IMMUNOLOGICAL MECHANISMS OF PANCREATIC ISLET TRANSPLANT REJECTION IN AUTOIMMUNE DIABETIC MICE

by

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Immunological Mechanisms of Pancreatic Islet Transplant Rejection in Autoimmune Diabetic Mice

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ABSTRACT

Type 1 diabetes (T1D) is a T cell-dependent autoimmune disease resulting from the destruction of insulin producing beta cells in the pancreas. Pancreatic islet transplantation is a potentially curative therapy for this lifelong disease. Unfortunately, transplanted insulin-producing cells in these recipients are subject to both autoimmune T cell responses as well as conventional anti-transplant responses. I focused on T cell-mediated rejection of pancreatic islet transplants in autoimmune non-obese diabetic (NOD) mice. This mouse model provides an excellent system that recapitulates many of the relevant clinical features of T1D.

It has been unclear whether autoimmune or anti-transplant T cell responses predominate in the autoimmune diabetic islet transplant recipient. Therefore, we tested whether expression of MHC on transplanted islet cells in the diabetic NOD mouse recipient was required for transplant rejection. Our results suggest that CD4 T cells in NOD mice do not require T cell receptor (TCR)-mediated MHC contact with their target cells to mediate transplant rejection if other non-self proteins are expressed.

We tested whether autoimmune T cells associated with islet allograft rejection were enriched for MHC-cross-reactive cells. Briefly, we harvested T cells infiltrating rejected islet allografts in NOD mice, isolated RNA, and sequenced TCR alpha and TCR beta chains. We then recapitulated allograft-enriched TCRs. Strikingly, we characterized four TCRs which confer both
islet-responsiveness and allogeneic cell responsiveness, demonstrating heterologous alloimmunity.

We tested whether a standard transplant tolerance-promoting co-stimulation blockade reagent would delay islet transplant rejection in autoimmune NOD mice. We observed moderately decreased autoreactive CD4+ T cell responses. In combination with peri-transplant CD8+ T cell depletion, we observed significantly prolonged islet transplant survival in autoimmune NOD mice. Unfortunately, when applied to the clinically relevant scenario of genetically unrelated pancreas donors the combination therapy was much less efficacious.

Overall, the research conducted for this dissertation has shown that (1) autoreactive T cells have the potential to cross-react with allogeneic MHC molecules, (2) autoreactive CD4 T cells can respond to and reject MHC-bald islet transplants, and (3) through interrogation of graft-infiltrating T cells, therapies can be developed which significantly delay islet transplant rejection.

The form and content of this abstract are approved. I recommend its publication.

Approved: Ronald G. Gill
DEDICATION

To those without whom I would not have completed this work:

To my father, who has always encouraged me to enjoy life.

To my mother, who has quietly reminded me to be humble, honest, and polite.

To my sister, who has persistently nudged me toward following my passion.

To my wife, who continues to remind me of all of the above.

Without you, none of what follows would have been possible.
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picture of the fall aspen trees around the 7-mile mark – and you toughed it out really well, even
though it was a brutal course and not the best day for either of us. I remember thinking then,
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together, particularly when you force me to think through my opinions and critically evaluate
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LIST OF ABBREVIATIONS

\( \alpha = \) alpha

APC = antigen presenting cell

\( \beta = \) beta

BG = blood glucose

CD = cluster of differentiation

ChgA = chromagranin A

DC = dendritic cell

ELISA = enzyme-linked immunosorbent assay

ER = endoplasmic reticulum

GAD = glutamic acid decarboxylase

HLA = human leukocyte antigen

IAPP = islet amyloid polypeptide

i.p. = intraperitoneal

IFN-\( \gamma \) = interferon gamma

IGRP = islet-specific glucose 6 phosphatase catalytic subunit-related protein

IL = interleukin

LCMV = lymphocytic choriomeningitis virus

MCSV = mouse stem cell virus

MST = median survival time

MHC = major histocompatibility complex

MLR = mixed lymphocyte reaction

NOD = non-obese diabetic

SZ = streptozotocin
T1D = type 1 diabetes

TCR = T cell receptor

NOD = non-obese diabetic mouse

PCR = polymerase chain reaction

PFA = paraformaldehyde

PLN = pancreas-draining lymph node

RAG = recombination activating gene

T2D = type 2 diabetes

TBE = tribromo ethanol

TNF-α = tumor necrosis factor alpha

ZnT8 = zinc transporter 8
CHAPTER I

INTRODUCTION

**Type 1 diabetes and type 2 diabetes**

Type 1 diabetes (T1D) is considered a classic example of an organ-specific T cell-mediated autoimmune disease. The best historical evidence in support of this contention are the classic 1986 study by Eisenbarth and colleagues demonstrating islet-specific autoantibody production in individuals with T1D (1–3), along with the islet transplantation studies from the University of Minnesota demonstrating islet-specific destruction in identical twin pancreas transplants in T1D patients (4). Clinical T1D is characterized by chronically elevated blood glucose levels due to autoimmune destruction of insulin-producing beta cells in the pancreatic islets. Current clinical understanding of the development of autoimmune diabetes is shown in figure 1-1, an update of the original Eisenbarth curve, which depicts the interaction of well-characterized genetic factors with environmental factors leading to T cell and B cell responses against beta cells. Following activation of T and B cells, gradual loss of beta cell mass ensues, along with detectable T cell and B cell responses against beta cell-derived peptides. Eventually C-peptide levels (a cleavage product of insulin production) become undetectable, correlating with dependence on injection of exogenous insulin to maintain euglycemia. At time of diagnosis with T1D, blood glucose levels are severely elevated, along with polyphagia, polydipsia, potential ketoacidosis, and other symptoms including blurred vision (5). Formerly termed juvenile diabetes, T1D is often diagnosed prior to the age of 5 years, and requires continuous exogenous replacement of the hormone insulin. Insulin replacement therapy was initiated in the 1920’s with semi-purified animal insulin, and has progressed through the use molecular biological techniques such that modern-day endocrinologists can choose among a multiple options of fast-acting and slow-acting recombinant human insulin analogs with which to treat
their patients (6). Encouragingly, ever-increasing numbers of patients with T1D survive >50 years with the disease with minimal development of diabetes-related complications, meeting the classification of 50-year ‘medalists’ (7). Intriguingly, there is a global trend of increasing prevalence of both early onset (<5 years of age) and later-life diagnoses of T1D (>20 years of age), throughout the world, with an average relative increase of 3-4% per year (8).

T1D differs from type 2 diabetes (T2D) in both physiological reasons for onset and baseline treatments. Unlike T1D, T2D is the result of peripheral target cell resistance to insulin. As a hormone, the primary physiologic role of insulin is to promote up-take of glucose into muscle and adipose cells via up-regulation of glucose transporters on the cell surface. The up-take of glucose into cells is a pre-requisite step for much of normal cellular metabolism to follow. For genetic and environmental (poor diet, lack of exercise) reasons that are not completely clear, the muscle and adipose cells of individuals who develop T2D develop resistance to the effects of insulin, leading to the outcome of chronically elevated blood glucose levels and derangements in metabolism of cells throughout the body including chronically systemic markers of inflammation (9). The primary treatments for T2D are lifestyle interventions (eg. dietary control, increased levels of moderate exercise), as well as pharmaceutical agents that promote insulin production by beta cells or promote target cell sensitivity. Secretagogues promote increased beta cell metabolic activity and insulin secretion, which are not a suitable treatment for T1D due to lack of endogenous beta cells. Furthermore, these drugs may not be ideal treatments for T2D, because peripheral cell resistance to insulin, not insufficient insulin production, is the primary pathology. Insulin sensitizing agents, including metformin, represent a more rational therapeutic approach to pharmacologic management of T2D primarily because beta cells in a T2D patient may undergo apoptosis due to the increased levels of metabolic stress, and this agent promotes increased levels of peripheral sensitivity to the insulin that is
produced by endogenous beta cells, leading to decreased requirements for insulin injections (10). As there are many metabolic abnormalities in T2D, metformin has been a component of multiple combination therapies, with successful metabolic outcomes for T2D patients (11, 12). An interesting current clinical trend in T1D management, which will not be further discussed in this review, is the recent trend of applying drugs developed to treat T2D to patients with T1D, including metformin (13) and pharmacologic kidney-tubule-specific glucose re-uptake transporter inhibition (14), in attempts to augment insulin injection-based diabetes management and prevent the development of the disease-related complications mentioned below. The rationale behind using insulin-sensitizing agents is to combat the development of insulin resistance in long-term T1D patients, which is a recently observed phenomenon (15). The rationale behind using glucose re-uptake inhibitors is to promote glucose excretion at the kidneys and glucose secretion in the urine of T1D patients. Promoting excretion of glucose is not without mild risks, including enhanced potential for urinary tract infections (16). In contrast to these pharmacologic strategies to decrease blood glucose levels, this dissertation focuses on the immune-mediated mechanisms of pancreatic islet transplant rejection in autoimmune diabetic mouse recipients.

**Diabetic complications requiring islet cell replacement therapy**

Once approximately >80% of the endogenous insulin-producing beta cells are destroyed, disease becomes symptomatic and T1D remains a lifelong disease requiring daily, and often hourly, blood glucose level management (17). Clinical management of T1D focuses on injecting a variety of insulin formulations in an effort to better promote glucose up-take by cells, lowering blood concentrations of glucose and making glucose available for intracellular metabolism. Several hormones (glucagon, epinephrine, and cortisol) act in opposition to insulin, with the primary effect of elevating blood glucose levels. Historically none of these latter agents
Briefly, well-characterized genetic predispositions to developing autoimmunity combine with environmental events (possibly including viral infections) to promote beta cell-specific attacks by T cells of the immune system. As these immune responses develop, a step-wise decrease in C-peptide levels in the serum occurs and detectable levels of autoantibody production against multiple beta cell-derived peptides increase. Overt diabetes occurs when fasting BG levels increase well over 150 mg/dl, multiple different autoantibodies are detectable, and C-peptide levels become undetectable.

have been deemed clinically relevant for treatment purposes, with the exception of glucagon.

During episodes of acute severe hypoglycemia, glucagon administration can be a life-saving treatment. Unfortunately, but not surprisingly given the ‘blunt’ tools available, T1D often results in large swings in blood glucose levels outside the normal physiologic range of 70-110 mg/dl.

