoptively transfer disease (77-80). However, they are probably not the cell type that initiates disease in the intact NOD host, but develop at about the time of disease onset (11, 81).
Regulatory CD4+CD25+ T cells, a cell type important in the control of peripheral self-tolerance, appear to be defective in NOD mice. Their role in the progression of the autoimmune process was elegantly documented using a stock of NOD mice deficient in both CD80 (B7.1) and CD86 (B7.2) (45). CD80/CD86 deficient NOD mice develop a rapid and severe autoimmune condition and have a paucity of CD4+CD25+ T cells. Additional evidence for the role of regulatory CD4+CD25+ T cells in autoimmunity has come from studies using anti-CD3 antibody. Chatenoud and colleagues (62, 82) used low doses of anti-CD3 mAb to reverse spontaneous diabetes and autoimmune recurrence in NOD mice. Herold et al. have treated recently diabetic humans with a humanized, non-depleting anti-CD3 mAb (83). In these studies, treatment with anti-CD3 mAb immediately after the diagnosis of type 1 diabetes is able to prolong the "honeymoon" period where patients revert into an insulin-independent state. Recent reports have begun to shed light on the mechanism of action of anti-CD3 mAb in mice and humans. These studies use newer, modified versions of non-mitogenic anti-CD3 antibodies that cause a Th1 to Th2 shift in cytokine production. This cytokine shift is believed to be protective from diabetes and induce an immunoregulatory T cell population (84, 85).

Role in transplantation tolerance

CD4+ T cells are also important in the survival of allografts after transplantation tolerance induction. CD4+ T cells can recognize alloantigens via two mechanisms: direct recognition of the allo-MHC on the graft or indirect presentation of alloantigens captured and processed by host APCs then subsequently presented in the context of self-MHC class II molecules (9). Recognition of alloantigen by CD4+ T cells likely plays a role in
targeting a graft for destruction or alternatively, maintaining tolerance to the tissue. In support of a role for CD4+ T cells in allograft survival, depletion of CD4+ T cells during the maintenance phase of allograft survival causes rapid rejection (26). Furthermore, mice genetically deficient in CD4+ T cells are completely resistant to costimulation blockade-based tolerance induction protocols (27). In a series of ex vivo studies, alloantigen tolerance induced by costimulatory blockade was maintained by CD4+CD25+ T cells (86). In a recent report, peripheral transplantation tolerance based on costimulation blockade was shown to be dependent on a population of CD4+CD25+ T cells that regulated the activity of CD154-independent CD8+ T cells (87). It will be important to determine if defects in NOD CD4+ T cells also mediate their resistance to transplantation tolerance. Experiments in this dissertation begin to address this point.

The role of CD8+ T cells in autoimmune diabetes and transplantation tolerance

CD8+ T cells or cytotoxic T lymphocytes (CTL), are the cytolytic arm of an adaptive cell-mediated immune response (73). They have the ability to directly lyse a target cell based on recognition of peptides presented in the context of MHC class I molecules. Upon receiving help from CD4+ T cells, naive CD8+ T cells are activated and release perforin and granzymes, two types of cytotoxic granules that damage the cell membrane leading to cytolysis. Additionally, CTLs express the membrane bound molecule CD95 (Fas ligand), which can lead to target cell destruction via capsase-induced apoptosis on CD90 (Fas)-expressing cells (88). An important quality of CD8+ T cells is the generation of memory CTL after an immune challenge (89). Memory CTL
have the ability to respond to their specific antigen in the absence of CD4+ T cell help and rapidly mount a secondary immune response. CD8+ T cells are important in the elimination of virus infected cells and tumor cells and are often a target to induce immunity via vaccination (90).

*Role in type 1 diabetes in NOD mice*

The importance of CD8+ T cells in the pathogenesis of type 1 diabetes was long overlooked, in part because the NOD mouse expresses the common MHC class I molecules K\(^d\) and D\(^\beta\), in contrast to the rare MHC class II molecule I-A\(^\beta\) (33). However, in an elegant series of experiments conducted by DiLorenzo and coworkers, it was demonstrated that the earliest islet-infiltrating cells are CD8+ T cells and these cells are required for all but the end stages of disease progression (81). More recently, autoreactive CD8+ T cell clones have been described, many of which have a conserved TCR V\(\alpha\) chain gene rearrangement (81, 91-93). A number of these autoreactive CD8+ T cell specificities can cause diabetes independent of CD4+ T cell help (94, 95).

*Role in transplantation tolerance*

CD8+ T cells are capable of rejecting an allograft by both cytotoxic granules and FasL-mediated mechanisms (9). Direct recognition of the allo-MHC expressed on the transplanted tissue is probably the major mechanism by which CTLs target a graft for destruction. As described below, elimination of alloreactive CD8+ T cells from the circulation appears to be a crucial step in establishing transplantation tolerance in a number of costimulatory blockade protocols (26, 96, 97).

In the unique situation of islet transplantation into autoimmune diabetics, it is
unknown if both autoreactive and alloreactive CTLs will respond equally well to tolerization strategies. Presumably, both types of CTLs must be targeted to cure type 1 diabetes by this strategy.

The role of T cell death in autoimmune diabetes and transplantation tolerance

Although the function of T cells in autoimmune diabetes and transplantation tolerance induction was described above, special consideration should be paid to T cell death. T cells undergo apoptosis at many stages of their life cycle. It is important for the cessation of the immune response and the ability of T cells to respond appropriately to "death" signals is crucial for the proper regulation of immune homeostasis.

Role in type 1 diabetes in NOD mice

Abnormal responses of peripheral T cells in NOD mice have been described and implicated in the pathogenesis of type 1 diabetes. For example, resistance of CD4+ T cells in NOD mice to activation induced cell death (AICD) after superantigen treatment has been demonstrated (42, 52, 53). Also, resistance to T cell apoptosis in NOD mice has been attributed to disregulated Bcl-x expression and has been implicated in disease pathogenesis (98). Not surprisingly, NOD mice with a ongoing, smoldering autoimmune process have elevated levels of IL-2R+ T cells in their periphery, and these activated cells are believed to be important in autoimmune diabetes (99). Therapies focused on elimination of these activated T cells. A cytolytic IL-2/Fc fusion protein that selectively targets activated T cells has been shown to block autoimmunity in NOD mice (100). Similarly, a combination therapy of the immunosuppressive sirolimus plus the
administration of IL-2 has been shown to induce apoptosis in NOD T cells, lead to a Th1 to Th2 cytokine profile shift, and most importantly, prevent spontaneous and recurrent autoimmunity (60).

*Role in transplantation tolerance*

Strategies that induce autoreactive T cell death to restore self-tolerance have also been applied to the induction of allo-specific tolerance (97). For example, therapies that block signals necessary for apoptosis also prevent the induction of peripheral transplantation tolerance (101, 102). Also, our laboratory has shown a requirement for the elimination from the periphery of both alloreactive CD8\(^+\) (26, 96) and CD4\(^+\) T cells (unpublished observations) in tolerance induced by DST plus anti-CD154 mAb, presumably by inducing apoptosis of the activated alloreactive T cells.

*The role of natural killer and natural killer T cells in autoimmune diabetes and transplantation tolerance*

Natural killer (NK) and natural killer T (NKT) cells are two related cell types that bridge the innate and adaptive immune response. NK cells were originally described based on their ability to lyse tumor and virus infected cells without prior activation or T cell help (73). Their effector mechanisms are similar to CTLs, in that they utilize perforin and granzymes, but they do not express a TCR or CD3 complex. Instead, NK cells express a complex set of receptors, both activating and inhibitory. The summation of signals through these receptors determines whether or not an NK cell will kill a target cell (103). NK cells can also mediate antibody dependent cellular cytotoxicity (ADCC) of antibody coated targets through the expression of Fc receptors on their cell surface.
Additionally, NK cells secrete IFN$\gamma$ during the early phase of an immune response, thereby activating T cells (104).

NKT cells share characteristics of both NK cells and T cells. They express a number of NK cell surface proteins, but also express T cell membrane receptors, including the TCR/CD3 complex (73). A major subset of mouse NKT cells are restricted to the non-classical MHC class I molecule, CD1d and have a heavily biased TCR rearrangement (105). CD1 molecules present glycolipid antigens, and the marine sponge-derived glycolipid $\alpha$-galactosylceramide ($\alpha$GalCer) that also binds CD1 is a strong NKT cell agonist. The effector functions of NKT cells are characterized by the rapid secretion of the cytokines IL-4 and/or IFN$\gamma$ early in an immune response and this is believed to be one mechanism by which the adaptive arm of the immune system is turned on (106).

**Role in type 1 diabetes in NOD mice**

NOD mice have deficiencies in NK cell number and function (35, 36, 107). However, a role for NK abnormalities in the pathogenesis of diabetes in this strain has been difficult to determine, in part because NOD mice are NK1.1$^{null}$ at the NKRP-1 complex, making the identification and analysis of both NK and NKT cells difficult. The congenic introgression of a resistance allele at $Idd6$, which harbors genes important in NK and NKT cell function, including the NK1.1 allele at the NKRP-1 complex, improves NK and NKT cell function in NOD mice and renders them modestly protected from diabetes (36).
NKT cells have been hypothesized to be important in the pathogenesis of type 1 diabetes in both humans (47) and NOD mice (37-40). As such, many investigators have been attempting to determine the consequences of the NKT cell defect in NOD mice and then develop strategies that improve function of this subset of cells. The importance of NKT cells in the progression of autoimmune diabetes has been demonstrated by genetic disruption of CD1 in NOD mice, which results in the absence of CD1-restricted invariant NKT cells (38, 108). In these mice, an accelerated onset and increased prevalence of disease is seen which is associated with an increase in the numbers of activated and memory T cells. Understandably, a number of investigators have attempted to exploit the function of NKT cells in hopes of restoring self-tolerance and preventing the autoimmune attack. Administration of the NKT cell receptor agonist αGalCer dramatically reduces the spontaneous onset of diabetes in NOD mice to 10% to 40% (vs. ~90% in untreated NOD mice) (38, 109, 110). Furthermore, αGalCer treatment also reduces the severity of insulitis and can prevent autoimmune recurrence in overtly diabetic mice given syngeneic islet grafts. The mechanism of action of this treatment is not completely known, but a shift to a Th2 type cytokine profile as well as an accumulation of NKT cells in the pancreatic lymph nodes that regulate tolerogenic myeloid dendritic cells (57, 110, 111) have been implicated.

Role in transplantation tolerance

NK cells likely have only a minor role in the survival of tissue allografts in tolerized recipients. NK cells participate in graft versus host disease (GVHD) (112) as well as in the rejection of hematopoietic grafts (113). Interestingly, human NK cells
express CD154 and could be one target of treatment with DST and anti-CD154 mAb (114).

A role for NKT cells in transplantation tolerance induction has also been suggested. NKT cell deficient mice rapidly reject corneal allografts and this correlates with an absence of regulatory NKT cells in the anterior chamber of the eye (115). Others have demonstrated the importance of NKT cells in cardiac and skin allograft models (116). Mice deficient in NKT cells are resistant to tolerance induced by anti-LFA-1 plus anti-B7.1/2 mAbs, and this phenomenon was dependent on IFNγ production by NKT cells (117). It is currently uncertain if the defects in NK and NKT cells in NOD mice mediate their underlying resistance to transplantation tolerance.

**The role of B cells in autoimmune diabetes and transplantation tolerance**

B cells are the effector cells of humoral immunity, secreting antibodies in response to a variety of extracellular challenges (73). Activation of B cells occurs through the B cell receptor (BCR), which consists of a membrane-bound immunoglobulin associated with two Ig-α/Ig-β heterodimers. B cells express a co-receptor complex as well as CD40, which help achieve full activation. Upon activation, B cells undergo class switching and affinity maturation of immunoglobulin genes, resulting in high affinity, antigen-specific antibody responses. Additionally, activated B cells express MHC class II and B7 molecules and can function as antigen presenting cells to T cells, efficiently presenting antigenic determinants that are specific for the BCR (118).

*Role in type 1 diabetes in NOD mice*
An important role of B cells in diabetes in NOD mice has been demonstrated. B cell deficient NOD mice are completely protected from disease (41). Antibodies against the putative diabetes autoantigens, GAD and insulin, can be detected in NOD mice, but the importance of autoantibodies in diabetes pathogenesis is not well understood. One pathogenic role of B cells that has been described is to function as antigen presenting cells, specifically by capturing islet antigens via the BCR and subsequently presenting antigenic determinants on MHC class II molecules (119).

Role in transplantation tolerance

Antibodies potentially can play a role in graft rejection. Natural antibodies in part mediate hyperacute rejection of xenografts, while the generation of antibody responses against allo-MHC antigens from prior blood transfusions or a previous allograft can impact the outcome of a tissue transplant, perhaps by targeting it for ADCC (9).

With respect to the induction of transplantation tolerance, the role of B cells is not well understood. In our two element protocol, B cells are present in the DST and presumably anti-CD154 mAb blocks the activation and subsequent upregulation of costimulatory molecules on these cells. Others have reported that host B cells must be tolerized to alloantigens and that administration of soluble antigen (120), anti-CD45 (121) and anti-CD154 mAbs (122, 123) can be used to achieve this.

The role of antigen presenting cells in autoimmune diabetes and transplantation tolerance

Antigen presenting cells are mediators of self-tolerance and immunity (124). They are not one specific cell, but rather a collection of cell types that function to activate
effector T cell responses. Dendritic cells of both lymphoid and myeloid origin function as “professional” APCs, but a number of other cell types, including macrophages and B cells also have the ability to efficiently activate T cell responses. The hallmarks of APC function are the ability acquire antigen and then process and present it in the context of MHC class I and class II molecules to T cells, while expressing costimulatory molecules such as B7 family members to allow full T cell activation (73). Additionally, cytokine secretion by APCs serves to further modulate T cell responses.

Role in type 1 diabetes in NOD mice

NOD mice have well-documented abnormalities in various APC compartments that are believed to be important in the expression of autoimmune diabetes (125). Individual consideration was given to B cells above, while two other cell populations are described below.

Defective maturation of NOD macrophages cultured from bone marrow progenitors in the presence of CSF-1 has been determined to be due to defective regulation of the CSF-1 receptor. Additionally, those macrophages that do mature respond abnormally to mitogenic stimulation and produce low levels of IL-1β compared to macrophages derived from other strains. Defective IL-1β secretion has been implicated in the pathogenesis of autoimmune diabetes in NOD mice (126-128).