Moderate to severe disease-related complications can develop over years of disease course in individuals with T1D with particularly labile blood glucose level management. These chronic complications can affect essentially every organ system, and are particularly pronounced in the
microvasculature. Diabetes – both T1D and T2D – are the leading cause of adult blindness (diabetic retinopathy, (18)) and end-stage renal failure (diabetic nephropathy, (19)), as well as a leading cause of lower-leg amputations (diabetic peripheral neuropathy, (20)), and heart disease (diabetic cardiomyopathy, (21, 22)). As such, T1D and T2D together represent a significant cost to the US health care system, recently estimated to be $17 billion annually by the NIH. Perhaps the most debilitating diabetic complication is hypoglycemic unawareness: when an individual with T1D is not aware their blood glucose levels are dangerously low (<50 mg/dl). This condition can result in seizures, diabetic coma, and in the most severe cases, death. Treatment with the hormone glucagon is a critical stopgap measure to prevent the development of diabetic comas in patients who have hypoglycemia unawareness. Patients with hypoglycemic unawareness often are not allowed to operate motor vehicles, due to the inherent risk to the public of operating heavy machinery with dangerously low and unpredictable blood glucose levels. In addition, the possibility of severe lows disqualifies individuals with T1D from employment as commercial pilots or long-haul truck drivers. The development of hypoglycemia unawareness is thought to be avoidable by patients with T1D through careful avoidance of hypoglycemia and prompt treatment with fast-acting carbohydrates upon recognition of blood glucose levels falling below 60 mg/dl.

Pharmacologic treatment with insulin analogs alone is not sufficient for some individuals with T1D to maintain blood glucose levels in the near-normal range. Whole pancreas or isolated pancreatic islet transplantation provides a potential path to restoration of biologically-controlled blood glucose level homeostasis for these individuals. However, the primary source of pancreata for transplantation purposes, cadavers, is severely limited. Therefore, T1D patients with severe disease-related complications, described above, are the only candidates currently considered for transplantation. Looking to the future, stem cell-derived insulin-producing beta cells may
become a viable alternative to cadaveric sources of pancreatic islets. Despite advances in directed differentiation of stem cells to insulin-producing beta cells, there are no current clinical-grade islet replacement therapies (23). Therefore, to ameliorate these complications – in particular with hypoglycemic unawareness – the current clinical strategy is to replace the lost beta cell function with whole pancreas or isolated pancreatic islet transplantation from genetically unrelated donors. Due to lack of genetically matched twins and the necessity of the exocrine pancreas in digestion, pancreas donors have generally been deceased cadaveric donors following permission from surviving kin. In addition to replacing lost beta cells, pancreas or isolated islet transplantation also replaces lost alpha cell function, which addresses the primary cause of severe hypoglycemia – lost glucagon production by alpha cells. Glucagon is the primary hormone which acts in opposition to insulin, promoting glycogen breakdown at the liver and therefore promoting increased levels of blood glucose for use by cells of the body. Therefore, pancreas or islet transplantation addresses two major needs for patients with hypoglycemic unawareness. Unfortunately, due to their autoimmune disease status, recipients of pancreas or isolated islet transplants must be treated with immune system-suppressive drugs following transplantation. In the absence of these treatments, transplanted beta cells in these patients would be subject to at least two categories of T cell responses: (a) autoimmune (islet-specific) responses by descendants of the T cells which destroyed the endogenous beta cells (24, 25), and (b) conventional anti-transplant-reactive T cell responses which would occur in non-autoimmune transplant recipients.

The non-obese diabetic (NOD) mouse model of T1D

The goal of this thesis project was to study pancreatic islet transplant rejection in diabetic NOD female mice as a model for the human transplantation scenario described above. The NOD mouse was first characterized in Japan, as a sub-strain of the ICR mouse originally
developed to study cataract development (11, 12). Depending on the colony/subline (Denver, Yale, San Diego, Miami, etc) approximately 50-90% of female NOD mice develop autoimmune diabetes within 30 weeks of age. The majority of new-onset diabetic NOD mice manifest blood glucose readings consistently above 250 mg/dl, between the ages of 10-26 weeks (28).

Generally, diabetes onset in male NOD mice is much less frequent, roughly 20% in the same age range, such that the vast majority of studies of autoimmune diabetes utilizing this strain of mice use female, not male, diabetic mice.

Over greater than 25 years of study, the NOD mouse has yielded much information regarding the genetics of autoimmunity (29), including several risk-associated alleles which have human orthologs related to T1D onset, including the IL-2 receptor (30, 31) and the regulatory T cell marker CTLA4 (32). Disease onset in female NOD mice is CD4 T cell-dependent (33–35) but also requires MHC class I expression on the islets themselves (36, 37), suggesting that both break-downs in CD4 T cell self-tolerance (addressed below) and CD8 T cell interaction with beta cell MHC class I are required for diabetes onset. However, the above is controversial with some reports indicating that CD8 T cell/MHC class I interactions are required at all but late stages of disease development (38), whereas others have concluded that MHC class I expression — and therefore physical interaction with autoreactive CD8 T cells — is required late in diabetes pathogenesis as well (39). Interestingly, transfer of disease from (non-TCR-transgenic) diabetic NOD mice into NOD.SCID recipients requires transfer of both CD4 and CD8 T cells (40). In addition, B cells appear to be the critical (autoantigen-specific) antigen-presenting cells in the initiation of autoreactive T cell responses in NOD mice (41). Despite the requirements of CD4 T cells, CD8 T cells, and B cells described above, in models of transferred CD4-mediated diabetes (including the BDC2.5 mouse, described below), macrophages may be the ‘final executioner’ of
beta cells, (42) meaning that autoreactive CD4 T cells may not require physical contact with their target cell to mediate cell death through the intermediary of activated macrophages.

At the Barbara Davis Center in Denver, Colorado, in the late 1980s, Haskins (43, 44), Wegman (45), et al utilized the NOD mouse to generate a series of pancreatic islet secretory granule-specific autoreactive CD4 T cell lines (43, 44). One CD4 T cell line in particular, the BDC2.5 cell line, has been extensively used in the cellular immunology community as a model of antigen-specific autoimmunity (46, 47). Since the development of these T cell lines, including the BDC2.5 TCR transgenic mouse by the Mathis group (48), as well as the study of other NOD-derived T cell clones, including IGRP-specific CD8 T cells first characterized by Santamaria’s group (49), the NOD mouse has proven to be a useful ‘work horse’ model system in which to study the pathogenesis and cellular immunology of T1D.

As mentioned above, B cells of the adaptive immune system may also play a critical role in development of T1D in mouse and man. Depletion of B cells by anti-CD20 antibody may prevent diabetes onset in NOD mice, and appears to delay clinical development of human T1D (50). As an antigen presenting cell, by virtue of the B cell receptor, B cells could activate T cells in an auto antigen-specific manner (51), and therefore have therapeutic potential as a method of (re)promoting self-tolerance to islet-derived peptides. Auto antibody levels have been used for >20 years as markers of clinical development of T1D (1, 52), and the development of auto antibodies specific for multiple different insulin secretory granule-associated peptides is used diagnostically as a sign of impending development of dysregulated blood glucose level management (53). However, auto antibodies, by themselves in serum from diabetic NOD mice, do not transfer diabetes and therefore are not considered intrinsically pathogenic. In addition, prevention of tertiary lymphoid organ formation in the pancreatic islets via genetic deletion of the chemokine CXCL13 – which is critical for directing trafficking and migration of B cells – has
recently demonstrated that tertiary lymphoid organ formation in islet is not required for development of T1D in NOD mice (54). In sum, B cells appear to be required for diabetes onset, but since auto antibodies alone do not transfer diabetes, the interaction between B cells and T cells appears to be critical for development of T1D.

In addition, the Lafferty and Gill labs at the Barbara Davis Center (33) and others including Ali Naji at Penn (55), Terry Strom at Harvard (56), Dale Greiner at UMass (57), Roland Tisch at UNC (58), the University of Miami group including Norma Sue Kenyon (59), and the group led by Thomas Kay at St Vincent’s Institute in Melbourne (60) have utilized the NOD mouse as a model system to study both autoimmune disease onset or islet transplant rejection (either self – syngeneic – or non-self – allogeneic, transplants). The NOD mouse was quickly recognized as an excellent model system in which to study autoimmune disease recurrence and islet allograft rejection (61). Due to its autoimmune disease status, the diabetic NOD female islet transplant recipient has historically represented a very stringent model to test islet transplant tolerance-promoting therapies. Several studies have demonstrated the requirement for CD4 T cells in diabetes recurrence in NOD mice (62, 63). Less data are available in the islet allograft scenario in NOD mice. **Figure 1-2** summarizes our current understanding of beta cell targeting and destruction in the NOD mouse. This model has historically been so stringent that the degree of technical skill and the sheer number of transplanted islets to restore euglycemia have been considered cost-and-effort-prohibitive.

Interestingly, the NOD mouse may not only be of use as a model of beta cell-specific autoimmunity. Recent studies have suggested that endoplasmic reticulum (ER) stress within ever-decreasing numbers of beta cells may play a significant role in signaling beta cell damage to the innate immune system through interleukin (IL)-1, promoting antigen-presenting cell (APC) activation and consequent T cell-mediated autoimmune diabetes (64). In this way, the NOD
Figure 1-2: Current understanding of T cell-mediated islet transplant rejection in NOD mice

mouse may be more similar to the Akita mouse model of diabetes than previously appreciated (65). The Akita mouse is a non-autoimmune model of ER stress-induced beta apoptosis, which develops diabetes on RAG−/− genetic backgrounds, in the absence of both T cells and B cells. Akita mice possess a dominant-negative mutation in the Insulin2 gene (mice have 2 insulin genes, whereas humans have 1 insulin gene) which renders insulin secretory granules incapable of being secreted, leading to ER stress and eventual apoptosis of the majority of beta cells. However, unlike NOD mice, Akita mice live for months with extremely elevated blood glucose levels (>400 mg/dl). As such, Akita mice are a useful model system to study the development of various diabetes-related complications (30) independent of autoimmunity and the degree to
which these complications may be reversed following normalization of blood glucose levels through islet transplantation.

Two other models of rodent autoimmune diabetes which deserve mention are the BB rat and the RIP-LCMV mouse. The BB rat, first characterized in 1985 (66), demonstrates approximately 50% disease onset (67), and appears to have immunological characteristics in common with human type 1 diabetes (68) – though the genetics of diabetes onset in this model are less well characterized. The RIP-LCMV mouse is derived from the C57BL/6 strain, and expresses the immunodominant CD8 T cell epitope of lymphocytic choriomeningitis virus (LCMV) under the control of the rat insulin promoter (69). As such, these mice provide an excellent model of T cell-mediated autoimmune destruction of pancreatic beta cells. However, there is no role for B cells in beta cell destruction (70), and RIP-LCMV mice are not spontaneously autoimmune – they destroy LCMV peptide-expressing beta cells only when infected with LCMV. In contrast, the NOD mouse is spontaneously autoreactive against beta cell-derived peptides and shares several important genetic similarities with human T1D, including an HLA/MHC bias and multiple autoantigenic peptides. Therefore, to study autoimmune T cell responses to beta cell-derived antigens, the NOD mouse is our model of choice.

Potential roles of innate immunity in the development of T1D

In addition to the above discussion of the roles of T cells and B cells in T1D onset in the NOD mouse, the cells of the innate immune system appear to play a less critical role in T1D onset. Natural killer (NK) cells are not required for disease onset (71). In addition, NKT cells (72, 73) and gamma-delta T cells (74, 75) may either accelerate or delay diabetes onset depending on the experimental conditions. The role of macrophages in T1D onset is controversial, with evidence for potential of macrophages to exacerbate disease onset (76) or for M2 macrophages to prevent diabetes onset (77). As mentioned above, in models of transferred diabetes using...
CD4-expressing TCR-transgenic cells, macrophages may be the final effector cells mediating both beta cell death and clearance of dead cells. In addition, some human patients with T1D demonstrate clinical-grade neutropenia (78), arguing against a critical role for neutrophils specifically and for innate immune cells generally in T1D onset. These data argue against a dominant protective role for innate immune cells to be harnessed therapeutically in attempts to prevent beta cell destruction by adaptive immune cells, with the potential exception of M2 macrophages.

T cells and B cells interact with each other and with another critical antigen presenting cell type, dendritic cells, in several important ways during the development of T1D. In particular, dendritic cells present peptides to T cells via HLA or MHC molecules to ‘prime’ T cells to respond to foreign antigens and not to respond to self-derived antigens. An example in the NOD mouse model of T1D are ‘merocytic’ dendritic cells, which appear to interact with T cells for longer durations of time than conventional dendritic cells, and are more prevalent in NOD mice than in other strains (79). Interestingly, the prevalence of this sub-type of dendritic cell in mouse models correlates with the presence of the Idd3 diabetes susceptibility loci (80). These data suggest that duration of the T cell-APC interaction is genetically influenced in the NOD mouse, and which sets the stage for diabetes development. Lastly, CD4 T cell-APC interaction in the pancreas-draining lymph node (PLN) are required for diabetes development in BDC2.5 TCR transgenic mice (81), demonstrating both the required cell-cell interaction and the anatomic location at which this interaction must occur for subsequent diabetes development. Two additional questions, addressed below, are (a) what are the antigenic specificities required for diabetes development? And (b) how does self-tolerance fail leading to the leakage of these autoreactive T cells into the periphery?
**Breakdown in normal self-tolerance mechanisms leading to T1D development**

Development of T1D is a prime example in the break-down of normal self-tolerance. Tolerance to self-derived peptides is established in the thymus as T cells develop and are exposed to self-derived peptides via the AIRE transcription factor and thymic epithelial medullary cells (82). Briefly, some developing thymocytes interact too strongly with self-peptides, receive too strong of signals through the T cell receptor, and undergo signaling which promotes apoptosis. This process is termed negative selection (83). Critical contributions to the current view of thymic T cell development, including the role of thymic epithelial cells in T cell selection and characterization of the role of strength of TCR signaling in the T cell survival ‘decision’, were made by the Kappler-Marrack lab in the UC-Denver immunology department (84–86). Alternatively, other T cells receive too weak a TCR-mediated signal and do not up-regulate TCR signaling to the level of a ‘survive’, or positive selection signal. However, T cells that will go on to survive both positive selection and negative selection receive a ‘strong enough, but not too strong’ TCR-mediated signal, termed the Goldilocks selection model (87, 88). Recent evidence has also demonstrated the presence of AIRE-expressing epithelial cells in peripheral secondary lymphoid organs (89), along with functional studies demonstrating that these lymph node-resistant epithelial cells also mediate T cell selection processes, further pruning the peripheral T cell population in attempts to decrease the presence of autoreactive T cells.

In addition to pruning the potentially autoreactive T cell repertoire, there is also a thymic selection of regulatory T cells that serve an additional protective role against the development of autoimmunity. Current opinion in the field is that the T cells that go on to become regulatory CD4+ T cells are derived from the relatively strong TCR signaling in developing T cells that survive the Goldilocks selection process (90). The requirement of
regulatory T cells in protection from systemic autoimmunity has been demonstrated through mouse models in which the transcription factor Foxp3 has been deleted (91, 92). This transcription factor is a master regulator of many regulatory T cell-associated genes (including the IL-2 receptor, decreased expression of which was mentioned above as a diabetes risk factor), as well as a negative regulator of many effector T cell-associated genes. Mice lacking this transcription factor, including in inducible deletion models in which T cells expressing this transcription factor can be deleted in adult mice that have developed normally (93) can develop severe systemic autoimmunity.

Despite clear roles for MHC/HLA polymorphisms and ineffective regulatory T cells, T1D is a polygenic disorder that involves the contribution of many different genes. Interestingly, the strongest genetic correlations with T1D onset are polymorphisms in HLA molecules themselves, the molecules which present peptides to T cells of the immune system (94). HLA molecules, by virtue of their peptide presentation role during thymic development of T cells, play a significant part in determining the three-dimensional structure (MHC + peptide) which T cell receptors in the thymus ‘see’ and have the option to respond to with intracellular survival signals. In the mouse model of T1D, MHC molecules are also critical to diabetes onset: NOD mice which are genetically engineered to express non-disease-associated MHC molecules do not develop diabetes due to apparently beneficial polymorphisms in the CD30, TNFR2 and CD137 genes (95). However, MHC alone does not confer disease either: non-NOD mice which are genetically engineered to express disease-associated MHC do not develop diabetes including B6.H-2g7 mice (96). Many other T cell activation (for example, IL-2 receptor, PTPN22) and antigen presentation-associated genes have been associated with T1D risk in human populations (97), however none of these other associations are as strong as the association disease-related HLA polymorphisms.
(DQ8 and DR4 in particular, (98)), and have not been studied as extensively in the murine T1D model system.

**Potential role of the gut microbiome in the development of T1D**

There has recently been a high degree of interest in the potential role(s) the gut flora may influence the development of digestive tract-related autoimmune disorders. Microbes within the gut lumen play a critical role in the development of the immune system, and in particular in IgA antibody response development (99). Gut-directed immune disorders, including irritable bowel syndrome, Crohn’s disease, and colitis, have been associated with changes in the bacterial content of the intestine (100–102). Since the pancreas is closely associated with the gut lumen, it is reasonable to speculate whether the gut microbiome may positively or negatively affect the development of autoimmune diabetes. Along this line, MyD88−/− NOD mice do not develop autoimmune diabetes (103) and the intestinal microbiome of these NOD background mice is significantly altered from conventional NOD mice. MyD88 is a signaling molecule critical to Toll-like Receptor (TLR)-mediated signaling in most cells of the body (104, 105). Therefore MyD88-deficient mice have greatly decreased ability to respond to common bacterial and viral products, and most likely have greatly decreased levels of inflammation in the gut due to decreased innate immune responses to both commensal and pathogenic gut flora.

Unfortunately, follow-up studies have shown that this protection is not transferable to MyD88-sufficient NOD mice (106), indicating that gut microbiome responsiveness by immune cells may serve to accelerate diabetes onset. However, there is little evidence to suggest that decreased anti-microbiome responsiveness is not dominantly-protective against development of anti-islet autoimmunity in NOD mice. Moving forward, it will be a task for the field to determine what role the microbiome plays in accelerating T cell responses in autoimmune-prone settings, especially in the context of human patient samples. The interaction of diabetes-susceptible MHC
with pro-inflammatory gut microbiota may yet prove to be a crucial link in the chain of developmental events leading to autoimmune diabetes.

**Insulin secretory granule peptide-specific T cells in development of T1D**

To date, the majority of auto antigens characterized in the NOD mouse have been derived from the insulin secretory granule (Figure 1.3). CD4 T cell epitopes discovered to date include insulin B chain epitopes (107), chromagranin A epitopes (108), islet amyloid polypeptide (IAPP) epitopes (109, 110). CD8 T cell epitopes include peptides derived from islet-specific glucose 6 phosphatase catalytic subunit-related protein (IGRP) (111), Zinc transporter 8 (112, 113), and glutamic acid decarboxylase (GAD)65 (114). Of particular importance in both the NOD mouse model system and for translation to the human disease, is a peptide derived from amino acids 10-23 of the insulin B chain (InsB10:23). This peptide is absolutely required for the development of autoimmune diabetes in the NOD mouse (107). If the amino acid at position 16 is mutated to alanine, the mice will not develop diabetes. In addition, re-establishing immune tolerance to pro-insulin (PI), a CD4 T cell antigen, prevents diabetes onset, but re-establishing tolerance to IGRP206-214, a CD8 T cell antigen, does not prevent diabetes (60), emphasizing the primacy of anti-insulin T cell responses in the development of autoimmune diabetes. Figure 1-3 summarizes intracellular locations of beta cell-derived auto-antigens. As pioneered by the late George Eisenbarth, autoantibody responses against multiple different T cell antigens are highly predictive of diabetes onset within 12-36 months (5, 52, 115). As shown by sibling studies (DAISY, TEDDY), the presence of 1 known auto antibody response is a moderate risk level, with risk of imminent development of diabetes increasing exponentially with the detection of each additional auto antibody response. In this way diabetes risk can be stratified among the at-risk population, as determined by HLA typing.
Controversy over the nature of islet injury in the NOD mouse model of T1D

The data gathered to date in the NOD mouse during diabetes onset challenge the traditional view of T cell subset interaction. Classically, CD4 T cells are considered to serve as ‘helper’ cells which promote activation of CD8 T cells, and the CD8 T cells then go on to act as ‘final executioners’ in immune responses. The data to date regarding diabetes onset in the NOD mouse are consistent with a model in which early CD8 T cell infiltration – as shown in human pancreatic sections (116) – help to activate CD4 T cells, which may then act as the ‘final executioner’ of beta cells either through cytokines IFN-γ and TNF-α or through activation of innate cells including macrophages. Consistent with this model, in a recent study of autoimmune
disease recurrence in NOD mice (117), IGRP-specific CD8 T cells accumulated within rejected transplants only after islet transplant rejection, and IGRP expression in transplanted islet cells was not required for acute transplant rejection. As such, I propose the presence and activation of auto-aggressive CD8 T cells within diabetes onset lesions may serve as a ‘red herring’. I speculate that, while required for disease onset, CD8 T cells are not the cell driving this immune response. Instead, I propose that secretory granule-specific CD4 T cells drive diabetes onset, and are the critical cell type which must be “re-educated” to stop new-onset diabetes, or prevent the development of clinical hyperglycemia.