Dendritic cells have been studied extensively in NOD mice and various abnormalities have been described ranging from impaired maturation (129, 130) to hyper-activation (131, 132). One defect observed from ex vivo isolated NOD dendritic cells is low CD86 expression which is hypothesized to result in impaired self-tolerance due to a
failure to upregulate CTLA-4 on CD4+ T cells (133). Furthermore, the adoptive transfer of syngeneic freshly isolated DCs, peptide-pulsed DC, or in vitro cultured bone marrow-derived CD86+ dendritic cells prevents diabetes in NOD mice. This protection is likely due to the induction of a regulatory cell population (57, 58, 134, 135). Similar to the effects on DC maturation seen after administration of αGalCer, stimulation of NOD DC ex vivo with IFNγ before adoptive transfer results their accumulation in the pancreatic lymph nodes and the prevention of diabetes (136).

Role in transplantation tolerance

An important role for APCs in the induction of transplantation tolerance may seem obvious, but in the case of B cells (as mentioned earlier) and macrophages, the relationship remains poorly understood. Mixed lymphocyte cultures carried out while blocking B7/CD28 interactions results in the generation of macrophages that can suppress T cell activation, implicating a potential tolerogenic role for macrophages in tolerance induced by costimulatory blockade (137). In a study from our group, the NOD macrophage maturation defect was shown to correlate with this strain’s generalized resistance to transplantation tolerance induction (12).

The use of “tolerogenic” dendritic cells is an appealing approach for the induction of transplantation tolerance. Numerous transplantation protocols utilize anti-CD154 mAb, an intervention believed to block the T cell dependent maturation of DC, rendering them “immature” or “tolerogenic.” This phenomenon can be mimicked by co-culture of T cells with recipient-origin immature DC pulsed with allogeneic peptides, a system which tolerizes T cells (138). Additionally, DC that have captured apoptotic debris are believed
to be tolerogenic (139) and the injection of apoptotic cells improves the acceptance of allogeneic bone marrow grafts, presumably through the generation of tolerogenic DC (140). Finally, others investigators have utilized gene therapy or other *in vitro* manipulations to generate donor-derived DC expressing tolerogenic molecules such as IL-10, TGFβ, CTLA4 Ig or FasL to induce transplantation tolerance (141-144).

**The importance of considering the cellular mechanisms of autoimmune diabetes and transplantation tolerance together**

There are many cellular immune abnormalities that are important in the progression of autoimmune diabetes in NOD mice. A number of these cell types are also believed to be important in the induction of transplantation tolerance (Table 1). It is interesting to speculate that the cellular defects that result in autoimmune diabetes in NOD mice also render it resistant to transplantation tolerance induction. To investigate this possibility, an understanding of the genetic basis of these two phenotypes must be obtained.
Table 1

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>Defects in NOD Mice</th>
<th>Role in Transplantation Tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effector CD4⁺ and CD8⁺ T cells</td>
<td>Abnormal deletion of activated T cells</td>
<td>Deletion required for transplantation tolerance</td>
</tr>
<tr>
<td>CD4⁺(CD25⁺) regulatory T cells</td>
<td>Abnormal function</td>
<td>Required for transplantation tolerance</td>
</tr>
<tr>
<td>NKT cells</td>
<td>Defective cytokine secretion and ability to mature dendritic cells</td>
<td>Suggested to be important in transplantation tolerance</td>
</tr>
<tr>
<td>NK cells</td>
<td>Defective, not required for expression of diabetes</td>
<td>Role in transplantation tolerance unknown</td>
</tr>
<tr>
<td>Dendritic Cells</td>
<td>Abnormal maturation</td>
<td>Tolerogenic dendritic cells important in tolerance induction, required for induction of regulatory T cells</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Abnormal maturation</td>
<td>Role in transplantation tolerance unknown</td>
</tr>
<tr>
<td>B Cells</td>
<td>Required as APCs for expression of diabetes; secrete autoantibodies</td>
<td>Suggested to be important in transplantation tolerance</td>
</tr>
</tbody>
</table>
Legend to Table 1: Immune cells hypothesized to be important in the pathogenesis of diabetes and in the induction of transplantation tolerance.
Genetic basis of autoimmune diabetes in NOD mice and the induction of transplantation tolerance

Idd loci and autoimmunity

The immunogenetics of autoimmune diabetes in NOD mice has revealed no fewer than 27 diabetes susceptibility loci (termed Idd loci) on 15 chromosomes of the mouse genome (11, 32). The vast majority of diabetes susceptibility alleles are derived from the NOD, but it is important to note that control strains harbor susceptibility alleles and that the NOD harbors some diabetes resistance loci (11). Many NOD Idd loci have human orthologues, including the most powerful susceptibility loci in mice, Idd1 which maps to the major histocompatibility complex (MHC) (11, 145). Indeed, NOD mice express a rare MHC class II molecule, I-A^K that is very similar to non-asp57 class II alleles that map to IDDM1 in humans (146-148). Other non-MHC Idd susceptibility loci have been well characterized and include candidate genes that are important in immune function (145). Determining the actual pathogenic genes in a given locus has proven difficult, and to date, β2 microglobulin (β2m) is the only non-MHC gene that has been definitively shown to confer diabetes susceptibility or resistance (149). Other candidate genes include Il2 (interleukin-2 gene), Cd152 (CTLA-4), Casp8 (caspase 8 gene), Ins-1 (insulin gene) and others (145). Currently, identifying candidate genes is essentially a process of elimination by continued narrowing of congenic intervals containing resistance alleles by extensive backcrossing, with transgenic rescue or gene “knock-in” technology providing the only definitive proof of the function of the gene (150). The sequencing of the mouse genome
and will aid in the identification of the actual diabetogenic genes and their products within each Idd interval.

It is reasonable to think that genes in Idd intervals that mediate expression of diabetes might also control the response to transplantation tolerance induction in NOD mice. As summarized in Table 2, there are a number of candidate genes within Idd loci that either have been shown to be or could be important in transplantation tolerance induction.

**The role of the genetic background on transplantation tolerance induction**

Very few data are available on the potential effects that genetic background plays in a strain’s response to transplantation tolerance induction. Indeed, it is likely that an examination across many inbred strains will demonstrate varying responses to costimulation blockade transplantation tolerance induction. In agreement with this, Larsen, Pearson and co-workers have observed such a phenomenon (151). Their protocol induces prolonged allograft survival by simultaneous blockade of CD40 and CD28 signaling. This treatment is effective in C3H/HeJ recipients, but not C57BL/6 recipients. Using a series of recombinant inbred strains between C3H/HeJ and C57BL/6, they observed a number of linked markers that associated with allograft survival in mice treated with costimulatory blockade.

**The role of the MHC in tolerance induction**

A genetic background effect that is likely to be important is the MHC haplotype of the recipient and its interaction with the donor haplotype. The NOD H2^e^ haplotype is neither necessary nor sufficient to result in resistance to transplantation tolerance (12),
but it seems possible that it may impact an animal’s response to tolerance induction. Indeed, from the results of Markees and coworkers it can be conjectured that the $H2^{b7}$ haplotype diminishes the survival of allografts on C57BL/6.$H2^{b7}$ mice (i.e. ~75 days vs >100 for wild type C57BL/6). Furthermore, it has recently been shown that the donor/recipient pairing is important in NOD recipients of full and partial MHC mismatched grafts. When transplanting cardiac allografts, better matching of the donor MHC improves survival in NOD recipients. However, when transplanting islets (i.e. the target of the autoimmune attack), better matching of the donor MHC actually worsened graft survival, likely because of autoimmune recurrence in addition to the allospecific response (152).
Table 2

<table>
<thead>
<tr>
<th>Locus</th>
<th>Candidate Genes</th>
<th>Candidate Genes in Transplantation Tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idd1</td>
<td>MHC class II and class I genes</td>
<td>Unknown; may confer resistance</td>
</tr>
<tr>
<td>Idd3</td>
<td>Il2, Fgf, Il21</td>
<td>Il2</td>
</tr>
<tr>
<td>Idd5.1</td>
<td>Cd152 (CTLA-4), Cd28</td>
<td>Cd152, Cd28</td>
</tr>
<tr>
<td>Idd5.2</td>
<td>Nramp1, Cmcar2</td>
<td>Unknown</td>
</tr>
<tr>
<td>Idd6</td>
<td>Nkrp1</td>
<td>Unknown</td>
</tr>
<tr>
<td>Idd9.1</td>
<td>Lck</td>
<td>Lck</td>
</tr>
<tr>
<td>Idd9.2</td>
<td>Tnfr2, Cd30</td>
<td>Tnfr2</td>
</tr>
<tr>
<td>Idd9.3</td>
<td>Cd137 (4-1BB)</td>
<td>Cd137</td>
</tr>
<tr>
<td>Idd10</td>
<td>Csfr, Cd53, Kcna2, Nras, Rap1a</td>
<td>Unknown</td>
</tr>
<tr>
<td>Idd13</td>
<td>B2m (β2 microglobulin)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Idd16</td>
<td>Proximal to MHC class I K gene</td>
<td>Unknown</td>
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<td>Idd17</td>
<td>Unknown</td>
<td>Unknown</td>
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<tr>
<td>Idd18</td>
<td>Csfr, Cd53, Kcna3, Nras, Rap1a</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
Table 2: Partial list of Idd loci, candidate genes within the Idd loci, and their relationship, if known, in transplantation tolerance.
Thesis goals and specific aims

The NOD mouse continues to be the model of choice for developing therapies to cure type 1 diabetes, including islet transplantation. However, the basis for this strain’s resistance to costimulation blockade-based transplantation tolerance induction protocols remains elusive. Determining if this resistance is unique to NOD mice or alternatively represents a condition that might be found in human type 1 diabetics has important consequences for the study of transplantation tolerance in this mouse model of autoimmune diabetes. To better understand this problem, three specific aims were addressed:

Specific aim 1: Tests the hypothesis that the genetic loci that mediate expression of diabetes also control resistance to transplantation tolerance in NOD mice. Chapter I is comprised of the data testing this hypothesis.

Specific aim 2: Hypothesizes that increasing the “threshold” of self-tolerance will improve the response to transplantation induction in (NOD x C57BL/6)F1 mice. The results of experiments exploring this hypothesis are presented in Chapter II.

Specific aim 3: To identify the genetic intervals mediating resistance to transplantation tolerance in NOD mice. The approach for this aim is a backcross genome wide scan using quantitative trait linkage (QTL) analysis to identify loci that control skin allograft survival on mice treated with DST and anti-CD154 mAb. The data are presented in Chapter III.

It should be pointed out in advance that the majority of transplantation experiments reported in this dissertation are skin grafts. This is done for both practical
and scientific reasons. In a practical sense, skin grafting is less costly, less labor intensive and graft survival is easier to monitor. Scientifically, skin is considered to be the most stringent test of allotolerance, because of its potent immunogenicity. Therefore, if a tolerance induction protocol can achieve prolonged skin allograft survival, it is likely that other transplanted tissues will succeed with the same protocol.
METHODS

Animals

C57BL/6 (H2b), C3H/HeJ (H2b), and BALB/c (H2d) mice were obtained from the National Cancer Institute (Frederick, MD). NOD/MrkTacfBR, NOD.B6 Idd3R450, NOD.B10 Idd5R444, NOD.B6 Idd3R450 B10 Idd5R8, NOD.B10 Idd9R28, NOD.B6 Idd10Idd18R2, NOD.B6 Idd3Idd10Idd18R323 (all H2g7) and C57BL/6NTac (H2b) were obtained from Taconic Farms (Germantown, NY). NOD/Lt (H2g7) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6.NODc1c, C57BL/6.NODc1t, C57BL/6.NODc3 and C57BL/6.NODc6 mice (H2b, 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). C57BL/6.NODc17 mice (H2g7, developed by Edward Wakeland) were the gift of Dr. David Serreze (The Jackson Laboratory, Bar Harbor, ME) and are hereafter referred to as C57BL/6.H2g7. Table 3 summarizes the congenic interval(s) and diabetes incidence of each of the congenic mouse strains listed above that were used in these experiments. B10.D2-HC0 (genetic designation: B10.D2-H2d H2-T188 Hc0/cSnJ) and B10D2-HC1 mice (genetic designation: B10.D2-H2d H2-T188 Hc1/nSnJ) were obtained from The Jackson Laboratory. C57BL/6.CD8α cH2 (H2b, official designation: C57BL/6.129S2-Cd8a tm1Mak) were obtained from The Jackson Laboratory (Bar Harbor, ME). NOD.CD8α cH2 mice (H2g7, official designation: NOD.129S2(B6)-Cd8a tm1Mak/DVS) were the gift of Dr. David Serreze. This stock has the CD8α cH2 mutation introduced by congenic transfer and have been backcrossed to the N9 generation. NOD.CD8α cH2 mice are homozygous for linkage
markers delineating all known Idd loci of NOD origin and are very strongly protected from diabetes (1/17 females diabetic by 30 weeks of age).

To generate first filial (F1) generation progeny, a single outcross was performed and the resulting hybrids are designated with standard nomenclature: (mother x father)F1. The F1 progeny used in these studies include: (NOD x C57BL/6)F1, (C57BL/6 x NOD)F1 and (NOD.CD8α−/− x C57BL/6.CD8α−/−)F1 (all H2d2d); (NOD x C57BL/6.H2g7)F1, (NOD.B6 Idd3 R450 B10 Idd5 R8 x C57BL/6.H2g7)F1 and (NOD.B10 Idd9 R28 x C57BL/6.H2g7)F1 (All H2g7). To generate N2 generation animals homozygous for the H2g7 haplotype, (NOD x C57BL/6.H2g7)F1 females were backcrossed to a C57BL/6.H2g7 male and the resulting progeny are designated (NOD x C57BL/6.H2g7)F1 x C57BL/6.H2g7 (H2g7).