**Islet allograft tolerance in non-autoimmune-prone streptozotocin-induced diabetic mice:**

**proof of principle**

As mentioned above, a major goal in the treatment of T1D is beta cell replacement. However, as discussed above, such transplants are subject to autoimmune disease recurrence and/or allograft rejection. Therefore, therapies that induce immune system tolerance to transplanted beta cells would be preferable to the current standard of care following pancreas and islet transplantation: immune suppression protocols. To test whether candidate T cell-directed therapies are capable of promoting islet allograft tolerance in the absence of autoimmune diabetes, the Gill lab (118) and others (119) have made extensive use of a chemically-induced model of diabetes through the use of a beta cell toxin (120, 121).

Streptozotocin induces diabetes – within a narrow dose range characteristic for each strain of mice – due to the relative lack of free radical scavenging enzymes expressed in pancreatic beta cells relative to other cell types. At too high a dose of streptozotocin damage to other organs, in particular the kidneys, will occur. Following induction of diabetes with this chemical agent, mice can be transplanted with allogeneic (MHC-disparate) pancreatic islets and treated with candidate transplant tolerance-promoting therapies.
Multiple different general immune suppressive therapies have been tested in mouse models and are used clinically (122). Used primarily in conjunction with whole pancreas transplantation in the clinic, these therapies can include anti-CD3/anti-thymocyte globulin (ATG), calcineurin inhibitors, mTOR inhibitors, tacrolimus, or mycophenolate mofetil (123). In contrast to these general immune suppression or cell depletion treatments, in this dissertation the attempt was to focus on more specific, mechanistic, therapeutic approaches which directly inhibited the most relevant T cell populations. Of particular interest for this dissertation, short-term monoclonal antibody therapy directed against the T cell-expressed co-stimulation molecule CD154 (MR-1) has been shown by our group (118) and others (124) to induce long-term (>100 day) islet allograft tolerance across full MHC mismatch donor/recipient pairs (BALB/c islets transplanted into strept-treated B6 male mice). This tolerance resides in the CD4 T cell compartment, and can be transferred from treated and tolerant mice to naïve mice (118). It is controversial whether this therapy induces allo-specific regulatory T cells de novo (125) or inhibits reactivity of naïve alloreactive CD8 T cells through killing mediated by NK cells (126), or if these effects are simultaneous. In addition, the combination of anti-CD154 antibody with other therapies has been highly efficacious in strep-induced diabetic islet allograft tolerance induction. The Gill lab combined anti-CD154 perturbation with blockade of leukocyte functional antigen (LFA) using anti-LFA1 antibody treatment (118). LFA1 is an adhesion molecule expressed on most lymphocytes, in particular on neutrophils, macrophages, and activated T cells, the inhibition of which appears to delay and/or prevent islet allograft rejection as a single therapy. In combination with anti-CD154 therapy, anti-LFA1 mediated virtually impenetrable islet allograft tolerance in STZ-induced diabetic mice. Similar to anti-CD154-induced transplant tolerance, tolerance induced by this combination therapy resided in the CD4 T cell compartment and was serially transferable to multiple recipients. The Miami Diabetes Center group extended
these studies to the NOD mouse, with less impressive results (127), highlighting the stringency of the NOD model for islet transplantation tolerance. In summary, STZ-induced diabetes represents a useful, non-autoimmune model system in which one can test candidate islet allograft tolerance-promoting therapies. However, the more clinically-relevant test of therapeutic efficacy, and the reason for validating these agents in animal models, is of course the autoimmune diabetic recipient.

**Islet transplant tolerance in the NOD mouse: where reductionist models fail**

As mentioned above, a promising combination therapy developed in the STZ-induced diabetic mouse/islet allograft model system yielded less impressive results in the autoimmune NOD mouse/islet allograft model system. This general observation has been a relatively consistent trend in the NOD mouse – the autoimmune recipient represents a stringent barrier to T cell-directed tolerance-promoting therapies (57, 124, 127). It has been a point of contention in the autoimmunity and transplantation fields whether this stringency was due to resistant to therapeutic intervention in the autoimmune primed/memory T cell compartment, the alloreactive T cell response in NOD mice, or in both populations.

General immune suppression is used in autoimmune diabetic recipients of whole pancreas or isolated islet transplants because mouse model and human clinical reports have suggested that autoimmune T cells resist therapeutic intervention (24, 127). Other evidence supports the existence of an accelerated and therapy-resistant anti-graft cell MHC-directed T cell response in NOD mice (128). Further muddying the waters on this issue, studies in the BB rat (a controversial and less-used model of T1D) have suggested that autoimmune T cells strongly resist therapeutic intervention – but are amenable to bone marrow transplantation with disease-resistant bone marrow – whereas the anti-allograft response can be made tolerant (129–132).
Due in part to the above confusion, clinical pancreas and islet transplantation protocols currently rely on global immune suppressive treatments. While these suppressive treatments promote survival of transplanted beta cells, it is challenging to interpret effects on specific T cell populations in the presence of non-specific treatments. In addition, in the autoimmune recipient there at least two concurrent immune responses occurring, adding further confusion in attempts to interpret therapeutic effect on recurrent autoimmunity or anti-allograft responses. In addition, the human recipient of a potentially life-altering transplanted organ is not the ethically appropriate scenario for controlled experimentation. We therefore chose to turn to the NOD mouse model of T1D to systematically test whether autoimmune T cells are amenable to pancreatic islets tolerance-promoting therapies, and to further test whether alloreactive T cells in autoimmune recipients are amenable to the same therapies. Once we have begun to develop evidence-based answers to this set of questions, we can then move forward to test whether allograft-reactive T cells in autoimmune mice are amenable to the same therapies. As a pre-requisite, however, it would be highly beneficial to develop a mechanistic understanding of which autoimmune T cells must interact with which graft cell-derived MHC molecules in order to mediate islet transplant rejection in autoimmune recipients.

Questions specifically addressed in this dissertation

The experiments in this dissertation in autoimmunity and transplantation were designed to address disconnects between model systems (SZ-induced diabetic mice) and islet allograft rejection in autoimmune diabetic NOD mice, which are the most clinically relevant model system available. We used diabetic female NOD mice as recipients of pancreatic islet transplants to test the MHC expression requirements for graft rejection and to test the effects of transplant tolerance-promoting therapies in the autoimmune recipient. Figure 1-4 illustrates the general workflow for the experiments which tested the hypotheses described below.
In chapter III, I describe the results of experiments in which we tested the **hypothesis** that islet graft cell MHC expression is required for transplant rejection in autoimmune NOD recipients. As preliminary data, we were aware from others that NOD MHC class I-deficient transplants are not rejected in diabetic NOD mice (133), strongly suggesting that direct CD8 T cell TCR interactions with islet MHC class I molecules was required for autoimmune disease recurrence. We were also aware from our own studies that CD4 T cells are required for autoimmune disease recurrence (134). In addition, B6 islets lacking either MHC class I expression or MHC class II expression are rejected in strep-induced diabetic BALB/c mice, but B6 background islets lacking both MHC class I and class II are not rejected, indicating that expression of an allogeneic MHC (either class I or class II) is required for islet transplant rejection in the non-autoimmune recipient (135). Based on these preliminary data, we predicted that B6 islets lacking both MHC class I and class II would not be rejected in autoimmune recipients, due to lack of ability to physically interact through TCR-MHC interactions with the transplant.

In chapter IV, I describe results in which we studied whether non-MHC-derived, ‘minor’ transplantation antigens could accelerate islet transplant rejection in NOD mice. Specifically, we hypothesized that NOD mice, in the absence of overt autoimmune disease status, would **rapidly respond to non-MHC graft cell differences.** If true, this hypothesis would predict that NOD MHC-matched but minor antigen disparate islet transplants would be rejected in strep-induced diabetic NOD mice in the same time frame as MHC-disparate islets. Furthermore, an implication of this hypothesis is that diabetes-associated MHC, I-Ag7, may mediate accelerated minor-antigen-directed islet transplant rejection outside the context of NOD background genes. To test this intriguing possibility we utilized MHC congenic mice on the B6 and BALB/c genetic backgrounds. If this hypothesis were correct, it would imply that the resistance of the NOD to the induction of transplantation tolerance in the absence of autoimmune diabetes may be due
Spontaneous onset diabetic NOD mice were grafted with 500 handpicked syngeneic (NOD.RAG\textsuperscript{-/-}) or allogeneic (B6, B10.BR, C3D) pancreatic islets. Transplant recipients that reached euglycemic blood glucose levels (>70 mg/dl and <200 mg/dl) within 48 hours of transplantation were observed for return to hyperglycemia (BG>300 mg/dl), as a proxy of transplant rejection. Within 48-72 hours of return to hyperglycemia, mice were euthanized and graft-infiltrating T cells were analyzed or the graft-bearing kidney was harvested for histology.

In chapter V, I describe experiments where we tested the hypothesis that individual TCRs isolated from islet allograft rejection lesions in autoimmune diabetic recipients would exhibit enriched ability to cross-react with allogeneic MHC molecules. We predicted that T cells which demonstrated the ability to cross-react with graft cell MHC would be enriched within rejected islet allografts in NOD mice compared to the endogenous pancreas of the same mice and compared to autoimmune disease recurrence lesions (rejection of syngeneic islets) in NOD mice. To pursue these experiments we transplanted autoimmune diabetic NOD mice with fully...
MHC mismatched C3H (H-2k) islets, or with NOD.RAG-s islets as disease recurrence controls, isolated both pancreas-infiltrating and transplant-infiltrating T cells at the time of transplant rejection – measured by return to hyperglycemia – and subjected both T cell populations to 454 deep sequencing. We then recapitulated highly graft-enriched TCR alpha/TCR beta pairs \textit{ex vivo} in hybridoma cells, and tested the degree of reactivity of cells bearing these recapitulated TCRs to NOD.RAG-s islets and C3H spleen cells. Our prediction at the outset of these experiments was that we would detect enrichment of autoreactive TCR sequences within rejected islet allografts in NOD mice, and further that the most highly enriched of these TCR sequences would demonstrate heterologous alloimmunity, as shown in figure 1-5b. An important clinical correlate of these predictions is that TCRs demonstrating this category of dual-reactivity may be a cell type which is partly responsible for the observed rapid and therapy-resistant islet allograft rejection in autoimmune recipients. Furthermore, the characterization of this category of T cell reactivity would provide a potential partial explanation for the general resistance that autoimmune humans and NOD mice demonstrate to tolerance-promoting therapies for transplants other than insulin-producing cells, including kidney transplants.

In chapter VI, I describe results in which \textbf{we tested the hypothesis that autoimmune T cells would resist the standard islet allograft tolerance-promoting therapy, anti-CD154 antibody treatment}. To test this hypothesis we treated autoimmune NOD mice with anti-CD154 peri-transplant, and grafted the mice with syngeneic islets. At the time of transplant rejection we harvested graft infiltrate T cells and re-stimulated \textit{ex vivo} to determine recent activation status. For comparison, we harvested and re-stimulated graft-infiltrating T cells following
Figure 1-5: Models of heterologous alloimmunity leading to pancreatic islet transplant rejection in diabetic NOD mice. Figure 1-5a: Indirect recognition of peptides derived from donor-derived MHC molecules is recipient MHC-restricted, meaning that self APCs present peptides derived from non-self MHC molecules to T cells, leading to T cell activation. Importantly, this pathway may include recognition of cognate auto antigen or modified auto antigen peptides. Figure 1-5b: Heterologous alloimmunity is defined as the situation when an autoreactive (memory) T cell is activated by interaction with allogeneic MHC molecules presenting peptides other than the cognate peptide. Figure 1-5c: Cross-restriction is defined as an autoreactive (memory) T cell recognizing its cognate peptide in the context of an allogeneic (transplant-derived) MHC molecule, leading to T cell activation.

Untreated autoimmune disease recurrence in NOD mice. Surprisingly, anti-CD154 therapy decreased autoreactive CD4 T cell responses, nearly doubling islet transplant survival time.

When combined with peri-transplant CD8 T cell depletion – used as a second component because anti-CD154 therapy showed comparatively little down-regulatory affect on autoreactive CD8 T cell responses – transplant survival time more than doubled again, to a mean survival time of 54 days. Greatly encouraged by this result, and keeping in mind our earlier results, we applied this combination therapy to transplantation into the autoimmune recipient with MHC-matched islets (B6.H-2g7), minor-matched islets (NOD.B10), and minor- and major-mismatched islets (B6).
CHAPTER II
MATERIALS AND METHODS

Mice

Mice were purchased from Jackson labs and bred in-house: NOD.BDC colony (mice transplanted were from generation 99-116 generations of sibling breeding), C57BL/6J (Jackson strain #000664), B6.B2M^-/- (Jackson strain #002439), B6.C2D (Jackson strain #003374), NOD.RAG^-/- (Jackson strain #003729), NOD.B2M^-/- (Jackson strain #002309), NOD.CIITA^-/- (Jackson strain #004448), NOD.Ca^-/- (Jackson strain #004444), NOD.Cb^-/- (Jackson strain #023082), B10.BR (Jackson strain #004804), C3H/HeJ (Jackson strain #000659), NOD.B10 (Jackson strain #002591), NOD.RAG^-/- (Jackson strain #003729), NOD.Ca^-/- (Jackson strain #004444), NOD.Cb^-/- (Jackson strain #023082) and BALB/cJ (Jackson strain #000651). Congenic BALB.H-2^g7 mice were generated by backcrossing B6.H-2g7 mice with BALB/c mice for >14 generations and selecting for the NOD.H-2g7 genetic region microsatellite via DNA screening (R Gill, personal communication). Congenic lab strain B6.H-2^g7 (Jackson strain #003300) was maintained by in-house breeding.

B6.B2M^-/- mice were interbred with B6.C2D to generate B6.MHC-bald mice. Progeny were screened for lack of both B2M expression and lack of MHC class II expression. Breeders were selected to ensure lack of expression of both MHC loci via PCR screening. It required >7 generations of DNA screening and selection to produce true-breeding double MHC-deficient B6 mice. Similarly, NOD.B2M^-/- mice were bred with NOD.CIITA^-/- (lacking expression of class II trans-activator) to yield NOD.MHC-bald mice. Progeny were screened via PCR for lack of both B2M expression and lack of CIITA expression. Breeders were selected to ensure lack of expression of both genes of interest via PCR screening. It required >7 generations of DNA screening and selection to produce true-breeding double MHC-deficient NOD background mice. All animal
experiments were approved by the University of Colorado Denver Institutional Animal Care and Use Committee (IACUC).

**Autoimmune diabetes onset and severity**

To screen for pancreatic islet transplantation candidate mice, blood glucose level of female NOD mice (NODbdc) in the Barbara Davis Center NOD colony (28) were monitored 2-3 times weekly for diabetes onset using Embrace blood glucose meters (Omnis Health). Screening for spontaneous onset of diabetes in female NOD mice began from at 10 weeks of age until 26 weeks of age, at which time non-diabetic female NODs which were not in breeding pairs were euthanized. Transplant recipient candidates were female NOD mice between 10 and 26 weeks of age with at least three consecutive days of blood glucose readings >250 mg/dl. Because of higher frequencies of spontaneous diabetes onset in female NOD mice (55-65% in the NOD.BDC colony) relative to male NOD mice (<20% in other colonies historically, including the BDC colony), only females were monitored for diabetes onset. Transplant recipients were grafted within 7 days of diabetes onset using procedures described below.

**Streptozotocin (SZ) induction of diabetes**

To chemically induce diabetes, 8-12 week old male NOD mice were injected retro-orbitally with 180 mg/kg of the free radical generator streptozotocin (Sigma-Aldrich) (136). Beginning approximately 72 hours after treatment, diabetic disease status was assessed using Embrace blood glucose meters (Omnis Health). Diabetes was defined as consecutive days with blood glucose readings >300 mg/dl, after which islet transplantation was performed to normalize blood glucose levels using procedures described below. In additional studies, 8-12 week old male B6.g7 mice were also treated with 180 mg/kg of SZ, 8-12 week old male BALB.g7 mice were treated with 225 mg/kg of SZ.
**T cell depletion protocols**

To determine requirement for T cell sub-sets in pancreatic islet transplant rejection in NOD recipients we depleted peripheral CD4 or CD8 T cells peri-transplant. Unless otherwise noted in figure legends, CD4+ T cells were depleted using a dose of 20 mg/kg of GK1.5 anti-CD4 monoclonal antibody (137) delivered via the intraperitoneal (ip.) route on days -2, 0, 2, 7, and 14 relative to islet transplantation. Unless otherwise noted in figure legends, CD8 T cells were depleted using a dose of 20 mg/kg of 53.6-7 anti-CD8a monoclonal antibody (138) delivered ip. on days -2, 0, 2, 7, and 14 relative to transplantation. Longer course depletions were used where noted in figure legends. T cell depletion regimens successfully depleted >90% of the relevant T cell populations as determined by analysis of T cells in the spleen on day of islet transplant harvest (data not shown).

**Regulatory T cell inhibition protocols**

Regulatory T cells delay diabetes onset and were hypothesized to delay autoimmune disease recurrence in NOD mice (see chapter VI). Therefore we pursued two strategies to inhibit regulatory T cell function in autoimmune recipients of NOD-background pancreatic islets. Regulatory T cell function was inhibited treating mice with anti-CD25 PC61 antibody (139) at 125 µg per dose of delivered i.p. on days -1, 2 relative to islet transplantation (BioXCell BE0012) or with anti-PD1 antibody clone J43 (140) at 125 µg per dose delivered i.p. days -2, 0, 2, 4, 6, and 8 relative to islet transplantation (BioXCell BE0033-2).

**IL-7 Receptor blockade**

Following two published reports (141, 142) demonstrating that IL-7 receptor blockade can stop new onset diabetes in NOD mice, we extended this treatment to studies of autoimmune disease recurrence. Toward this end, we treated a cohort of autoimmune diabetic syngeneic islet transplant recipients with IL-7 receptor blockade antibody (clone A7R34) at a
dose of 125 µg anti-IL-7 receptor antibody on days -2, 0, 2, 4, 6, 8 relative to transplantation (see chapter VI).

**Co-stimulation blockade treatment**

To investigate the effect of co-stimulation perturbation treatment on (1) autoimmune disease recurrence, (2) rejection of MHC-matched transplants in autoimmune recipients, and (3) rejection of MHC-disparate islet transplants in autoimmune recipients, we administered co-stimulation perturbation treatments peri-transplant. Transplant recipient mice were treated with 125 mg/kg of anti-CD154 antibody clone MR-1 (143) days -1, 2, 7, and 9 relative to islet transplantation (see chapter VI). MR-1 was purchased from BioXCell (#BE0017-1). This treatment regimen has been shown by our group and others to promote islet allograft tolerance in non-autoimmune recipients (59, 118, 127, 144). In this dissertation, we extended the analysis of the effects of this treatment beyond graft survival time, to include analysis of graft-infiltrating T cells, as described below.

**Islet isolation**

Treatment of pancreas donor mice was in accordance with University of Colorado – Anschutz Medical Campus IACUC regulations. Briefly, islet donor mice were anesthetized to surgical plane with tri-bromo ethanol (TBE), euthanized via severing the aorta, and infused with 4 ml of an ice-cold 2.5 mg/ml collagenase V (Sigma Chemical) solution via the common bile duct. Following distension with collagenase solution, two pancreata per 50 ml conical were incubated in a 37°C water bath for 8-10 minutes, depending on strain of islet donor mice. Following 37°C incubation, pancreata were vigorously shaken 4-6 times, then washed 3 times for 5 minutes each with HBSS/HEPES, 10 µg/ml DNase, 0.1% bovine serum albumin, 1% penicillin/streptomycin, and passed through a 500 µm metal filter to separate the islets from surrounding undigested acinar tissue. Islets were purified using lympholyte 1.1 gradient
(Cedarlane Labs) centrifugation at 1800 rpm for 15 minutes. The media-lympholyte interface was removed, and the islets were further washed 3 times for 5 minutes each in HBSS/HEPES, 10% fetal bovine serum and 1% penicillin/streptomycin, before handpicking and counting to yield 450-500 islet aliquots. In general, it required 5 NOD-background mice, 3-4 B6 background mice, and 2-3 BALB/c, B10.BR, or C3H mice to yield 500 islets.