All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, LDH 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 (IACUC) 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).
<table>
<thead>
<tr>
<th>Congenic Strain</th>
<th>Chromosomal Location</th>
<th>Insulitis</th>
<th>Diabetes</th>
<th>Partial List of Candidate Genes</th>
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</thead>
<tbody>
<tr>
<td>NOD.B6 Idd3</td>
<td>Chr. 3</td>
<td>70%</td>
<td>20%</td>
<td>II2, II-21, Fgf2,</td>
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<tr>
<td>NOD.B10 Idd5 (Idd5.1 and Idd5.2)</td>
<td>Chr. 1</td>
<td>78%</td>
<td>40%</td>
<td>Idd5.1: Cd152, Cd28, Casp8, Flip; Idd5.2: Cxcr2, Il-8r</td>
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<tr>
<td>NOD.B6 Idd3 B10 Idd5</td>
<td>See Idd3/Idd5 above</td>
<td>10%</td>
<td>1%</td>
<td>(see Idd3 and Idd5 above)</td>
</tr>
<tr>
<td>NOD.B6 Idd10Idd18</td>
<td>Chr. 3</td>
<td>50%</td>
<td></td>
<td>Csfm1, Cd53, Kcna3, Nras, Rap1a</td>
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<tr>
<td>NOD.B6 Idd3Idd10Idd18</td>
<td>Chr. 3</td>
<td>19%</td>
<td>9%</td>
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<tr>
<td>NOD.B10 Idd9</td>
<td>Chr. 4</td>
<td>90%</td>
<td>3%</td>
<td>Cd30, Cd137, Tnfr2</td>
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<tr>
<td>C57BL/6.NODelt</td>
<td>Chr. 1</td>
<td>0%</td>
<td>0%</td>
<td>(see Idd5.1 and Idd5.2 above)</td>
</tr>
<tr>
<td>C57BL/6.NODelc</td>
<td>Chr. 1</td>
<td>0%</td>
<td>0%</td>
<td>(see Idd5.1 above)</td>
</tr>
<tr>
<td>C57BL/6.NODc3</td>
<td>Chr. 3</td>
<td>0%</td>
<td>0%</td>
<td>(see Idd3, Idd10, Idd18 above); Idd17</td>
</tr>
<tr>
<td>C57BL/6.NODc6</td>
<td>Chr. 6</td>
<td>0%</td>
<td>0%</td>
<td>nkrp1 (NKR-P1 complex)</td>
</tr>
</tbody>
</table>
Legend to Table 3: Congenic strains, chromosomal location, frequency of diabetes and insulitis and candidate genes in each interval. The specific genetic intervals and a more complete listing of candidate genes contained within each interval can be found in (153-157). ¹Percent of females scoring positive for moderate (10% to 50% of islets positive for lymphocytic infiltrate) to severe (>50% islets positive for lymphocytic infiltrate) insulitis at 6-8 months of age. ²Diabetes prevalence in females at seven months of age.
Tolerance induction and skin or islet allograft transplantation

Graft recipient mice were treated with DST and anti-CD154 mAb and transplanted with skin or islet allografts as described (27). Briefly, the DST consisted of a single intravenous injection of $10^7$ C3H/HeJ or BALB/c female spleen cells obtained from 5 to 10 week old donors. The DST was injected on day $-7$ relative to skin transplantation. Clone MR1 hamster anti-mouse CD154 mAb (158) was produced as ascites in scid mice and purified by Protein A affinity chromatography (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Antibody concentration was determined by measurement of optical density and confirmed by ELISA as described previously (26). The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC) and was uniformly $<10$ units EU per mg of mAb. Mice were injected intraperitoneally with anti-CD154 mAb (0.25 or 0.5 mg/dose as indicated in text) on days $-7$, $-4$, $0$, and $+4$ relative to skin transplantation. Full thickness skin grafts 1-2 cm in diameter were obtained from the flanks of donor mice and transplanted onto the dorsal flanks of recipients as described (27). Grafts were examined 3 times weekly, and rejection was defined as the first day on which the entire graft surface appeared necrotic (27). Grafts adherent to the bandage or fully necrotic on day 7 were deemed technical failures and were excluded from analysis (27).

Islets were isolated by collagenase digestion followed by density gradient separation as described (12, 63). Handpicked islets (20 islets/gram body weight) were transplanted into the renal subcapsular space of recipients.
Diabetes was induced in male mice by a single intraperitoneal injection of streptozotocin (150 mg/kg). Hyperglycemia was verified by 2 consecutive days of plasma glucose levels >250 mg/dL (Glucose Analyzer 2, Beckman Instruments, Fullerton, CA). Diabetes was induced at least one week before initiating the tolerance induction and transplantation procedures. Rejection of functional islet allografts was monitored by plasma glucose and defined as a return to hyperglycemia (>250 mg/dL) on at least 2 consecutive days.
Figure 3

Recipient

Anti-CD154 mAb

Days: -7

+4

Observe

Spleen Cell DST

Donor

Skin

Donor
Legend to Figure 3: Schematic diagram of transplantation tolerance induction with DST and anti-CD154 mAb. Recipient mice receive a DST consisting of $1 \times 10^7$ donor splenocytes intravenously on day $-7$ relative to transplantation as well as four intraperitoneal injections of 0.5 mg of anti-CD154 mAb on days $-7$, $-4$, 0 and +4 relative to transplantation. A skin allograft is placed on the recipients dorsal flank on day 0, the day of transplantation. After surgery, recipients are monitored periodically for graft survival. Rejection is defined as the first day that the entire graft appears necrotic.
Serum levels of hamster anti-CD154 mAb

Clearance kinetics of the anti-CD154 mAb was determined in groups of NOD and C57BL/6 mice that were given a single intraperitoneal injection of 2.0 mg of antibody. Serum was collected from blood obtained prior to antibody injection and periodically thereafter, up to 40 days after injection. Hamster IgG levels were quantified in the serum samples by ELISA (26).

Flow cytometry

Purified rabbit anti-asialo GM-1 polyclonal antibody was obtained from Wako Chemicals USA (Richmond, VA). FITC-conjugated goat anti-rabbit Ig polyclonal antibody obtained from BD PharMingen (San Diego, CA) was used to visualize cell bound anti-asialo GM-1 antibody. Biotinylated anti-mouse pan-NK cell mAb (clone DX5), FITC-conjugated anti-NKR-P1C mAb (clone PK136), PerCep®-conjugated anti-CD3ε mAb (clone 145-2C11), PE conjugated anti-CD25 mAb (clone PC61), PerCep®-conjugated anti-CD4 mAb (clone RM4-5), FITC-conjugated anti-CD86 mAb (clone GL1), APC-conjugated anti-CD11b mAb (clone M1/70) and PE-conjugated anti-CD11c mAb (clone HL3) were obtained from BD PharMingen. APC-conjugated streptavidin was used to visualize bound DX5 as well as any bound biotinylated rat IgM, κ (clone R4-22) isotype control. Additional isotype controls included PerCep®-conjugated hamster IgG1, κ (clone A19-3), FITC-conjugated mouse IgG2a, κ (clone G155-178), PE-conjugated rat IgM, κ (clone R4-22), PerCep®-conjugated rat IgG2a, κ (clone R35-95), FITC-conjugated rat IgG2a, λ (clone B39-4), APC-conjugated rat IgG2b, κ (clone A95-1)
and PE-conjugated hamster IgG1, λ (clone G235-2356). All isotype controls were obtained from BD PharMingen.

Two and three color flow cytometry analyses of freshly isolated spleen cells or cultured dendritic cells were performed as previously described (96). Briefly, $1 \times 10^6$ viable cells were first 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. In some cases, a third incubation with APC-conjugated streptavidin or FITC-conjugated goat anti-rabbit Ig was performed. Stained cells were washed, suspended in 1% paraformaldehyde-PBS, and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Viable lymphoid cells were gated according to their light-scattering properties, and approximately 2.5 to $5.0 \times 10^4$ events were acquired for each analysis.

**Natural killer cell cytotoxicity assay**

Cytotoxic activity of NK splenic cells was quantified using a previously described $^{51}$Cr-release microcytotoxicity assay (111). NK-sensitive YAC-1 virus-induced mouse T cell lymphoma (159) target cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in our laboratory in RPMI media (Gibco Life Technologies, Gaithersburg, MD) supplemented with 10% Fetal Clone serum (FC, HyClone, Logan, Utah). YAC-1 target cells in growth phase were labeled with $^{51}$Cr as sodium chromate (100 μCi/million cells, New England Nuclear, Boston, MA), and $^{51}$Cr-labeled cells ($1.0 \times 10^4$) were added to each well of a 96 well microtiter plate. Freshly isolated spleen cells obtained from animals injected intraperitoneally with 100 μg
poly I:C 24 hours earlier were added at effector to target (E:T) cell ratios ranging from 100, 50, 25 and 12.5:1 and incubated for 4 hours at 37°C in a humidified atmosphere of 95% air-5% CO2 (160).

Total releasable radioactivity ("cpm_{maximal}") was determined by incubating an aliquot of $^{51}$Cr-labeled target cells with 5% Triton X-100. After incubation, cells were pelleted by centrifugation, and 100 μl aliquots of supernatant were transferred to a separate microtiter plate containing 100 μl Optiphase Supermix β-scintillation fluid (Wallac, Inc. Gaithersburg, MD) and counted ("cpm_{test}") using a 1450 Microbeta Trilux instrument (Wallac). Spontaneous release was uniformly <15% of maximal release. All assays were performed in triplicate and averaged and each assay was performed at least twice. Specific cytotoxicity was calculated as a percentage using the raw counts per minute (cpm) and the formula:

$$\text{Specific Lysis (\%)} = \left( \frac{\text{cpm}_{\text{test}} - \text{cpm}_{\text{spontaneous}}}{\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}}} \right) \times 100\%$$

**Macrophage IL-1β production assay**

Bone marrow-derived macrophages were generated as previously described (128). Briefly, 5 x $10^6$ bone marrow cells were cultured for 4 days at 37°C in RPMI-10% FC plus 500 U/ml human CSF-1 (Sigma, St. Louis, MO) and 10 U/ml rat IFNγ (R & D Systems, Minneapolis, MN). The cells were cultured an additional 16 hours in fresh media containing 10 μg/ml bacterial lipopolysaccharide (LPS, Sigma, St. Louis, MO) in the absence of additional growth factors. Supernatants were harvested and levels of
biologically active IL-1β were quantified in a C3H/HeJ thymocyte co-mitogenic assay as described (128). Data are presented as mean cpm of [\textsuperscript{3}H]Thymidine incorporation.

**Dendritic cell maturation assay**

Bone marrow was flushed from the femurs and tibias of euthanized donors into RPMI-10% FC serum. Erthrocytes were lysed with 0.85% hypotonic NH\textsubscript{4}Cl 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\textsuperscript{5} 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\textsuperscript{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\textsubscript{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 IL-4. On day four, non-adherent cells were removed and fresh medium supplemented with cytokines was added as on day two and 5 μg/ml of an agonist anti-CD40 mAb (clone HM40-3, BD PharMingen) was added to the appropriate wells. Cultures were incubated an additional 48 or 96 hours. After the final 48 hour incubation, all cells (adherent and non-adherent) were harvested and the supernatants were saved for cytokine ELISA. Adherent cells were removed by gentle scraping and pooled with the non-adherent cells. In a separate set of experiments, adherent and non-adherent cells from each well were collected separately. 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.

**Genome-wide scan**

(NOD x C57BL/6.H2\textsuperscript{g7})F1 x C57BL/6.H2\textsuperscript{g7} N2 generation mice were generated by backcrossing female F1s with male C57BL/6.H2\textsuperscript{g7} mice. N2 generation mice as well as (NOD x C57BL/6.H2\textsuperscript{g7})F1 and C57BL/6.H2\textsuperscript{g7} control mice, aged 7-8 weeks were treated with a single DST and four 0.5mg does anti-CD154 mAb as described above, followed by placement of a C3H/HeJ skin allografts and monitored for allograft survival. Upon rejection, liver and tail tissue was collected and frozen immediately on dry ice. Genomic DNA processed from the frozen tissue was used for 2 cM microsatellite mapping at the Jackson Laboratory. Results were analyzed using the R/qtl software package (http://www.biostat.jhsph.edu/~kbroman/qtl).

**Statistics**

Average duration of graft survival is presented as the median. Graft survival among groups was compared using the method of Kaplan and Meier (161). The equality of allograft survival distributions for animals in different treatment groups was tested using the log rank statistic (161). P values <0.05 were considered statistically significant. Comparisons of two means used unpaired t-tests (162). Comparisons of three or more means used one-way analysis of variance and the LSD procedure for *a posteriori* contrasts (163). To determine the half-life of MR1 in sera of treated animals, single-phase exponential decay curves were fitted by regression analysis using Prism software (Version 3.0, Graphpad Software, Inc., San Diego, CA).
CHAPTER I

NOD MICE GENETICALLY PROTECTED FROM TYPE 1 DIABETES REMAIN RESISTANT TO TRANSPLANTATION TOLERANCE INDUCTION

Introduction to Chapter I

Our laboratory has demonstrated a generalized resistance to transplantation tolerance in the NOD mouse. This conclusion was reached because both spontaneously diabetic and prediabetic NOD mice treated with DST and anti-CD154 mAb rapidly reject a skin allograft that is not the target of the autoimmune attack (12). This treatment induces long-term allograft survival in non-autoimmune prone strains (27, 164, 165). It remains unknown, but generally assumed that the generalized defect to transplantation tolerance induction in the NOD mouse is caused by the ongoing autoimmune process and/or controlled by the same genetic (Idd) loci that mediate autoimmune diabetes in this strain (152). To test this hypothesis, we studied various congenic stocks of NOD mice that bear one or a few resistance alleles at powerful non-H2 Idd loci. These mice were tested for their response to transplantation tolerance induction with DST and anti-CD154 mAb. The congenic mice have varying degrees of diabetes expression and some are almost completely protected from disease (Table 3). We reasoned that if NOD-origin Idd loci are also responsible for the generalized defect to transplantation tolerance induction, the congenic introgression of one or a few diabetes resistance alleles from a transplantation tolerance susceptible strain might improve allograft survival. Therefore, if the ongoing condition of autoimmunity is responsible for the defective response to
transplantation tolerance induction, the congenic stocks with a genetic attenuation of the disease may respond better to our transplantation tolerance induction protocol.

In the results presented below, we first eliminated two more trivial explanations for the NOD defect in transplantation tolerance induction: the NOD's absence of hemolytic complement and faster clearance of the anti-CD154 mAb. We showed that tolerance susceptible C57BL/6 mice harboring NOD derived Idd susceptibility alleles responded to tolerance induction identical to the wild type C57BL/6 strain. We then demonstrated that NOD Idd congenic strains that are genetically protected from autoimmune diabetes still remained resistant to transplantation tolerance induction.
Chapter I Results

1a. Costimulation Blockade Prolongs Skin Allograft Survival in Mice Genetically Deficient in C5a and Hemolytic Complement

The NOD mouse has numerous immune defects that may be involved in their resistance to the induction of transplantation tolerance. One defect that could impact this strain’s response to in vivo antibody therapy is the absence of hemolytic complement due to their genetic deficiency in C5a (44). To test this, we attempted to tolerize B10.D2 congenic mice that genetically differ in the presence or absence of C5a (166). These congenic mice were treated with DST, anti-CD154 mAb, and C3H/HeJ skin allografts. As shown in Figure 4A, the genetic absence of C5a in B10.D2-HC0 mice did not impair prolongation of skin allograft survival induced by costimulation blockade (MST=54 days), and in fact, slightly prolonged skin allograft survival as compared to similarly treated B10.D2-HC1 mice in which C5a was present (MST=39 days, p<0.01).