**Islet transplantation**

Islets were hand-counted as described (118, 126). Each transplant recipient mouse received 450-500 normalized by size to deliver a standard volume of islets per recipient upon spinning in PE-50 tubing. Islets were drawn into PE-50 tubing using a hand-machined micrometer (kindly provided by Dr. Ray Rajotte, Edmonton, Alberta, Canada). Recipient mice were anesthetized using 3% oxygen flow rate and isoflurane (VetOne). Recipient animals were also treated with oxymorphone (Endo Pharmaceuticals) according to body weight at 0.2 to 0.5 mg/kg for analgesia. Recipient mice were shaved to remove fur from the surgical plane. Next, an incision was made in the left flank superior to the left kidney. The kidney was then pushed through the incision. A dissecting microscope (Leica) allowed fine inspection of the surgical field. The kidney capsule was carefully cut using a 1ml 25G TB syringe (BD), the tubing containing pelleted islets inserted into the opening, and the islets were dialed underneath the capsule with the micrometer. The animal was closed using 9 mm surgical wound clips (Stoelting) in which both the peritoneum and the epidermis were sealed. Following this closure procedure, recipient mice were hydrated with 1 ml normal saline (Hospira, Inc) using 25G TB syringe (BD).

Following transplantation, blood glucose levels were monitored with Embrace blood glucose meters (Omnis Health) daily. Recipient animals were considered euglycemic following two consecutive days with blood glucose readings <180 mg/dl. When transplantation was successful, euglycemia was established within 24-72 hours and was maintained for 5-7 days in
untreated allograft recipients or 11-15 days in untreated recipients of NOD.RAG\(^+\) islets. Unless indicated in figure legends, recipient animals were not further manipulated until grafts were destroyed by disease recurrence or allograft rejection or 100 days post-transplant was reached, whichever came first. In groups where recipients reached 100 days post-transplant euglycemic, graft-dependent euglycemia was confirmed by removal of the graft-bearing kidney, described below.

**Survival nephrectomy**

If transplanted recipients remained euglycemic for >100 days post-transplant, it became necessary to establish that euglycemia was graft-dependent. We therefore performed survival nephrectomies of the graft-bearing kidney on a subset of mice from each group in which members remained euglycemic >100 days post-transplant. Twenty four to forty-eight hours prior to surgery, animals were pre-loaded with 2 mg/ml acetaminophen (PenCol compounding pharmacy) through their water supply. As with islet transplantation, mice undergoing survival nephrectomy were anesthetized using isoflurane (Vetone) with an oxygen flow rate of 2-5%. Animals were shaved on the left flank and a similar left flank incision was made to that made prior to islet transplantation. Any adhesions between the kidney and the peritoneum were severed, the kidney was pulled through the incision, and the renal artery was clamped using a hemostat. Through the aid of a dissecting microscope (Leica), the renal artery was tied off using silk suture (Teleflex Medical) and the renal artery severed. Graft-bearing kidneys were retained for histology or cellular analysis, described below. Following removal of the kidney, the peritoneum and epidermis were closed with surgical wound clips (Stoelting) and hydrated with 1 ml of normal saline (Hospira, Inc). Blood glucose levels were monitored daily using Embrace blood glucose meters (Omnis Health). Demonstration of graft-dependent euglycemia was
established by return to hyperglycemia following survival nephrectomy: mice were euthanized upon 2-3 consecutive days with blood glucose readings >300 mg/dl.

**Ex vivo PMA/ionomycin T cell re-stimulation**

To determine the degree of intra-graft T cell activation and cytokine expression associated with islet transplant rejection in autoimmune NOD mice, on day 2-3 following rejection – defined as at least 2 consecutive days of blood glucose readings > 300 mg/dl – mice were euthanized using CO₂ asphyxiation and cervical dislocation. Following euthanasia, graft-bearing kidneys were excised. Remaining graft tissue adherent to the kidney capsule was removed and incubated at 37°C for 30-40 minutes in 2.4 U/ml dispase II (Roche Diagnostics). Following incubation, graft infiltrate was gently dissociated to single cells using double-frosted glass slides (Fisherbrand). T cells were re-stimulated in 96-well plate (Corning, Inc, Costar) format for 4-6 hours in EMEM (Gibco) containing 1 µg/ml PMA (Sigma-Aldrich) and 40 µg/ml ionomycin (Sigma-Aldrich), in the presence of 50 µg/ml brefeldin A (Sigma Life Science).

**Flow cytometry: cell surface and intracellular staining**

To investigate cell surface phenotypes and cytokine production of lymphocytes following ex vivo re-stimulation or in vitro MLR assays we used the following protocol.

Responding cell populations were washed twice in FACS buffer (made in-house, composed of PBS, 0.5% fetal calf serum, and containing 1% sodium azide) and blocked with anti-Fc-gamma receptor. Anti-Fc-gamma receptor antibody was produced using the 2.4G2 cell line (ATCC #HB-197) to produce ascites in ICR.SCID mice, followed by quantification via ELISA assay. To determine the proportions of lymphocytes infiltrating rejected islet transplants in NOD mice, we used the following antibodies αCD4 (GK1.5) conjugated to FITC (Biolegend), αCD8a (53-6.7) conjugated to PerCP Cy 5.5 (BD biosciences), anti-CD19 (eBio1D3) conjugated to PE (eBioscience) and anti-F4/80 (BM8) conjugated to APC (Biolegend) all at 1:500 dilutions. If cells
were not analyzed for cytokine production, samples were fixed with 1% paraformaldehyde (PFA) solution and stored at 4°C overnight before data acquisition.

To characterize cytokine production by graft infiltrate T cells following ex vivo re-stimulation, we used the following surface antibodies at 1:500 and cytokine antibodies at 1:300 concentrations. Anti-CD45.1 (A20) conjugated to PerCP Cy 5.5 (BD biosciences), anti-CD4 (RM4.5) conjugated to PE Cy7 (Biolegend), anti-CD8a (53-6.7) conjugated to Pacific Blue (Biolegend), and anti-CD19 (eBio1D3) conjugated to PE (eBiosciences). Following cell surface staining, cells were permeabilized and fixed using 1% PFA as described (145). To quantify ex vivo cytokine production, eBioscience anti-cytokine antibodies to IFN-γ (XMG1.2) conjugated to APC and anti-TNF-α (MP6.XT22) conjugated to FITC were used. Following intracellular cytokine staining, cells were fixed using 1% PFA solution and stored at 4°C overnight before data acquisition.

To characterize in vitro MLR responses the following cell surface antibodies were used: αCD45.1 (A20) conjugated to PerCP Cy 5.5 (BD), αCD8a (53-6.7) conjugated to Pacific Blue (Biolegend), and αCD4 (GK1.5) conjugated to FITC (Biolegend). We used anti-cytokine antibodies to IFN-γ (XMG1.2) conjugated to PE Cy7 (BD biosciences) and TNF-α conjugated to PE (rat anti-mouse, BD biosciences). To quantify cell division in MLR reactions we used efluor 670 cell proliferation dye (eBioscience). Following intracellular cytokine staining, cells were fixed in 1% PFA solution and stored at 4°C overnight before data acquisition.

**Auto antigen MHC tetramer staining**

To determine proportion of spleen or graft infiltrate CD4 T cells specific for insulin B10:23-derived or chromagranin A-derived peptides, we utilized reagents developed by the Kappler group (146). Insulin peptide tetramers were conjugated to PE, whereas ChgA tetramer was conjugated to brilliant violet 421. Briefly, MHC II-loaded auto antigen tetramers were
diluted to 0.02 mg/ml in anti-mouse TCR Cβ (HAM57-597). T cells were stained at 37°C for 2 hours in a volume 25 µl per well in 96-well plates. Following tetramer stain, cell surface αCD45.1, αCD4 and αCD8 antibodies were added as described above for an additional 20 minutes at 4°C in the dark. Cells were then washed in 2% FBS-containing medium, fixed with 1% PFA solution for 20 minutes at room temperature, and stored at 4°C overnight prior to data acquisition. Control tetramers used were derived from the HEL peptide and loaded in I-Ag7.

To determine spleen or graft infiltrate CD8 T cells specific for IGRP206-214, we used the K<sup>d</sup>-restricted tetramer reagent recently developed and characterized by the Santamaria group (117). Briefly, cells were incubated in the presence of K<sup>d</sup>-IGRP tetramer and anti-CD8a antibody (clone 53-6.7) at a volume of 50 µl per well for 60 minutes at 37°C, followed by counter-staining with αCD4 (GK1.5) or αCD19 (eBio1D3) for 20 minutes at 4°C to establish dump gates. Cells were fixed with 1% paraformaldehyde (PFA) solution and stored overnight at 4°C prior to data acquisition. Control peptide was TUM loaded in H-2k<sup>d</sup>.

**Flow cytometric data acquisition and data analysis**

Data acquired on FACS Calibur using CellQuest software or on LSRII with FACSDiva software (BD) and analyzed using FlowJo version 9.5.2 (TreeStar).

**Mixed lymphocyte reactions**

Alloreactive mixed lymphocyte reaction (MLR) was performed in which irradiated stimulating cells were plated in co-culture with responding NOD-derived cells. NOD T cell proliferation in response to co-culture with B6-background cells was assessed by efluor proliferation dye dilution and cytokine production (eBioscience). Prior to MLR set-up, responding cells were labeled with efluor proliferation dye for 10 minutes at 37°C, according to manufacturer’s instructions. Briefly, 2 x 10^6 responding NOD cells were stimulated with 3 x 10^6 irradiated (2000 rads) B6, B6.B2M<sup>−/−</sup>, B6.C2D, or B6.MHC-bald cells for 4 days in MEM.
(Gibco) containing 10% fetal bovine serum, 1 ml L-glutamine, 1 ml non-essential amino acids, 2 ml HEPES, and 1 ml penicillin/streptomycin, along with 100 µl of 0.1 M betamercaptoethanol (Sigma M7522). Cells were incubated at 37°C in 10% CO₂ in air for 4 days. Final reaction volume was 2 ml in 24-well plates, with brefeldin A (Sigma Life Science) added at 50 µg/ml for the final 12 hours of culture to capture cytokines within the Golgi apparatus.

In additional experiments comparing the strength of NOD T cell responses against minor antigens and MHC antigens, the above experimental set-up was utilized, with the substitution of MHC-matched B6.g7 cells and minor-matched NOD.B10 cells for MHC-deficient B6-background cells.