1b. Clearance Kinetics of Anti-CD154 mAb Are Normal in NOD Mice

In a related experiment, we sought to determine if NOD mice are resistant to tolerance induction because of an accelerated clearance rate of anti-CD154 mAb from the circulation. We have previously documented that skin allograft rejection in tolerized mice is inversely correlated with the level of circulating anti-CD154 mAb (26). Cohorts of NOD and C57BL/6 mice were administered a single 2.0 mg intraperitoneal injection of anti-CD154 mAb and circulating antibody concentrations were determined beginning on day 13 and occasionally thereafter until 40 days after treatment. The antibody levels plotted over time and the decay curves are presented in Figure 4B. The half-life of
antibody in circulation as determined by regression analysis for NOD and C57BL/6 is 10.7 days and 10.4 days, respectively. These data document that neither the absence of hemolytic complement or an accelerated clearance of anti-CD154 mAb in NOD mice accounts for their defect in transplantation tolerance induction.
Figure 4

A

% Surviving Skin Allografts

Days Post Skin Transplantation

B

Serum Hamster IgG (µg/ml)

Days After Injection

- - - - NOD

- - - - B6
Legend to Figure 4: Skin allograft survival in mice genetically deficient in hemolytic complement and clearance kinetics of anti-CD154 mAb in NOD and C57BL/6 mice.

Panel A: Skin allograft survival was determined in B10.D2 mice (H2b) treated with DST and anti-CD154 mAb that have (HC) or are genetically deficient (HC') in C5a and hemolytic complement. Male mice 6-10 weeks of age were treated with 4 doses of 0.25 mg anti-CD154 mAb, a single DST, and given a C3H/HeJ skin graft as described in the Methods. B10.D2 HC' recipients genetically deficient in C5a had significantly prolonged skin allograft survival as compared to mice with C5a (p <0.01). Panel B: In vivo clearance of anti-CD154 mAb in NOD and C57BL/6 mice. Cohorts of NOD and C57BL/6 mice received a single intraperitoneal injection of 2.0 mg of anti-CD154 mAb. On day +14 and periodically thereafter, mice were bled and serum hamster IgG concentration was determined by ELISA as described in the methods. Clearance rates were determined by regression analysis for single phase exponential decay. The half-life of hamster IgG in NOD mice (10.7 days) was not significantly different than in C57BL/6 mice (10.4 days, p=N.S.).
2. Costimulation Blockade Prolongs Skin Allograft Survival in C57BL/6 Congenic Mice With NOD Susceptibility Loci

We next tested the hypothesis that selected NOD derived Idd susceptibility loci are responsible for their defect in peripheral tolerance induction by costimulation blockade. This was first assessed by determining if the ability to prolong skin allograft survival was abrogated in C57BL/6 mice congenic for various NOD derived Idd loci (153). None of the C57BL/6 congenic mice develop insulitis or diabetes (Table 3). We tested four congenic strains. C57BL/6.NODc1c mice carry the NOD derived Idd5.1 region which includes genes encoding CD152 (CTLA4) and CD28, costimulatory molecules that are important for tolerance induction and immune activation. C57BL/6.NODc1t mice carry the NOD derived Idd5.1 as well as the Idd5.2 region that includes the CXCR2 and IL-8 receptor genes. C57BL/6.NODc3 mice carry the NOD derived Idd3, Idd17, Idd10 and Idd18 loci, which include genes that control cell activation. C57BL/6.NODc6 mice carry the NOD Idd6 locus that includes genes contained within the NKR-P1 complex (Table 3).

The median skin allograft survival time of each of these C57BL/6 congenic strains ranged from 62 to >93 days and were statistically similar to that observed in C57BL/6 wild-type mice (MST=81 to >94 days, Table 4). Skin allograft survival in the C57BL/6 congenic mice was significantly greater than that observed in similarly treated NOD mice (MST=20 to 24 days, Tables 5 and 6).
Table 4

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Skin Graft</th>
<th>Anti-CD154</th>
<th>Skin Graft Survival (Days)</th>
<th>Median Survival</th>
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<tbody>
<tr>
<td></td>
<td>Donor</td>
<td>mAb (mg)</td>
<td>Time (Days)</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
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<td>0.25</td>
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<td>BALB/c</td>
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<td>29, &gt;101, &gt;101, &gt;101, 105, &gt;106</td>
<td>&gt;101</td>
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</table>
Legend to Table 4: Skin allograft survival on C57BL/6 mice congenic for NOD Idd intervals. Recipient mice of either sex 6-10 weeks of age were treated with 4 doses of anti-CD154 mAb (0.25 or 0.5 mg/dose, as indicated), a single DST, and skin grafted as described in the Methods. No significant differences in skin allograft survival were observed between any of the groups (p = N.S.).
3. Diabetes Resistant NOD Congenic Mice Treated with DST and anti-CD154 mAb Rapidly Reject Skin Allografts

We next determined if NOD stocks congenic for selected C57BL/6- or C57BL/10-derived Idd loci that mediate protection from insulitis and diabetes would also exhibit prolonged skin allograft survival after treatment with DST and anti-CD154 mAb. Three groups of NOD congenic mice with intervals that encompass the non-MHC Idd loci with the greatest effect on diabetes expression were tested (154-157). The first of these were NOD congenic stocks carrying a C57BL/6 derived Idd3 or C57BL/10 derived Idd5 locus alone or in combination. These NOD single Idd congenic mice each have a reduced frequency of diabetes, and when the Idd3 and Idd5 resistance loci are combined, the frequency of diabetes is reduced to 1% and insulitis is absent in most mice (154) (see Table 3). The median survival time of C3H/HeJ skin allografts in these NOD congenic mice treated with DST plus anti-CD154 mAb (24 to 31 days) was statistically significantly shorter than that of C57BL/6 mice (>115 days, Table 5). Skin allograft survival in these NOD congenic mice was similar to that of NOD/Lt (MST=20 days) or NOD/MrkTac (MST=23 days, Table 5) mice.

We next tested NOD congenic mice that carried various combinations of C57BL/6 derived Idd3, Idd10, and Idd18 resistance loci. The incidence of diabetes in these NOD congenic mice varies from 9 to 50% (Table 3). Significant reduction in the frequency of insulitis is also observed in NOD.B6 Idd3/10/18 triple congenic mice (157, 167). Again, all of the NOD congenic strains with various combinations of these genetic
intervals were resistant to tolerance induction, and had median skin allograft survival times of 17 to 29 days (Table 6).

Finally, we tested a NOD stock congenic for the C57BL/10 derived Idd9 locus. This locus contains molecular variants of Cd30, Tnfr2, and Cd137 (155) and these mice have a diabetes incidence of only 3% (Table 3). The median survival time of skin allografts in NOD.B10 Idd9 mice was 20 days, similar to that observed in NOD/Lt mice (MST=24 days, Table 6).
<table>
<thead>
<tr>
<th>Recipient</th>
<th>Skin Donor</th>
<th>Skin Graft Survival (Days)</th>
<th>Median Survival Time (Days)</th>
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Legend to Table 5: skin allograft survival on Idd3/Idd5 congenic NOD mice. Recipient mice of either sex 6-10 weeks of age were treated with 4 doses of 0.5 mg anti-CD154 mAb, a single DST, and skin grafted as described in the Methods. *Significant from all other groups (p <0.001).
<table>
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<tr>
<th>Recipient</th>
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<th>Skin Graft Survival (Days)</th>
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Legend to Table 6: skin allograft survival on Idd3/Idd10/Idd18 and Idd9 congenic NOD mice. Recipient mice of either sex 6-10 weeks of age were treated with 4 doses of 0.5 mg anti-CD154 mAb, a single DST, and skin grafted as described in the Methods.

*Significant from all other groups (p <0.001).
Summary of Chapter I

The data presented in Chapter I eliminate a number of candidates that could control the resistance of NOD mice to transplantation tolerance induction in NOD mice. We demonstrate that powerful Idd loci that can profoundly reduce the prevalence of diabetes in NOD mice are unable to restore susceptibility to transplantation tolerance induction and reciprocally, NOD-derived Idd susceptibility loci are unable to reduce skin allograft survival on the tolerance-susceptible C57BL/6 background. We further showed that resistance is not due to the absence of hemolytic complement, as skin allograft survival in tolerized B10.D2 congenic mice that either have or lack hemolytic complement is similar. Additionally, more rapid clearance of anti-CD154 mAb in NOD mice is not responsible for their resistance to tolerance induction. NOD and C57BL/6 mice treated with a single 2.0 mg injection of anti-CD154 mAb cleared the antibody with the same kinetics. The half-lives observed in this study are similar to a previously published report from our laboratory (26).

There are at least two possible interpretations of our data. First, the response of NOD mice to DST plus anti-CD154 mAb is controlled by genetic intervals distinct from the Idd loci that control their expression of autoimmune. A second interpretation is that transplantation tolerance induction and autoimmunity are controlled by the same genetic pathway, but the threshold for restoration of self tolerance and prevention of autoimmunity is lower than the threshold required for the induction of transplantation tolerance. This explanation predicts that while the evaluated single Idd loci or small combinations of Idd loci are able to improve self-tolerance, the establishment of
transplantation tolerance in NOD mice may require complex combinations of \textit{Idd} resistance loci. Distinguishing between these two hypotheses is the focus of Chapter II.
CHAPTER II

A DOMINANT RESISTANCE TO TRANSPLANTATION TOLERANCE INDUCTION IN (NOD X C57BL/6)F1 MICE

Introduction to Chapter II

The results obtained in Chapter I using Idd congenic stocks that are genetically protected from autoimmune diabetes furthered our understanding of the defect in transplantation tolerance induction in NOD mice. However, it remained unknown from these studies if this defect is genetically uncoupled from the expression of autoimmunity or controlled by the same pathway but at a higher threshold for tolerance (72). To test the "threshold" hypothesis, we chose to study a more self-tolerant, NOD-related stock of mice: first filial generation (F1) progeny between the NOD and C57BL/6. The C57BL/6 mouse was chosen as a parental strain because it is both susceptible to transplantation tolerance induction and resistant to autoimmune diabetes. Additionally, it has been used as the congenic donor in a majority of the Idd congenic stocks, so its diabetes resistance/susceptibility loci have been well established. We reasoned, based on several criteria, that these F1 hybrids have achieved a higher level of self-tolerance. First, (NOD x C57BL/6)F1 mice are fully heterozygous at all Idd loci and the vast majority of diabetes susceptibility alleles are recessive (145, 168). Second, these F1 mice are fully protected from spontaneous diabetes and remain insulitis-free. Third, (NOD x C57BL/6)F1 mice are completely resistant to diabetes induction with cyclophosphamide, an agent that can induce an autoimmune diabetes-like condition on a susceptible background (169).
In this chapter, we demonstrate that (NOD x C57BL/6)F1 hybrids are resistant to tolerance induction, even though they are completely protected from autoimmune diabetes. This resistance to tolerance induction is not a maternally inherited trait and not altered by homozygous expression of some of the most protective Idd resistance loci. Although a vast majority of the NOD cellular immunological defects are restored, we document that (NOD x C57BL/6)F1 mice inherit the NOD defects in bone marrow derived DC maturation as well as CD4+ T cell tolerance. Finally, we show that although these F1 hybrids are resistant to prolonged skin allograft survival, prolonged islet allograft survival can be established in these mice.
Chapter II Results

1. (NOD x C57BL/6)F1 mice are resistant to co-stimulation blockade-based transplantation tolerance

Our laboratory has documented that NOD mice have a generalized defect in their response to peripheral tolerance induction (12), and that NOD congenic mice bearing strongly protective Idd diabetes-resistance loci which ameliorate autoimmunity also resist the induction of prolonged skin allograft survival (Chapter 1)(72). To analyze the genetic basis for these observations, we generated (NOD x C57BL/6)F1 progeny and measured skin allograft survival after treatment with co-stimulation blockade.

Consistent with previous reports (12), survival of C3H/HeJ skin allografts on NOD/Lt mice treated with anti-CD154 mAb plus a single C3H/HeJ donor-specific transfusion (DST) was brief (MST=25 d, Figure 5), whereas on age-matched C57BL/6 mice it was, as expected (12, 27), prolonged (MST >98d, p<0.001). Unexpectedly, survival of C3H/HeJ skin grafts on age-matched (NOD x C57BL/6)F1 mice (MST=34 d), was much shorter than on C57BL/6 mice (p<0.001), and only slightly longer than on NOD/Lt mice (p<0.001, Figure 5).

NOD mice are ancestrally related to Alloxan-resistant (ALR) mice (170), a strain that expresses a maternally-inherited genetic resistance to beta-cytotoxic cytokines and alloxan (171). To exclude the possibility that resistance to transplantation tolerance in (NOD x C57BL/6)F1 mice was dependent on the strain of the maternal parent, we generated and tested (C57BL/6 x NOD)F1 mice. The median survival time of skin
allografts in (C57BL/6 x NOD)F1 mice (MST=34 d) was not statistically different than that observed in (NOD x C57BL/6)F1 mice (MST=34 d, p=N.S., Figure 5).
Legend to Figure 5: Skin allograft survival in NOD, C57BL/6 and (NOD x C57BL/6)F1 mice treated with DST plus anti-CD154 mAb. All mice received 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, as described in the methods. Graft survival is significantly longer in C57BL/6 mice (p<0.001) than in any other strain. (NOD x C57BL/6)F1 and (C57BL/6 x NOD)F1 are statistically indistinguishable (p=N.S.). Both (NOD x C57BL/6)F1 and (C57BL/6 x NOD)F1 have modestly prolonged graft survival (~10 days) compared to NOD mice (p<0.001). The experiment was arbitrarily terminated on day 120; a total of 7 mice with intact grafts were removed from the study between days 98 and 112 for use in other experiments. Vertical bars indicate mice removed from the study with intact grafts or alive with intact grafts on day 120.
2. Fixation of strong diabetes resistance *Idd* loci to homozygosity does not increase skin allograft survival in (NOD x C57BL/6)F1 mice treated with costimulation blockade

In Chapter I (72), we demonstrated that NOD congenic mice bearing resistance alleles at various *Idd* loci were still resistant to transplantation tolerance, even though expression of autoimmunity was reduced. In (NOD x C57BL/6)F1 mice, all *Idd* loci are heterozygous. To determine if transplantation tolerance would be restored in the presence of homozygous *Idd* diabetes-resistant loci, we generated (NOD.B6 *Idd3 B10 *Idd5 x C57BL/6.H2<sup>87</sup>)F1 and (NOD.B10 *Idd9 x C57BL/6.H2<sup>87</sup>)F1 mice. Both F1 hybrids are homozygous for several diabetes-resistance alleles: *Idd3*, *Idd5.1* and *Idd5.2* in (NOD.B6 *Idd3 B10 *Idd5 x C57BL/6.H2<sup>87</sup>)F1 mice and *Idd9.1*, *Idd9.2* and *Idd9.3* in (NOD.B10 *Idd9 x C57BL/6.H2<sup>87</sup>)F1 mice. Both F1 strains, which are completely protected from autoimmune diabetes, are heterozygous for all other *Idd* diabetes-resistant loci distinguishing the two parental strains.

As shown in Table 7, homozygous expression of *Idd9* or *Idd3* plus *Idd5* variants in (NOD x C57BL/6.H2<sup>87</sup>)F1 mice does not lead to improved skin allograft survival following co-stimulation blockade. Skin allograft survival in (NOD.B6 *Idd3 B10 *Idd5 x C57BL/6.H2<sup>87</sup>)F1 (MST=42 d) and (NOD.B10 *Idd9 x C57BL/6.H2<sup>87</sup>)F1 (MST=37 d) mice are not statistically significantly different from that observed in (NOD x C57BL/6.H2<sup>87</sup>)F1 mice (MST=38 d). Skin allograft survival in C57BL/6.H2<sup>87</sup> mice (MST=77 days) is statistically significantly greater than in any of the F1 groups (p<0.01, Table 7).
### Table 7

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Legend to Table 7: Skin allograft survival on F1 hybrids of *Idd* congenic NOD mice mated with C57BL/6.J *H2*\(^{b}\). All mice received 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. Significantly longer than any other group (p<0.01) mice. No other paired comparisons were statistically different.
3. (NOD x C57BL/6)F1 mice express abnormalities in dendritic cell maturation

To begin to understand the cellular basis for the inability of co-stimulation blockade to prolong skin allograft survival in (NOD x C57BL/6)F1 mice, we investigated dendritic cell maturation. Dendritic cells are regulators of immunity and self tolerance (172, 173) and have an important role in transplantation tolerance (143, 174, 175). Maturation of dendritic cells is dependent on CD40-CD154 interaction (176), and NOD mice have abnormalities in dendritic cell maturation (129-133, 177-180).

The proportion of total bone marrow-derived anti-CD40 mAb-stimulated and unstimulated CD86\textsuperscript{high} dendritic cells is low in both (NOD x C57BL/6)F1 and NOD/Lt mice

We cultured bone marrow in the presence of GM-CSF and IL-4 for 6 days, in some cases adding an agonist anti-CD40 mAb for the last 2 days. The total cell population (adherent and non-adherent) was recovered on day 6. The proportions of CD40 mAb stimulated CD11b\textsuperscript{+}CD11c\textsuperscript{+} dendritic cells expressing high levels of CD86 were high in C57BL/6 mice (36.3 ± 4.0%, N=4) and significantly lower in both NOD (16.5 ± 8.0%, p<0.01, N=4) and (NOD x C57BL/6)F1 mice (23.7 ± 8.3%, p<0.05, N=3). Levels in the NOD and (NOD x C57BL/6)F1 mice were similar (p=N.S.). Representative histograms are shown in Figure 6.

These overall results using pools of adherent and non-adherent cells suggest that both NOD and (NOD x C57BL/6)F1 dendritic cells fail to mature and are consistent with several previous analyses of NOD dendritic cells (129, 130, 133, 177, 179). However,
others have reported that the abnormality of NOD dendritic cells is not immaturity but rather hyper-activation (131, 132, 181). These discrepancies could be related both to the developing autoimmune state in the NOD mouse and/or to the particular subpopulation of cultured cells that was analyzed. To confirm that dendritic cells from (NOD x C57BL/6)F1 mice (which are free of autoimmunity) are truly NOD-like, we proceeded to evaluate comprehensively the phenotype of adherent (immature) and non-adherent (mature) dendritic cells generated in the presence and absence of anti-CD40 mAb stimulation.

The proportion of non-adherent stimulated CD86\textsuperscript{high} dendritic cells in (NOD x C57BL/6)F1 mice is intermediate between that of NOD/Lt mice (low) and C57BL/6 mice (high)

Following incubation in the presence of an agonist anti-CD40 mAb, the percentage of non-adherent dendritic cells that expressed CD86 was higher in C57BL/6 (51.7 ± 13.3%; N=3) than in NOD (26.5 ± 1.6%; N=3, p<0.005) or (NOD x C57BL/6)F1 (37.8 ± 4.4%; N=3, p<0.01) bone marrow cultures. While trending towards higher levels, the proportion of CD86 positive non-adherent dendritic cells generated from (NOD x C57BL/6)F1 marrow was not significantly different from that of the NOD cultures. Representative histograms are shown in Figure 7 (right column, solid lines). The level of CD86 expression on the non-adherent cells was significantly higher in NOD (MFI=1468 ± 174; p<0.03) and in (NOD x C57BL/6)F1 (MFI=1397 ± 260; p<0.02) cultures than in C57BL/6 (MFI=1011 ± 189).
The proportion of non-adherent, unstimulated CD86\textsuperscript{high} dendritic cells in (NOD x C57BL/6)F1 mice is intermediate between that of NOD/Lt mice (low) and C57BL/6 mice (high).

Consistent with the data from stimulated cultures, we observed a similar pattern in unstimulated cultures. The percentage of CD86\textsuperscript{high} non-adherent cells in the C57BL/6 cultures (19.4 ± 3.7\%) was higher than that in cultures of NOD (7.3 ± 0.4\%; p<0.03) or (NOD x C57BL/6)F1 bone marrow (11.4 ± 0.6\%; p<0.02, Figure 7). However, the level of CD86 expression on non-stimulated, non-adherent dendritic cells was significantly less in the C57BL/6 (MFI=745 ± 177) than in NOD (MFI=1158 ± 26; p<0.01) or (NOD x C57BL/6)F1 cultures (MFI=1015 ± 152; p<0.01).

Bone marrow cultures of (NOD x C57BL/6)F1 and NOD mice generate larger numbers of immature adherent dendritic cells than do C57BL/6 mice.

We next determined the percentage and number of CD86\textsuperscript{high} adherent dendritic cells in cultures stimulated with anti-CD40 mAb. The percentage of CD86\textsuperscript{high} adherent dendritic cells was slightly higher in C57BL/6 (7.6 ± 1.0\%) than in NOD (3.9 ± 1.1\%; p<0.01) or (NOD x C57BL/6)F1 (3.8 ± 0.5\%; p<0.004) bone marrow cultures. Representative histograms are shown in Figure 7 (right column). C57BL/6 bone marrow (4.3 ±0.7 x 10\textsuperscript{5} cells/well) generated fewer adherent CD11b\textsuperscript{+}CD11c\textsuperscript{+} cells than did cultures of NOD (5.8 ± 0.6 x 10\textsuperscript{5} cells/well, p<0.05) or (NOD x C57BL/6)F1 (6.6 ± 0.8 x 10\textsuperscript{5} cells/well; p<0.02) origin (Figure 7). The number of adherent cells generated in cultures of NOD and (NOD x C57BL/6)F1 bone marrow was similar (p=N.S.).
This data are consistent across CD11b^+CD11c^+ dendritic cell subpopulations and reveals that there is an overall increase in the number of adherent dendritic cells expressing low levels of CD86 in cultures of NOD and (NOD x C57BL/6)F1 bone marrow. This leads to a decrease in the percentage of CD86^{high} dendritic cells that develop in the total culture (Figure 6). Although their percentages are decreased, it is important to note that the mature non-adherent dendritic cells that do develop from NOD and (NOD x C57BL/6)F1 bone marrow express higher levels of CD86 than those of C57BL/6 origin.
Figure 6

- NOD
- C57BL/6
- (NOD x C57BL/6)F1

- CD11b vs. CD11c
- Cell Number vs. CD86

- 15.6% ± 3.4%
- 40.5% ± 8.9%
- 23.0% ± 4.9%
Legend to Figure 6: Maturation of total dendritic cells recovered from bone marrow cultures of NOD, C57BL/6 and (NOD x C57BL/6)F1 mice. Bone marrow-derived dendritic cells from NOD (upper panels), C57BL/6 (middle panels), and (NOD x C57BL/6)F1 (lower panels) mice were incubated in the presence or absence of an agonist anti-CD40 mAb as described in the Methods. Each culture consisted of a pool of combined adherent and non-adherent cells from two mice. Cells recovered after culture were analyzed by flow cytometry. Representative dot plots of CD11b⁺ CD11c⁺ dendritic cells obtained from anti-CD40 mAb-stimulated cultures are shown in the left column. Gating was similar for plots for dendritic cells obtained from unstimulated cultures. The right column shows the distribution of CD86 (B7.2) expression on stimulated (solid lines) and unstimulated (dotted lines) dendritic cells. The horizontal bars in the right column indicate the gate used for counting CD86^{high} cells. The number above each horizontal bar indicates the percentage of CD86^{high} dendritic cells in the anti-CD40 mAb-stimulated cultures. The number below each horizontal bar indicates the percentage of CD86^{high} dendritic cells in the unstimulated cultures. Isotype controls are shown in the insets; levels of non-specific CD86 staining were uniformly <0.3%. Shown are representative histograms; the experiment was repeated 3 times with similar results. Average percentages for all three trials are given in the Results.
Figure 7

Adherent

Non-adherent

NOD

C57BL/6

(NOD x C57BL/6)F1

CD86

Cell Number

CD86

Cell Number

- 28.2%  
--- 6.8%

- 67.0%  
--- 23.6%

- 41.2%  
--- 11.7%
Legend to Figure 7: Maturation of adherent and non-adherent dendritic cells recovered from bone marrow cultures of NOD, C57BL/6 and (NOD x C57BL/6)F1 mice. Bone marrow-derived dendritic cells from NOD (upper panels), C57BL/6 (middle panels), and (NOD x C57BL/6)F1 (lower panels) mice were incubated in the presence or absence of an agonist anti-CD40 mAb as described in the Methods. Each culture consisted of a pool of cells from two mice. Cells recovered after culture were analyzed by flow cytometry. Histograms in the left column show the expression of CD86 on adherent CD11b+ CD11c+ cells. Histograms in the right column show the expression of CD86 on non-adherent CD11b+CD11c+ cells. The horizontal bars in each histogram indicate the gate used for counting CD86<sup>high</sup> cells. The number above each horizontal bar indicates the percentage of CD86<sup>high</sup> dendritic cells in the anti-CD40 mAb-stimulated cultures. The number below each horizontal bar indicates the percentage of CD86<sup>high</sup> dendritic cells in the unstimulated cultures. Isotype controls are shown in the insets; levels of non-specific CD86 staining were uniformly <0.3%. Shown are representative histograms; the experiment was repeated 3 times with similar results. Average percentages for all three trials are given in the Results.
4. (NOD x C57BL/6)F1 mice genetically deficient in CD8⁺ T cells are resistant to transplantation tolerance

Allograft survival in recipients treated with DST and anti-CD154 mAb requires the deletion of alloreactive CD8⁺ T cells (26, 96). As a part of the analysis of cellular defects that might be responsible for the resistance of (NOD x C57BL/6)F1 mice to costimulation blockade, we generated (NOD x C57BL/6)F1 CD8α⁻⁄ mice. These (NOD x C57BL/6)F1 CD8α⁻⁄ mice were used to determine if the failure to delete alloreactive CD8⁺ T cells in response to costimulation blockade was responsible for their resistance to transplantation tolerance induction.

Three groups of CD8α⁻⁄ mice (NOD, C57BL/6 and (NOD x C57BL/6)F1) were randomized and given DST plus anti-CD154 mAb. Median graft survival in C57BL/6 CD8α⁻⁄ mice was >101 days and 5 of 8 grafts were intact at the end of the experiment (Table 8). Duration of skin allograft survival in NOD CD8α⁻⁄ mice (MST=21 d) was significantly shorter than C57BL/6 CD8α⁻⁄ (p=0.005). Graft survival in (NOD x C57BL/6)F1 CD8α⁻⁄ mice (MST=35 d) was significantly less than that observed in C57BL/6 CD8α⁻⁄ mice (p<0.01) but slightly longer than in NOD CD8α⁻⁄ mice (p=0.03, Table 8). These data document that resistance of alloreactive CD8⁺ T cells to deletion by co-stimulation blockade cannot be the sole mechanism of resistance of NOD and (NOD x C57BL/6)F1 mice to transplantation tolerance.

We also tested anti-CD154 mAb monotherapy in these animals because we have previously observed that it somewhat prolongs skin allograft survival in C57BL/6 CD8α⁻⁄ mice (96). Using anti-CD154 mAb monotherapy, we observed uniformly brief
skin allograft survival in all three groups: NOD CD8α−/− mice (MST=17 d, N=8), C57BL/6 CD8α−/− mice (MST=23 d, N=12) and (NOD x C57BL/6)F1 CD8α−/− mice (MST=20 d, N=7). None of these MSTs were statistically different.
### Table 8

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Legend to Table 8: Survival of skin allografts on NOD, C57BL/6 and (NOD x C57BL/6)F1 mice genetically deficient in CD8α. Appropriate mice received a single C3H/HeJ DST consisting of $1 \times 10^7$ spleen cells intravenously on day-7, and/or 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. Some mice received a C3H/HeJ skin graft only. ¹No significant difference between groups. ²Significantly different from C57BL/6 mice (p<0.003). ³Significantly different from similarly treated NOD (p<0.01) and C57BL/6 (p<0.03).
5. Natural killer cell number and cytotoxic activity are similar in (NOD x C57BL/6)F1 and C57BL/6 mice

NK cells are important in the rejection of allogeneic hematopoietic grafts (113), and NOD mice have a deficiency in NK cell activity (35, 107). The role of NK cells in co-stimulation blockade-induced transplantation tolerance is unknown. We therefore compared NK cell number and activity in NOD (NOD x C57BL/6)F1 mice to determine if, like the NOD dendritic cell abnormality, it is a genetically dominant trait. Because NOD/Lt mice are NK1.1⁺, we quantified the number of NK cells by dual label analysis using the DX5 anti-NK cell and anti-ASGM-1 antibodies (35). The percentage of DX5⁺ASGM-1⁺ cells in the spleen of C57BL/6 mice (2.18 ± 0.43%, N=6) is greater than that in NOD/Lt mice (1.68 ± 0.31%, N=6); this difference was not statistically significant (p<0.06), but trended in the same direction as reported by others (35) (Figure 8A). The percentage of DX5⁺ASGM-1⁺ cells in the spleen of (NOD x C57BL/6)F1 mice (1.88 ± 0.28%, N=6) was similar to that detected in C57BL/6 mice (p=N.S.) and in NOD/Lt mice (p=N.S.).