**454 TCR sequencing and data analysis**

Following rejection of NOD.RAG⁻/⁻ or C3H islet transplants in NOD mice, RNA was isolated from endogenous pancreatic islets – which were harvested as described above – as well as from transplanted NOD or C3H islets using QiAgen RNeasy Mini kit. Single-strand cDNA was synthesized using the Clontech SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA) with oligo-dT primers according to manufacturer's instructions. To amplify TCR alpha and beta chains a two-step PCR was performed. Four-six PCR reactions were generated for individual samples using the Universal Primer A Mix supplied from the SMARTer RACE cDNA Amplification Kit, along with a primer designed on the constant region of alpha chains (5’ GGGTGCTGTCTCGAGACCGAGGATC) and beta chains (5’ AGCCCATGGAACTGCACTTGGCAGCG), respectively. The first PCR products were further amplified with nested primers ligated with the 454 adaptor sequences containing a multiple identifier sequence (5’ CCTATCCCCTGTGTGCTTGGCAGTCTCAGAAGCAGTGGTATCAACGCAGCG), 5’ CCATCTCATCCCCTGTGTGCTCGACTCAG-multiple identifier sequence- 5’ GTACACAGCAGGTCTGCTCGTGTCGAGGTC for alpha chains, 5’ CCATCTCATCCCCTGTGTGCTCGACTCAG-
multiple identifier sequence- CCTGGCCAAGCACACGAGGG for beta chains), agarose gel-purified followed by further purification with the AMPure XP Beads (Beckman Coulter), subject to emulsion PCR with the 454 GSJR titanium chemistry, and sequenced on the 454 GSJR instrument (Roche). Sequence reads were processed by the 454 GS junior amplicon pipeline in the absence of the primer trimming filter. To identify V(D)J sequences along with CDR3 junction sequences, and compared to known mouse TCR sequences sequence data were uploaded into the IMGT High V-quest (http://www.imgt.org/), followed by additional analysis by in-house software to determine frequencies of individual TCR sequences. TCR sequences which scored >1200 for the IMGT V-gene score (correlating with approximately 95% homology with reference sequences) were chosen for analyses. Frequencies of individual TCR α and β chain sequences determined by mean values from the 4-6 PCR reactions were analyzed for individual samples.

**Generation of retroviral vectors**

TCR sequences which were highly enriched from endogenous islets to rejected allograft lesions were cloned into the retrovirus mouse stem cell virus (MSCV). Briefly, the TCR alpha or beta chain variable regions were amplified directly from the first PCR products generated for sequencing above to add the restriction enzyme for oligonucleotides at the 5’ and 3’ ends (EcoRI and BspEI for alpha chains, Xhol and BglII for beta chains). The TCR α and β chain constructions were cloned in the MSCV-based retroviral vectors carrying the TCR α and β chain region genes using the appropriate restriction enzymes. The retroviral vector for α chains contains the internal ribosome entry sites (IRES) followed by the neomycin-resistant gene, and that for β chains the puromycin-resistant gene driven by the cytomegalovirus (CMV)-promoter.

**Generation of T cell transductants**

Phoenix cells were transfected with the retroviral vectors carrying the mouse CD8 α and β genes or a TCR α or β chain gene of interest along with the pCL-Eco packaging vector using
Lipofectamine 2000 (Life Technologies) to produce replication-incompetent retroviruses. The 5KC α-β- hybridoma T cell line lacking TCR α and β chain genes (147), which is derived from B10.BR CD4 cells, was used for host cells of TCR expression. To generate 5KC cells expressing the CD8 molecule, cells were spin-infected with supernatant containing the MSCV-based retroviruses encoding the mouse CD8 α and β genes followed by cell-sorting of CD8-positive and CD4-negative cells on the MoFlo cell sorter (Dakocytomation). The original (CD4-positive) and CD8-positive 5KC cells were then transduced with various combinations of TCR α and β chain genes followed by the culture in the presence of 750 μg/ml of G418 (Life Technologies) for 5 days and 3 μg/ml of puromycin (Life Technologies) for 3 days. All T cell transductants were analyzed on FACSCalibur (BD Biosciences) for the expression of CD4 (GK1.5, BD Pharmingen), CD8 (53-6.7, eBioscience), and TCR-β (H57-597, BD Pharmingen) and were confirmed that >90% of cells express TCR-β along with either the CD4 or CD8 molecule.

**Cell Lines, peptides, antibodies used in stimulation assays**

To identify autoreactive TCR α and β chain combinations reacting with islet antigens, CD4-positive T cell transductants bearing presumed autoreactive TCRs were stimulated with 200 μg/ml of the insulin B chain 9-23 peptide, the insulin B chain 13-23 peptide, the pS3 peptide mimotope of the ChgA WE14 peptides, the ChgA WE14 peptide, or the islet amyloid polypeptide (IAPP) KS20 peptide in the presence of NOD.Ca/or NOD.Cb/spleen cells (100,000 cells per well) in 96-well round bottom culture plates. CD8-positive T cell transductants bearing presumed autoreactive TCRs were stimulated with the NOD-derived NIT-1 insulinoma cell line (148) (400,000 cells per well) in 96-well flat bottom culture plates, or 200 μg/ml of the insulin B chain 6-22 peptide, the insulin B chain 9-23 peptide, the insulin B chain 15-23 peptide, IGRP_{206-214} peptide mimotope NRP-V7, or the MimA2 mimotope peptide (149) in the presence of NOD.Ca/or NOD.Cb/spleen cells (100,000 cells per well) in 96-well round bottom culture plates. All the
peptides were synthesized by Genemed Synthesis Inc. Once TCR combinations were determined, only cells expressing either CD4 or CD8 that responded more strongly were chosen for the further analysis testing the stimulation by islet cells. Islets were harvested from NOD.RAG\(^{-/-}\) mice as described above and were cultured in the presence of 1,000 units/ml of human IFN-\(\gamma\) (Life Technologies PHC4031) overnight. The following day, the islets were incubated in 0.25% Trypsin-EDTA (GIBCO 25200-056) for 5 minutes to be dispersed to single cells, and 600,000-900,000 single islet cells were added in a 96-well round bottom plate to stimulate 5KC cells.

To verify alloreactive responses mediated by allograft-enriched TCR sequences, we co-cultured CD4-positive or CD8-positive 5KC cells bearing TCRs with irradiated (3,000 rads) C3H spleen cells (400,000 cells per well). As a negative control, we co-cultured the cell lines with the same number of irradiated (3,000 rads) T cell-deficient NOD spleen cells harvested from either NOD.Ca\(^{-/-}\) or NOD.Cb\(^{-/-}\) mice. For subsequent assays to test the inhibition of stimulation by anti-MHC antibodies, 5KC cells expressing the 9860A1B1 or 9860A3B3 TCR were cultured with 40,000 or 1,000,000 C3H spleen cells respectively, which stimulates 5KC cells to secrete 500-1000 pg/ml of IL2, with or without 1, 10, or 40 \(\mu\)g/ml of anti-MHC-antibodies (anti-I-A\(\kappa\), 10-3.6, Biolegend 109902; anti-I-E\(\kappa\), M5/114.15.2, eBioscience 16-5321-85; anti-D\(\kappa\), 15-5-5, Biolegend 110302; anti-K\(\kappa\), 36-7-5, Biolegend 114902).

**In vitro stimulation assays**

5KC hybridoma cells bearing allograft-enriched TCR sequences (100,000 cells per well) were re-stimulated with NOD islets, candidate auto-antigen peptides, or allogeneic spleen cells, as described above. Co-culture assays were set up in 96-well plates in 37\(^{\circ}\)C incubators at 5% CO\(_2\) overnight, using S-MEM (Gibco 11380) supplemented with 10% FBS (Atlanta Biologics), 6% tumor cocktail. [A 500 ml aliquot of tumor cocktail contains 351 ml S-MEM (Gibco 11380), 7.5 g

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Dextrose (Sigma G-7021), 3.20 g Glutamine (Sigma G-8540), 75 ml of 50x Essential Amino Acids (Gibco 111-30051), 140 ml of 100x NEAA (Gibco 11140-050), and 100 ml of 100x sodium pyruvate (Gibco 11140-070). pH is then adjusted to 7.0 using 10 N NaOH. Following pH adjustment, 8.5 g of sodium bicarbonate (Sigma S-6297) is added, along with 500 mg Gentamycin (G-3632), and 34 µl betamercaptoethanol (Sigma M-7522), and 100 units/ml penicillin/streptomycin (GIBCO 15140). Following overnight incubation, quantification of IL-2 production the following day was conducted, as described below.

**ELISA quantification of hybridoma stimulation experiments**

IL-2 production in supernatant by SKC T-hybridoma cells bearing allograft-enriched TCRs in response to co-culture conditions was quantified via enzyme-linked immunosorbent assay (ELISA) using a set of anti-IL-2 antibodies (capture antibody BD 554424; detection antibody BD 554426) and europium-labeling streptavidin (Perkin Elmer 1244-360) by detection of time-resolved fluorescence on the Wallac 1420 VICTOR2 Multi-Label counter (Perkin Elmer). To establish reliable results, the assay was repeated three times with aliquots of different stimulating cells or peptides.

**Histology**

To investigate the degree of lymphocytic infiltration in various grafts (long-term treated mice or short-term acute rejection recipients), graft-bearing kidneys were excised as described above. To characterize beta cell-selective destruction during diabetes onset in NOD mice, several pancreata from diabetic female NOD mice were harvested. The tissues were fixed in 10% (v/v) formalin in aqueous phosphate buffer and embedded in paraffin and 5 µm sections were prepared. To evaluate the degree of lymphocytic infiltration sections were stained with H&E. To determine whether beta cells had been selectively destroyed or whether alpha cells had also
been destroyed, we stained for insulin and glucagon, respectively. Guinea pig anti-insulin and rabbit anti-glucagon (Dako) antibodies were used.

**Statistical analysis**

Transplant survival data are analyzed using Kaplan-Maier curves and log-rank tests. All other data, including cytokine expression by graft-infiltrating T cells and enrichment of autoantigen-specific T cells within rejection lesions, were analyzed using GraphPad Prism 5 software. Data were evaluated for statistically significant differences using a two-way ANOVA followed by a Bonferroni multiple comparison test. A p-value < 0.05 was considered statistically significant. All differences not specifically indicated to be significant were not significant (P > 0.05).
CHAPTER III
ISLET TRANSPLANT REJECTION IN NOD MICE INDEPENDENT OF GRAFT MHC EXPRESSION

Introduction

Type 1 diabetes (T1D) results from T cell-mediated destruction of insulin-producing beta cells in the pancreatic islets, and is characterized by dysregulated blood glucose levels (5). T1D currently represents an annual burden to the US health care system of approximately $17 billion, and the rate of disease incidence is increasing throughout the world (150). The front-line therapy is insulin replacement therapy via syringe injections or insulin pump infusion, coupled with careful monitoring of blood glucose levels. Despite insulin replacement therapy and intensive diabetes management (testing BG levels >4 times per day), many patients with T1D experience large variations in blood glucose levels and, over years of disease course, develop diabetes-related complications which significantly reduce quality and quantity of life. Whole pancreas or isolated islet transplantation is a potentially curative therapy for T1D (151, 152). Unfortunately, recurrence of autoimmune diabetes is a formidable barrier to long-term function of isolated pancreatic islet transplants in individual with T1D, as shown in recipients in the original Edmonton trials (153, 154).