To assess NK killing activity in these same mice, we measured the cytotoxic activity of spleen cells against NK-sensitive YAC-1 targets. Interestingly, the NK cell cytotoxic activity of spleen cells from (NOD x C57BL/6)F1 mice was similar to that of spleen cells from C57BL/6 mice, both of which were more significantly more potent than that of spleen cells from NOD mice (Figure 8B).
Figure 8

A

B

---

Asialo GM-1

Effector to Target Ratio

Percent Specific Lysis

C57BL/6 (N=6)

NOD (N=8)

(NOD x C57BL/6)F1 (N=8)

Effector to Target Ratio
Legend to Figure 8: Splenic NK cell percentages and function in NOD, C57BL/6 and (NOD x C57BL/6)F1 mice. **Panel A:** Spleen cells were obtained from 6-12 week old NOD, C57BL/6, and (NOD x C57BL/6)F1 and stained with DX5 (vertical axis) and anti-ASGM-1 (horizontal axis) antibodies as described in the Methods. The circular gates indicate the percentage of total splenic lymphocytes that were DX5$^+$ASGM-1$^+$ NK cells. Shown are representative contour plots. Each analysis was performed 6 times with similar results. Average percentages for all trials are given in the Results. Shown in the insets is staining of ASGM-1 and the isotype control for the DX-5 mAb. **Panel B:** Cytotoxic activity directed against NK-sensitive YAC-1 target cells as determined by $^{51}$Cr release. Effector cells were spleen cells recovered from 6-12 week old NOD, C57BL/6 and (NOD x C57BL/6)F1 mice and assayed as described in Methods. Mice were treated with a single injection of poly I:C 24 hours before spleen cell recovery. Shown are the results of three independent experiments.
6. Macrophage maturation in (NOD x C57BL/6)F1 mice is normal

NOD mice have defects in macrophage maturation that have been associated with their resistance to transplantation tolerance (12). Macrophage maturation can be quantified by measuring the secretion of IL-1β by bone marrow-derived macrophages stimulated with LPS (184). Bone marrow from NOD/Lt, C57BL/6, and (NOD x C57BL/6)F1 mice was cultured in the presence of CSF-1 and IFN-γ to generate macrophages. Cultures were then stimulated with LPS to induce secretion of IL-1β, which was in turn measured in a thymocyte co-stimulation proliferation assay (184).

As expected, LPS-stimulated macrophages derived from NOD/Lt cultures produced less IL-1β than did LPS-stimulated macrophages derived from C57BL/6 cultures (Figure 9, p<0.001). Surprisingly, (NOD x C57BL/6)F1-derived macrophages responded to LPS by secreting high levels of IL-1β that were comparable to those of C57BL/6 macrophages (p=N.S.). Results of two independent trials were similar. These results indicate that unlike the genetically dominant dendritic cell defect in the NOD mouse, the NOD macrophage maturation defect is not expressed in (NOD x C57BL/6)F1 mice.
Figure 9

Counts per Minute

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>NOD</th>
<th>(NOD x C57BL/6)F1</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>12000</td>
<td>10000</td>
<td>8000</td>
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Legend to Figure 9: Mitogen-stimulated production of IL-1β by cultures of bone marrow-derived macrophages from NOD, C57BL/6 and (NOD x C57BL/6)F1 mice. Pools of bone marrow-derived macrophages were obtained from NOD, (NOD x C57BL/6)F1, and C57BL/6 mice, stimulated with LPS, and assayed for production of IL-1β as described in the Methods. Vertical bars indicates incorporation of [3H]thymidine by C3H/HeJ thymocytes in a co-mitogenic assay. Shown is a representative experiment of supernatants from a bone marrow-derived macrophage culture generated from a pool of two mice. The data represent the mean ± 1 s.d. of [3H]thymidine incorporated in 6 replicate wells for each strain. The experiment was repeated 2 times with similar results.
7. (NOD x C57BL/6)F1 mice have normal percentages of CD4+CD25+ T cells

The poor allograft survival in NOD CD8α−/− and (NOD x C57BL/6)F1 CD8α−/− mice suggests that the resistance to transplantation tolerance could in part be due to an abnormal response of CD4+ T cells, in particular CD4+CD25+ regulatory T cells known to be important in transplantation tolerance (86, 185-187). To begin to address this possibility, we measured the percentage of splenic CD4+CD25+ T cells. We found that the percentage of CD4+ cells that co-expressed CD25 in C57BL/6 (10.64% ± 1.97%, N=6) was similar to that in both NOD (11.03% ± 1.67%, N=6) and (NOD x C57BL/6)F1 (11.04% ± 3.14%, N=6) mice. Representative dot plots are shown in Figure 10.

These results suggest that a deficiency in the percentage of CD4+CD25+ T cells is not responsible for the abnormal response of (NOD x C57BL/6)F1 mice to co-stimulation blockade. However, the data do not exclude the possibility of a functional abnormality in their CD4+CD25+ regulatory T cells.
Figure 10

NOD

10000

1000

100

10

1

10

100

1000

10000

10.5%

C57BL/6

10000

1000

100

10

1

10

100

1000

10000

11.7%

(NOD x C57BL/6)F1

10000

1000

100

10

1

10

100

1000

10000

12.9%
Legend to Figure 10: Splenic CD4+CD25+ T Cells in NOD, C57BL/6 and (NOD x C57BL/6)F1 mice. Spleen cells from 6-8 week old NOD (upper panel), C57BL/6 (middle panel) and (NOD x C57BL/6)F1 (lower panel) mice were stained with anti-CD4 (horizontal axis) and anti-CD25 (vertical axis) mAbs and analyzed by flow cytometry. The percentage of CD4+ cells that are also CD25+ is indicated in the upper right of each plot. Shown are representative dot plots; the experiment was repeated 2 times using a total of 6 individual mice in each group with similar results. Average percentages for all mice are given in the Results.
8. Transplantation tolerance to allogeneic islets is normal in (NODxC57BL/6)F1 mice

Finally, we hypothesized that the absence of autoimmunity in (NOD x C57BL/6)F1 mice may be sufficient for restoration of the induction of transplantation tolerance to islet allografts in mice, irrespective of their continued resistance to the induction of tolerance to skin allografts. This hypothesis is based in part on our observation that transplantation tolerance to islet allografts does not lead to transplantation tolerance to skin allografts (188). To test this hypothesis, we determined the ability of co-stimulation blockade to prolong islet allograft survival in chemically diabetic male NOD mice. We have previously shown that ~40% of islet allografts survive indefinitely in C57BL/6 mice treated with anti-CD154 mAb monotherapy, and essentially all survive following treatment with DST plus anti-CD154 mAb (189, 190).

Islet allograft survival in chemically diabetic NOD mice treated with anti-CD154 mAb monotherapy (MST=43 d) or DST plus anti-CD154 mAb (MST=43 d) was prolonged (Table 9) as compared to that previously observed for islet allograft survival in spontaneously diabetic NOD mice (12). However, the survival of islet allografts in chemically diabetic NOD mice remained significantly shorter than in comparably-treated C57BL/6 mice (MST=>98 d and >89 d, respectively, Table 9). Histological analysis revealed complete destruction of the NOD islet grafts instead of selective β cell loss. The histological data suggest that an alloresponse mediated the rejection of allogeneic islets in chemically diabetic NOD mice. However, an autoimmune component of islet graft destruction, can’t be ruled out.
In sharp contrast to the resistance of (NOD x C57BL/6)F1 mice to skin allograft tolerance induction, and in contrast to the short survival of islet allografts in chemically diabetic NOD mice, chemically diabetic (NOD x C57BL/6)F1 mice treated with anti-CD154 mAb monotherapy (MST=>96 d) or DST plus anti-CD154 mAb (MST=>96 d) exhibit long term islet graft survival (Table 9). The survival of islets in (NOD x C57BL/6)F1 mice is not statistically significantly different from that achieved in C57BL/6 mice (Table 9).

These data document that the genetically-dominant resistance of (NOD x C57BL/6)F1 mice to prolonged skin allograft survival by co-stimulation blockade does extend to resistance to induction of islet allograft tolerance. These data suggest that induction of transplantation tolerance to skin allografts requires cellular and genetic mechanisms that at least partially differ from those required for tolerance induction to islet allografts, or alternatively, that there is a common genetic basis for islet cell autoimmunity and resistance to the induction of islet allograft transplantation tolerance in NOD mice.
<table>
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<th>DST</th>
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<td>No</td>
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**Legend to Table 9:** Survival of islet allografts in chemically diabetic NOD, C57BL/6 and (NOD x C57BL/6)F1 mice. Recipient mice were rendered chemically diabetic by intraperitoneal administration of streptozotocin (150 mg/kg body weight) and verified to be hyperglycemic prior to tolerance induction. Appropriate animals received a single DST consisting of $1 \times 10^7$ C3H/HeJ spleen cells intravenously on day $-7$, four injections of 0.5 mg anti-CD154 mAb intraperitoneally on days $-7$, $-4$, $0$, $+4$ and a C3H/HeJ islet graft consisting of 20 islets/gram body weight placed under the kidney capsule on day 0. Graft survival was monitored by blood glucose levels and rejection was defined as a blood glucose level $>250$ mg/dL. N/D, not done.
Summary of Chapter II

In this series of experiments, we sought to test the hypothesis that resistance to transplantation tolerance in NOD mice is controlled by the same genetic pathways that control the breakdown of self tolerance leading to autoimmune diabetes. The hypothesis posited that a lower threshold is required to establish self tolerance compared to the ability to induce tolerance to a foreign tissue with costimulatory blockade. To test this hypothesis, F1 progeny between various NOD and C57BL/6 stocks were generated, and they all proved to be resistant to transplantaton tolerance induction even though they are completely diabetes resistant. The resistance to skin allograft tolerance induction in F1 mice correlates with dominant NOD defects in dendritic cells and CD4⁺ T cells. Surprisingly, (NOD x C57BL/6)F1 mice are readily tolerized to islet allografts with DST and anti-CD154 mAb, highlighting the tissue specific differences in the genetic control of transplantation tolerance induction.

While the data presented in this chapter argue against the higher “threshold” model for transplantation tolerance in NOD mice, the genetic separation of these two phenotypes cannot be proven by this series of experiments. The true test of the hypothesis that partially, but not completely overlapping genetic mechanisms control transplantation tolerance and autoimmunity requires the identification of the loci that mediate induction of transplantation tolerance induction, which is the focus of Chapter III.
CHAPTER III
IDENTIFICATION OF THE GENETIC INTERVALS THAT CONTROL PROLONGED SKIN ALLOGRAFT SURVIVAL IN NOD MICE TREATED WITH DONOR SPECIFIC TRANSFUSION AND ANTI-CD154 mAb

Introduction to Chapter III

To assess whether the genes that determine autoimmunity and resistance to transplantation tolerance are distinct or overlapping, we chose a genome wide scan approach. N2 generation mice, generated by backcrossing (NOD x C57BL/6.H2\textsuperscript{87})F1 mice to the tolerance-susceptible C57BL/6.H2\textsuperscript{87} strain were treated with DST and anti-CD154 mAb and the duration of skin allograft survival measured.

We chose to perform a backcross mating scheme for a few reasons. First, we were primarily interested in determining the genetic basis for the dominant NOD resistance to skin transplantation tolerance induction. Secondly, this approach is feasible because the diabetes-resistant C57BL/6.H2\textsuperscript{87} strain can be used as the backcross parent, thus avoiding the confounding effects of autoimmunity in the N2 generation. Finally, an F1 intercross strategy, while informative, is more difficult to analyze because recombination events can occur in both parents and is most useful for mapping recessive phenotypes; backcross mating strategies are amenable to QTL analysis (191).

Identification of the quantitative trait loci (QTLs) that control the duration of graft survival will allow us to localize dominant NOD-derived loci and to determine if these coincide with the location of previously-defined Idd loci or if new loci are identified.
QTL analysis has also been used to map a number of other genes in many polygenic diseases (192). Indeed, this strategy has identified Idd loci in mice, but in contrast to the experimental design for the experiments described in this chapter, the NOD strain was used as the backcross parent for the identification of Idd loci since diabetes is a recessive trait (145). This approach may discover new chromosomal regions that contain genes that control only the transplantation tolerance defect of NOD mice, but it is equally likely that these new regions may contain previously unknown dominant NOD-derived genes that may have overlapping function in autoimmune diabetes. The genome wide scan strategy is particularly advantageous in comparison with testing existing Idd congenic strains for tolerance induction (Chapter I) because it is unbiased.

In this chapter, the preliminary results of the genome wide scan on (NOD x C57BL/6.H2^b^) x C57BL/6.H2^b^ backcross progeny are reported. As expected, the skin allograft survival in response to DST and anti-CD154 mAb is a variable quantitative trait and four suggestive loci have been identified that control this phenotype: two near reported Idd loci, and two novel loci. Continuation of this study will allow for better resolution of these putative transplantation tolerance loci.
Chapter III Results

1. Skin allograft survival in (NOD x C57BL/6.H2\(\text{b}^7\))F1 x C57BL/6.H2\(\text{b}^7\) N2 generation animals is a polygenic trait.

In Chapter II, we documented a dominant resistance to transplantation tolerance in NOD mice that is inherited in F1 progeny. To map the genes independent of the MHC that control this phenotype, we generated N2 mice, treated them with DST and anti-CD154 mAb, and monitored skin allograft survival. In this initial study, we measured skin allograft survival on 64 (NOD x C57BL/6.H2\(\text{b}^7\))F1 x C57BL/6.H2\(\text{b}^7\) backcross mice, along with 16 C57BL/6.H2\(\text{b}^7\) and 8 (NOD x C57BL/6.H2\(\text{b}^7\))F1 mice. The median duration of allograft survival on C57BL/6.H2\(\text{b}^7\) and (NOD x C57BL/6.H2\(\text{b}^7\))F1 was 55 days and 35 days (p<0.001), respectively (Figure 11A). When the frequency distribution of allograft survival in the N2 generation mice is plotted, we observe that the data is widely distributed, with a high proportion of the data centered around the median, indicating that duration of skin allograft survival is a polygenic trait (Figure 11B).
Legend to Figure 11: Skin allograft survival on (NOD x C57BL/6.H2b7)F1 x C57BL/6.H2b7 backcross mice treated with DST and anti-CD154 mAb. **Panel A:** Comparison of the distribution of skin allograft survival in NOD, C57BL/6.H2b7, (NOD x C57BL/6.H2b7 and backcross mice. Horizontal bars in each scatter plot indicate the median value for that group. **Panel B:** Frequency distribution of skin allograft survival in backcross mice with the best-fit normal distribution curve for the data.
2. QTL analysis reveals four suggestive loci mediating the response to costimulation blockade.

Because polygenic traits are amenable to QTL analysis, we carried out a genome wide QTL analysis using the R/qtl software package (http://www.biostat.jhsph.edu/~kbroman/qtl). Days to rejection was used as the quantitative trait and, because N2 generation males and females showed a difference in the median time to rejection (MST = 48 days for males, 30 days for females, p<0.0001), sex was included as an additive covariate in the analysis, meaning that we are examining genetic effects after taking into account the differences between the two sexes.

After analyzing the data, four loci reach a suggestive, but not significant log of odds (LOD) score. Suggestive thresholds for the single sex genome scan and the combined scan with sex as a covariate are 1.4 and 2.8 LOD, respectively. Significant thresholds for the single sex genome scan and the combined scan with sex as a covariate are 2.8 and 4.9 LOD, respectively. The significance thresholds were computed using permutation analysis (193). A profile of LOD scores across the genome is shown in Figure 12A. Markers on chromosomes 2, 5 and X are suggestive regardless of sex and a marker on chromosome 4 is suggestive in males only. Also, the marker on the X chromosome is more suggestive when males are analyzed separately. The direction and magnitude of the effect for the four markers: D2Mit190, D4Mit26, D5Mit348 and DXMit64 are shown in Figure 12B. Two markers, D4Mit26 and DXMit64 have effects in the opposite direction expected, i.e. better allograft survival in heterozygous animals where a NOD-derived allele is present compared to homozygous expression of the
C57BL/6-derived allele. The other two markers, D2Mit190 and D5Mit348 have their effects in the expected direction, i.e. better allograft survival in the mice homozygous for C57BL/6-derived alleles at the marker.
Figure 12

A

Main scan: Days to Rejection w/ sex as ADD cov

Main scan: Days to rejection (Male only)

Main scan: Days to rejection (Female only)

B

Interaction plot for Sex and DXM1684

Interaction plot for Sex and D2Mit190

Interaction plot for Sex and D5Mit348

Interaction plot for Sex and D4Mit26
Legend to Figure 12: Results of the genome wide scan for QTL controlling transplantation tolerance induction in NOD mice. Panel A: Profile of LOD scores across all the chromosomes of the mouse genome from the cohort of mice in Figure 11. Suggestive and significant LOD scores in the single sex scans are 1.4 and 2.8, respectively. For the scan with sex as an added covariate, suggestive and significant LOD scores are 2.8 and 4.9, respectively. Panel B: Direction and magnitude of the QTL effects at each of the suggestive loci (bb = homozygous C57BL/6-derived; nb = heterozygous). Males, closed circles; females, open circles.
Summary of Chapter III

The genome wide scan analysis of (NOD x C57BL/6.J2-87)F1 x C57BL/6.J2-87 backcross progeny treated with DST and anti-CD154 mAb and a skin allograft is a polygenic trait, as demonstrated by the bell shaped curve of duration of survival in this preliminary cohort. Since this phenotype is polygenic, it is amenable to QTL analysis. Indeed, genome wide QTL analysis reveals four suggestive loci and while they don’t yet reach statistical significance, increasing the number of mice in the study will hopefully achieve significant thresholds. It is important to note that this study has been designed to include approximately 300 N2 generation animals and only 64 were included in this preliminary analysis.

Interestingly, two previously described Idd loci are located very near two of the suggestive QTL. The two other QTL are not near known Idd loci. The data provide unbiased support to the hypothesis of “distinct but partially overlapping” genetic control hypothesis. However, it remains to be determined if the QTL identified on chromosomes X and 4 also alter the expression of autoimmunity in NOD mice, even though previous screening by others failed to identify them as Idd loci. It is apparent from these preliminary studies that the control of transplantation tolerance in NOD mice is controlled by a complex genetic program.
DISCUSSION

The goal of this thesis project was to investigate the relationship between genes that control autoimmune diabetes expression in NOD mice with those controlling their resistance to transplantation tolerance induced by costimulation blockade. The results in Chapter I document that single or small combinations of the evaluated C57BL/6-derived Idd loci that dramatically alter diabetes expression are not able to correct the response of NOD mice to costimulation blockade and, conversely, that the evaluated NOD-derived Idd loci do not shorten skin allograft survival in C57BL/6 mice. Chapter II demonstrates that resistance to skin transplantation tolerance in NOD mice is a dominant trait. Resistance to skin transplantation tolerance in F1 hybrids correlates with inherited NOD-like defects in dendritic cells and CD4+ T cells. Finally, the exciting preliminary data in Chapter III implicates four loci that control resistance to skin allograft transplantation tolerance: two near known Idd loci and two intervals outside of currently known Idd loci.

The genetic basis of transplantation tolerance induction in NOD mice

Our first genetic approach tested the hypothesis that some of the loci associated with development of autoimmune diabetes in NOD mice would also be important in their resistance to induction of peripheral transplantation tolerance. This hypothesis predicts that in NOD congenic mice harboring C57BL/6 or C57BL/10-derived diabetes resistance alleles skin allograft survival will be prolonged and conversely, C57BL/6 congenic mice harboring NOD-derived diabetes susceptibility alleles will have abbreviated skin allograft survival after tolerance induction. Prolonged allograft survival was not abrogated in C57BL/6 stocks congenic for any of the analyzed NOD-derived Idd susceptibility loci.
We note, however, that no combination of \textit{Idd} susceptibility loci introgressed into C57BL/6 mice to date has rendered them susceptible to the spontaneous development of insulitis or autoimmune diabetes (153).

Similarly, none of the analyzed C57BL/6- or C57BL/10-derived \textit{Idd} congenic intervals that confer various degrees of diabetes resistance to NOD mice restored their ability to be tolerized to skin allografts by DST and anti-CD154 mAb treatment. This observation was surprising because congenic introgression of even a few of the \textit{Idd} resistance loci into NOD mice profoundly reduces the incidence of insulitis and diabetes (154, 155, 194, 195) (see Table 3). Furthermore, many of the tested \textit{Idd} congenic intervals are characterized by polymorphisms in genes important for costimulation and immune activation, giving a plausible explanation why \textit{Idd} loci could control the response to transplantation tolerance induction.

It is possible that the results from Chapter I reflect the fact that more \textit{Idd} resistance loci are required to genetically alter the phenotype of abnormal transplantation tolerance induction in NOD mice than are required to decrease the incidence of spontaneous autoimmune diabetes (i.e. a higher "threshold" must be achieved). Specifically, peripheral transplantation tolerance induction by costimulation blockade may be under the control of a complex combination of \textit{Idd} loci not yet tested. Additionally, there could be potential interactions of the unique NOD \textit{H2}MHC with these genetic loci that have an effect on tolerance induction. However, these data raise the possibility that the loss of self-tolerance leading to autoimmunity in NOD mice may be
mediated by mechanisms that differ, in part, from their resistance to peripheral transplantation tolerance.

The focus of Chapter II was to distinguish between the "genetic threshold" and the "genetic separation" hypotheses that arose out of the analysis of transplantation tolerance induction in Idd congenic mice (Chapter I). Previous studies have lent support to the genetic threshold hypothesis for autoimmunity in NOD mice. These studies used NOD mice mated with various non-autoimmune strains and treated the F1 mice with cyclophosphamide to induce diabetes (196, 197). Approximately 30% of NOD female mice crossed with the closely related but diabetes-resistant MHC-compatible NOR/Lt strain developed diabetes using this treatment protocol (198). In another study, NOD mice were crossed with diabetes- and insulitis-free NOD.H2b mice. Approximately 50% of (NOD x NOD.H2b)F1 mice developed insulitis, a low percentage (3%) of female F1 mice spontaneously developed diabetes, and ~20% become diabetic after treatment with cyclophosphamide (199). These data argue for a genetic "threshold" model for expression of autoimmunity.

In Chapter II, we generated (NOD x C57BL/6)F1 mice to begin to test the "genetic threshold" hypothesis and the possible role of Idd loci in transplantation tolerance. (NOD x C57BL/6)F1 mice are heterozygous at all Idd loci distinguishing the two parental strains (168). Only three NOD Idd diabetes-susceptibility loci, Idd13, Idd14, and Idd15 (145, 149, 200-202) are dominant, the remainder are recessive. (NOD x C57BL/6)F1 mice treated with cyclophosphamide remain free of diabetes (169), suggesting that they have a high genetic threshold of resistance to autoimmunity.
Therefore, if similar genes control autoimmunity and transplantation tolerance, (NOD x C57BL/6)F1 mice should have a higher “threshold” facilitating the induction of transplantation tolerance. Unexpectedly, (NOD x C57BL/6)F1 mice clearly remain resistant to the induction of prolonged skin allograft survival by costimulation blockade.

We further tested the genetic threshold model and a role for Idd loci by generating (NOD.B6 Idd3 B10 Idd5 x C57BL/6.H2\textsuperscript{g7})F1 and (NOD.B10 Idd9 x C57BL/6.H2\textsuperscript{g7})F1 mice, which are homozygous for strongly protective diabetes-resistance Idd loci. We nonetheless observed skin allograft survival in response to costimulation blockade was no better in these congenic F1 progeny than that in (NOD x C57BL/6)F1 mice.

Finally, we observed that the resistance of skin allograft survival is genetically uncoupled from islet allograft survival, as (NOD x C57BL/6)F1 mice treated with DST and anti-CD154 mAb have prolonged islet allograft survival. This phenotype resembles the C57BL/6 phenotype and is in contrast to brief islet allograft survival in similarly treated NOD mice. This phenotype could reflect that the “threshold” to achieve tolerance to islet allografts is intermediate between self-tolerance and skin allograft tolerance in (NOD x C57BL/6)F1 mice. Another possibility is that the genetic control of islet, as opposed to skin allograft survival is mediated by recessive Idd loci that are corrected in the (NOD x C57BL/6)F1 mice. Ongoing studies with the congenic mice described in Chapter I will help to test this possibility.

We conclude from this data that skin transplantation tolerance induction and susceptibility to autoimmune diabetes are not likely to be controlled by identical genetic pathways, since (NOD x C57BL/6)F1 mice have achieved a higher threshold of self-
tolerance, yet remain resistant to transplantation tolerance induction. Perhaps the strongest piece of data arguing for distinct genetic control is the fact that susceptibility to diabetes and response to transplantation tolerance induction are differentially inherited in these F1 hybrids. However, we cannot rule out the possibility that there is partial overlap between these phenotypes. Indeed, some of the Idd congenic stocks examined in Chapter I have modestly prolonged skin allograft survival compared to wild type NOD mice suggesting that diabetes resistance alleles may have a small but detectable influence on transplantation tolerance induction. Interestingly, skin allograft survival in (NOD x C57BL/6)F1 mice was also slightly but reproducibly longer than that observed in NOD mice. Speculatively, this difference could be controlled by a dominant C57BL/6-origin Idd locus/loci with an unknown phenotype. The modest prolongation of skin allograft survival in (NOD x C57BL/6)F1 mice compared to NOD mice is not what would be expected of a dominant or co-dominant phenotype. Rather it suggests that a complex set of genetic interactions involving several loci is needed for prolongation of allograft survival in response to costimulation blockade (203).

The data in Chapter III begin to confirm that the genetic control of prolonged skin allograft survival in response to costimulation blockade is indeed complex. QTL analysis on the N2 generation highlights four suggestive markers, two that are near the known diabetes loci, Idd13 on chromosome 2 and Idd15 on chromosome 5. The other two markers, DXMit64 and D4Mit26, on the X chromosome and chromosome 4, respectively, are not near any known Idd loci. Interestingly, loci on chromosomes 4 and X are restricted to male mice and opposite of the direction expected. A QTL in the
opposite direction is not uncommon and can lead to transgressive segregation, where the progeny have phenotypes that are more extreme than either of the parental lines. Indeed, *Idd* diabetes susceptibility alleles have been found to be harbored in the genome of otherwise diabetes resistant strains; introducing a tightly linked *Idd* locus is one of the complications of congenic transfer of a gene “knockout” generated on the 129 background to the NOD background (204).

The locus centered around D5Mit348 maps near *Idd15*, a poorly defined, dominant diabetes susceptibility locus (200). D2Mit190 is near *Idd13*, a recessive diabetes susceptibility locus. However, this interval is better defined, containing the both β2M (~2 cM from D2Mit190) and the *Il1* gene encoding IL-1. The NOD β2M<α> allele is the only non-MHC gene to date that has been definitively shown to be a diabetes susceptibility gene (149). Interestingly, the NOD alleles of these transplantation loci on chromosomes 2 and 5 exert their effects in a dominant manner, meaning that genes in these regions could be exerting their effects on diabetes pathogenesis and response to costimulation blockade in a similar fashion.

The preliminary data from the genome-wide scan also suggests that there is some degree of overlap in the genetic control of skin transplantation tolerance and autoimmunity, as markers both near *Idd* loci and markers distinct from known *Idd* loci suggestively control the transplantation tolerance phenotype in this study. These data could reflect that the two phenotypes are controlled at different set points along the same genetic pathway. However, as this analysis continues, the exact nature of the genetic control of the transplantation tolerance phenotype will be elucidated.
While the genome-wide scan described in this dissertation will eventually allow high-resolution genetic analysis, there are genetic interactions and variables not accounted for by this strategy. A genetic variable that was intentionally fixed in this backcross experiment is the MHC haplotype. We were specifically looking for loci that were independent of the MHC, but acknowledge that there probably are interactions between MHC and non-MHC genes that alter the response to transplantation tolerance induction. Indeed, the fact that the suggestive marker D2Mit190 is approximately 2 cM from a known diabetes susceptibility gene that most definitely interacts with genes within the MHC, i.e. the $\beta2M$ gene (149), indicates that transplantation tolerance induction is likely controlled by genetic interactions between MHC and non-MHC genes. To determine the interactions between MHC and non-MHC genes, the role of the $H2^g$ vs. $H2^b$ haplotypes, and other recessive phenotypes involved in transplantation tolerance induction, a second mapping experiment will need to be undertaken by our laboratory. This strategy involves intercrossing (NOD x C57BL/6)F1 (not fixed at the MHC) to generate F2 progeny that will have the MHC segregating as an additional variable. An F2 intercross mating strategy has the advantage over a backcross breeding strategy by picking up recessive phenotypes and epistatic interactions (191). Additionally, future experiments that mate C57BL/6.$H2^g$ mice with C57BL/6.NODc2 (D2Mit274-D2Mit343) that harbor the NOD $Idd13$ allele so that both the NOD MHC and $\beta2M$ are on the C57BL/6 background will also aid in determining a role for the interaction of genes in the MHC and $Idd13$ (perhaps $\beta2M$) in the response to costimulation blockade.
The cellular basis of transplantation tolerance induction in NOD mice

What is the cellular basis for autoimmune diabetes and transplantation tolerance induction in NOD mice? The data presented suggest a number of cellular abnormalities in the immune system of NOD mice that may be important. There is evidence that autoimmune diabetes in NOD mice is due primarily to defects in central tolerance (34). Bone marrow chimerism is known to prevent autoimmunity in NOD mice by this mechanism (205). In humans, bone marrow cells from diabetic donors have been documented to adoptively transfer disease to non-diabetic recipients, suggesting that central tolerance defects are also important in type 1 diabetes in humans (64). Additionally, there are data to suggest that manipulation of the peripheral immune system can affect self-tolerance and the expression of autoimmune diabetes in NOD mice (23, 206, 207). Mechanisms that control central and peripheral tolerance are different. Central tolerance is primarily mediated by intrathymic deletion of autoreactive T-cells during thymic development, whereas peripheral tolerance is mediated by multiple mechanisms, including deletion, anergy, and regulatory processes (9).

It is currently unknown whether improved central or peripheral tolerance is the mechanism by which the NOD congenic mice we studied in Chapter I were rendered resistant to autoimmune diabetes. Our data suggest, however, that if the mechanism of protection from diabetes is due to restoration of the factors that permit peripheral regulation of autoimmunity, these mechanisms are not sufficient for the induction of peripheral transplantation tolerance to skin allografts by costimulation blockade.
In Chapter I, we eliminated two additional possibilities for resistance to transplantation tolerance in NOD mice: absence of hemolytic complement and more rapid clearance of anti-CD154 mAb. Peripheral transplantation tolerance induction by DST and anti-CD154 mAb involves the deletion of alloreactive CD8+ T-cells (26, 96). Because CD8+ T-cells in NOD mice appear to be resistant to tolerance induction (54), this may be one mechanism by which NOD mice are resistant to costimulation blockade–induced tolerance. However, we have recently determined that the majority of high-affinity alloreactive CD4+ T-cells are also deleted by treatment with DST and anti-CD154 mAb (unpublished observations from our laboratory). CD4+ T-cells express CD154 when activated (158). This suggests that one possible mechanism by which DST and anti-CD154 mAb induces tolerance could involve deletion of alloreactive CD4+ T-cells by antibody-mediated, complement-dependent lysis. However, the ability to prolong skin allograft survival in congenic B10.D2 mice that lacked C5a and hemolytic complement argues that this defect does not prevent tolerance induction in NOD mice.

We have also documented that the circulating level of anti-CD154 mAb is inversely correlated with skin allograft survival in recipients treated with DST and anti-CD154 mAb (26). However, the clearance rate of anti-CD154 mAb from the circulation of NOD mice was similar to that of C57BL/6 mice. These data suggest that rapid clearance of anti-CD154 mAb, hence potentially lowering anti-CD154 mAb concentrations below effective tolerizing levels (~100 μg/ml) (26), was not the basis for the resistance of NOD mice to tolerance induction.
Examination of various cellular compartments in (NOD x C57BL/6)F1 mice for dominant NOD-like cellular abnormalities could explain our genetic observations. First, we found that (NOD x C57BL/6)F1 mice exhibit abnormal dendritic cell maturation. Second, we observed an abnormal response of (NOD x C57BL/6)F1 CD8α−/− mice to costimulation blockade, suggesting a defect in the response of CD4+ T cells in these animals.

An attractive candidate cell population to explain the resistance to transplantation tolerance is dendritic cells. Maturation of dendritic cells is abnormal in NOD mice (129-133, 177-179), and dendritic cells are the primary target of CD154 expressed by activated CD4+ T cells (176, 208, 209). We found that dendritic cells derived from NOD and (NOD x C57BL/6)F1 mouse bone marrow cultures mature abnormally, even when stimulated with an agonist anti-CD40 mAb. With respect to NOD dendritic cells, it is also interesting to note that, although reduced in number, the mature non-adherent dendritic cells generated by NOD bone marrow expressed higher levels of CD86 than did those from C57BL/6 controls. These apparently conflicting data on the maturation of NOD dendritic cells depending on the cell population analyzed may in part explain the differing reports in the literature that dendritic cells derived from NOD bone marrow cultures fail to mature normally (129, 130, 133, 177, 179) or are hyper-activated (131, 132).

We also observed that (NOD x C57BL/6)F1 CD8α−/− mice remain resistant to tolerance induction. A requirement for prolongation of skin allograft survival in mice treated with costimulation blockade is the deletion of host alloreactive CD8+ T cells (26,
The resistance of (NOD x C57BL/6)F1 CD8α⁻/⁻ mice to transplantation tolerance suggests that their CD4⁺ T cells respond abnormally to costimulation blockade.

At least three explanations for the brief skin allograft survival in tolerized (NOD x C57BL/6)F1 CD8α⁻/⁻ mice are possible. First, anti-CD154 mAb may fail to block alloreactive CD4⁺ T cell activity. Second, the absence of the CD8α⁺ dendritic cell subset in (NOD x C57BL/6)F1 CD8α⁻/⁻ mice may be important (124). This possibility seems unlikely, however, because we have shown that C57BL/6 CD8α⁻/⁻ knockout mice are susceptible to transplantation tolerance. Third, there may be defects in the function of regulatory CD4⁺CD25⁺ T cells; these cells are important both for the induction of transplantation tolerance (86, 87, 185-187) and for the expression of autoimmunity in NOD mice (45). We found that NOD/Lt, C57BL/6, and (NOD x C57BL/6)F1 mice have comparable percentages of small resting CD4⁺CD25⁺ spleen cells, but we recognize that functional defects in CD4⁺CD25⁺ T cells could nonetheless be present in (NOD x C57BL/6)F1 mice.

We speculate that dendritic cell maturation abnormalities and abnormal response of CD4⁺ T cells to costimulation blockade may be causally related in (NOD x C57BL/6)F1 mice. Costimulatory molecule expression by dendritic cells is important for modulating CD4⁺ T cell responses in both autoimmunity (139) and transplantation (141, 143, 175), and dendritic cells are thought to control the generation of regulatory CD4⁺CD25⁺ T cells (211-215). It has also been suggested that low expression of CD86 on NOD dendritic cells leads to the failure of CD4⁺ T cells to up-regulate CTLA-4,
contributing to impaired self-tolerance (133). CTLA-4 expression is also critical for the induction of peripheral transplantation tolerance and regulatory CD4^+CD25^+ T cells (27, 96, 216). Finally, NOD mice deficient in CD80 and CD86, the ligands for CTLA-4, rapidly develop diabetes, presumably due to deficiencies in regulatory CD4^+CD25^+ cells (45).

Additional cell populations defective in NOD mice are NK and NKT cells. Our observation that NK cell number and cytotoxic activity is normal in (NOD x C57BL/6)F1 mice suggests that NK cell defects are unlikely to be responsible for the resistance of (NOD x C57BL/6)F1 mice to tolerance induction. NKT cells appear to play a minor role in tolerance induced via DST and anti-CD154 mAb (unpublished observations) and cannot rule out a possible role for NKT cells in skin transplantation tolerance in (NOD x C57BL/6)F1 mice. Recently, work by our collaborators indicates that NKT cell function as assessed by in vivo activation with suboptimal doses of anti-CD3 mAb results in an improved IL-4 response, determined by quantitative RT-PCR (Laurence Peterson, personal communication). We recognize, however, that functions of both NK and NKT cells not measured in these studies (e.g. cytokine production) could be defective.

Defective macrophage function has previously been associated with impaired self tolerance and resistance of NOD mice to transplantation tolerance (12, 126, 184). Our analyses of macrophages derived from bone marrow cultures of (NOD x C57BL/6)F1 mice showed that their macrophage maturation appears to be normal. These data permit us to separate defects in macrophage maturation from defects in dendritic cell maturation and resistance to skin transplantation tolerance.
Finally, it remains to be determined if alloreactive CD8\(^+\) T cells in NOD and (NOD x C57BL/6)F1 mice resist deletion in response to costimulation blockade. Recent evidence suggests that NOD CD8\(^+\) T cells are resistant to deletion in response to peripheral tolerance induction to soluble antigens (54). In preliminary studies, we have obtained evidence that alloreactive TCR transgenic CD8\(^+\) T cells in (NOD x CBA)F1 mice are also relatively resistant to deletion following treatment with DST plus anti-CD154 mAb (unpublished observations).

It was surprising that transplantation tolerance to islet allografts is restored in (NOD x C57BL/6)F1 mice. This might reflect a difference in the requirement for a regulatory T cell population, as it has been suggested that this cell type is required for skin but not islet allograft tolerance (188). However, as noted above, the basal numbers of CD25\(^+\)CD4\(^+\) T cells appear to be normal in both NOD and (NOD x C57BL/6)F1 mice. Recently, others have also reported that basal numbers of CD4\(^+\)CD25\(^+\) T cells in prediabetic NOD mice are similar to other non-autoimmune strains (217). The exact cellular basis for the difference in skin versus islet allograft survival in (NOD x C57BL/6)F1 mice remains undetermined.

**Summary**

Our data documenting resistance to transplantation tolerance in (NOD x C57BL/6)F1 mice and in congeneric NOD mice bearing strongly protective Idd diabetes-resistant loci (72) (Chapter I) could be due to two different but not mutually exclusive genetic mechanisms. First, the transplantation tolerance defect could be controlled by the same genetic loci that control autoimmune diabetes, but the “genetic threshold” for
restoring susceptibility to transplantation tolerance could be higher than that required to prevent autoimmunity. Alternatively, the genes that control transplantation tolerance to skin allografts may be partially or even completely distinct from those that control autoimmunity.

The data in Chapter II unexpectedly reveal that poor skin allograft survival in response to costimulation blockade is a characteristic not only of autoimmune NOD mice (12, 218, 219) and NOD congenic mice that bear strongly protective Idd resistance loci (72) (Chapter I), but also of diabetes-free (NOD x C57BL/6)F1 mice (Chapter II). This dominant genetic resistance is not a maternally inherited trait and is not corrected by fixing to homozygosity strongly protective non-H2 Idd diabetes-resistant loci in the F1 NOD.\textit{Idd} x C57BL/6 intercross mice. What is most interesting is that transplantation tolerance to allogeneic islets appears to be normal in (NOD x C57BL/6)F1 mice, meaning that the genetic control of tolerance to skin versus islet allografts in these mice are different.

Finally, the preliminary results of Chapter III begin to shed light on the exact genetic basis of resistance to transplantation tolerance induction. The identification of two new loci unique from all currently described Idd loci as well as the implication of two suggestive markers near Idd13 and Idd15 will need to be verified and the intervals narrowed. However, this exciting preliminary data will hopefully lead to important contributions in the understanding of this confounding phenotype in NOD mice.

The exact genetic basis for resistance to skin allograft transplantation tolerance in NOD mice remains elusive, as does the exact genetic basis for their susceptibility to
autoimmunity. Our observations suggest that an ongoing autoimmune process, even one with low penetrance as measured by diabetes, is not responsible for the failure of tolerance induction in NOD mice. Although we have separated the skin transplantation tolerance resistance phenotype from the autoimmunity phenotype, we recognize that our data have not definitively falsified either the higher “threshold” hypothesis or the “distinct or overlapping genes” hypothesis.

Understanding the relationship between transplantation tolerance and autoimmunity represents an important area of research with implications for curing type 1 diabetes by islet transplantation. The assumption that autoimmunity and transplantation tolerance in NOD mice are controlled by exactly the same genes may be incorrect, and the data presented herein support this conclusion (Figure 13).

An immediate implication of the current findings concerns the way in which investigators evaluate tolerance induction protocols eventually intended for the clinic. If those protocols are evaluated in the NOD mouse, it must be asked if the defect in transplantation tolerance seen in these animals represents a defect that is likely to be encountered in people with type 1 diabetes or if it is a unique abnormality restricted to this mouse model. If the latter should prove true, this raises questions about the utility of the NOD mouse for modeling costimulation blockade-based transplantation tolerance induction in type 1 diabetes. Alternatively, the genome-wide scan for loci important in regulating transplantation tolerance induction may lead to the identification of human orthologues, much like the *Idd-IDDM* orthologous diabetes loci. In this event, we envision the development of more advanced therapies that take into account the specific
IDDM alleles, transplantation tolerance alleles and HLA haplotype when a person with type 1 diabetes is evaluated for a curative islet graft in the clinic.
Figure 13

ALTERNATE VIEW

Idd Loci

\[\Downarrow\]

Transplantation Tolerance Loci

\[\Downarrow\]

Cellular Abnormalities

\[\Downarrow\]

Cellular Abnormalities

\[?\]

Autoimmune Diabetes

\[?\]

Transplantation Tolerance Defect
Legend to Figure 13: A revised schematic diagram depicting the relationship between the genetic and cellular basis of autoimmunity and transplantation tolerance induction in NOD mice, based on the data presented in this dissertation. This alternative view proposes that different sets of genes contribute independently to the two phenotypes and that the ongoing autoimmune process does not control transplantation tolerance. The model does not exclude the possibility, as indicated by question marks, that the gene sets and the cellular abnormalities they cause overlap (See also Figure 2).
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Protected from Autoimmune Diabetes Remain Resistant to Transplantation