Autoimmune T cells in the T1D human or NOD mouse specifically attack insulin-producing beta cells due to insufficient negative selection in the thymus and/or insufficient regulatory activity followed by inappropriate peripheral activation. Otherwise, however, these T cells likely follow conventional rules by which T cells generally operate. Namely, autoreactive T cells presumably recognize peptides presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells in pancreatic islet-draining lymph nodes and are activated by this recognition process. Following activation these cells follow chemokine gradients to areas of inflammation and likely re-encounter their cognate antigen. Lastly, upon recognition of their
cognate antigen within a peripheral site, up-regulation of effector molecules mediating target cell death.

Previous data using the NOD mouse model of T1D has shown that MHC class I-deficient NOD-derived islets are not acutely rejected in autoimmune NOD recipients (133). It has also been demonstrated that chronic CD4 T cell depletion following transplant with MHC-bearing NOD islets prevents disease recurrence (134) but also that tolerance – including tolerance transferable to other mice – to islet allografts in non-autoimmune recipients requires CD4 T cells (118, 144). Clinically, MHC-matching is a common technique used in attempts to decrease anti-transplant T cell responses. A related phenomenon, the frequency of pre-existing donor-reactive allo-antibodies in transplant recipients, is used to narrow down potential HLA matches for kidney transplantation (155, 156). However, it has been suggested that MHC-disparate islet transplants in autoimmune diabetic recipients are subject to both recurrent autoimmunity (4) and conventional anti-MHC-directed T cell responses (128, 157). The critical question of which of these T cell responses predominates in the autoimmune diabetic pancreas or isolated islet transplant recipient has not been resolved. Assuming these two T cell responses are parallel and separate, one would expect the autoimmune memory T cell response to be more rapid than the naive anti-allograft T cell response, despite the relatively high frequency of potentially allograft-reactive T cells (approximately 1% of the peripheral T cell repertoire). One reason for lack of consensus is that self-MHC-restricted autoimmune cells could reasonably be expected to more effectively target MHC-matched transplants. Therefore, rapid rejection of MHC-mismatched allografts in autoimmune recipients appears paradoxical.

We sought to determine the recognition pathway whereby autoreactive T cells target allogeneic (MHC-disparate) islet transplants through genetic deletion of MHC on pancreas donors and T cell depletion in autoimmune diabetic NOD recipients. Specifically, we tested the
hypothesis that MHC expression on islet allografts – the clinically relevant model of islet transplantation – would be required for rapid transplant rejection in the autoimmune recipient. Since autoimmune T cells in NOD survive thymic development based on self-MHC reactivity, it was our expectation that autoimmune T cell responses would predominate within rejection allograft lesions. Because of this expectation, we anticipated relatively few alloreactive TCR sequences to be isolated from freshly rejected islet allografts in NOD mice.

Results

**MHC-disparate transplants are rapidly rejected in autoimmune recipients**

We first demonstrate the beta cell-specific nature of both disease onset ([Figure 3-1c](#)) and disease recurrence ([figure 3-1d](#)) in NOD mice. In neither scenario – diseased pancreas or disease recurrence lesion – do we observe significant destruction of glucagon-producing alpha cells. In contrast, within islet allograft rejection lesions in autoimmune diabetic recipients, both beta cells and alpha cells have been destroyed ([figure 3-1e](#)) due to MHC disparity with the recipient. To avoid islet transplant rejection by autoimmune CD4 T cells, it has been suggested that intentionally MHC class II mismatching pancreatic islet would confer protection from self-MHC-restricted autoimmune memory T cells (89, 108). However, others, including our group (159) have shown that MHC-disparate islet transplants in NOD mice are rapidly rejected by autoreactive CD4 T cells. Figure 3-2a directly tested this question. MHC-disparate islet transplants from either B6 (partial MHC class I match) or B10.BR (full MHC mismatch) donors are rejected in the autoimmune recipient in 5-7 days, in contrast to rejection of syngeneic islets in MST 13 days. In addition, NOD.B10 islets (minor match, MHC mismatch) are rejected in median survival time (MST) 7 days. Furthermore, we predicted that expression of an allogeneic MHC, either class I or class II, would be both sufficient and required in order for autoimmune T cells to physically interact with islet beta cells and mediate transplant rejection. We found that while T
cells in non-autoimmune, chemically-induced diabetic recipient mice appear to follow the canonical understanding of TCR/MHC interaction-mediated recognition, T cells in autoimmune diabetic NOD mice responding to MHC-deficient pancreatic islet transplants appear to operate by a differing set of rules.

![Figure 3-1: Pancreatic islet histology of diabetes onset and islet transplant rejection in NOD mice.](image)

- **Figure 3-1a**: Normal islet architecture from C57BL/6 mice. Figure 3-1b: 500 hand picked islets were transplanted under the left kidney capsule in STZ-induced diabetic B6 mice. We show minimal lymphocytes infiltrating and islet staining for insulin and glucagon following 100-day function of B6 islets. Figure 3-1c: Islets in diabetic NOD mice do not stain for insulin but do stain for glucagon – indicating selective destruction of beta cells. Figure 3-1d: Transplant lesions in NOD mice stain positive for glucagon but not for insulin following return to hyperglycemia in recipients of syngeneic islets – indicating selective destruction of beta cells. Figure 3-1e: Transplant lesions in NOD mice demonstrate minimal staining for either insulin or glucagon following rejection of allogeneic islets – indicating destruction of both beta cells and alpha cells.
Figure 3-2: Islet allograft rejection is more rapid than autoimmune disease recurrence in NOD mice and requires CD4 T cells. Figure 3-2a: Transplantation of 500 hand-counted syngeneic (NOD.RAG) islets maintains euglycemia significantly longer than 500 hand-counted allogeneic (B6) islets in autoimmune diabetic female NOD recipients, p <0.0001. Figure 3-2b: Depletion of CD4 T cells, but not CD8 T cells, significantly delays allograft rejection in autoimmune recipients (p<0.05). Depletion treatments were administered with either GK1.5 (αCD4) or 53-6.72 (αCD8) antibodies at a dose of 20 mg/kg, on days -2, 0, 2, 7, and 14 relative to islet transplantation. Depletion therapies described herein yielded depletion of >90% of T cells (data not shown) in autoimmune NOD mice. Statistical comparisons between islet transplantation groups are log-rank tests. Figure 3-2a and 3-2b represent independent experiments. Untreated controls referenced in subsequent figures are using the data shown in Figure 3-2b. Time scales differ in figure 3-2a and 3-2b to highlight the statistically significant different in time to allograft rejection and time to disease recurrence in autoimmune NOD mice.

Results shown in. Figure 3-2b shows that for rejection of allogeneic B6 islets in autoimmune NOD mice: CD4 T cells are required whereas CD8 T cells are not required.

Autoreactive CD4 and CD8 T cells are associated with rejection of both syngeneic and allogeneic islet transplants in NOD mice

After determining which T cell subsets were required for autoimmune disease recurrence and islet allograft rejection in NOD mice, we next investigated graft-infiltrating T cell activation status (figure 3-3). Figure 3-3a illustrates very similar proportions of CD4+, CD8+, CD19+, and F4/80+ cells within rejected syngeneic (NOD.RAG) or allogeneic (B6) islets at the
time of transplant destruction, defined by return to hyperglycemia. Flow cytometric analysis following ex vivo re-stimulation in figure 3-3b and 3-3c, demonstrate very similar activation status for CD4+ and CD8+ T cells in the different transplant rejection scenarios in autoimmune recipients. Using recently characterized tetramer reagents which allow the tracking of both autoreactive CD4 T cells (146) and autoreactive CD8 T cells (117), we demonstrate enrichment of three populations of autoreactive T cells in autoimmune disease recurrence lesions. Interestingly, we could not establish enrichment of autoreactive T cells, relative to NOD spleen, within islet allograft rejection lesions. These data demonstrate that the rapidity of islet allograft destruction in these mice is not mediated solely by autoreactive T cells, and suggests that alloreactive T cells play a significant role in this biological outcome.

**MHC class I expression on transplanted islets is required for autoimmune disease recurrence but not for allograft rejection in NOD mice**

Whether autoreactive CD8 T cell contact with transplanted beta cells is required for islet transplant rejection is an area of active interest in autoimmune pathogenesis. In figure 3-5, we confirmed that MHC class I expression on NOD islets is required for autoimmune disease recurrence (figure 3-5a) and demonstrate that MHC class I expression on otherwise allogeneic (B6) islets is not required for CD4 T cell-dependent islet transplant destruction in autoimmune recipients (figure 3-5d). These data further confirm that CD8 T cells are dispensable for islet allograft rejection in autoimmune recipients, which is in contrast to our data in autoimmune disease recurrence. In figure 3-6 we investigated the critical question of whether islet graft MHC class II expression is required for islet transplant rejection by CD4 T cells in NOD mice. Figure 3-5a demonstrates that NOD.CIITA−/− islets are acutely rejected in a CD8 T cell-dependent manner. Figure 3-6b shows that B6-background MHC class II deficient islets are rejected in a CD4 T cell-dependent manner. In summary, we found that MHC class I expression is required for
Figure 3-3: Lymphocytic infiltrate at the time of either autoimmune disease recurrence or islet allograft rejection in NOD mice. Figure 3-3a: representative flow cytometric analysis of lymphocytic infiltrate at the time of either autoimmune disease recurrence or rejection of B6 islets in autoimmune diabetic NOD mice. Figure 3-3b: Representative data of graft infiltrate CD4+ T cell ex vivo re-stimulation with PMA/ionomycin following either autoimmune disease recurrence (top) or B6 islet transplant rejection (bottom). Figure 3-3c: Representative data of graft infiltrate CD8+ T cell ex vivo re-stimulation with PMA/ionomycin following either autoimmune disease recurrence (top) or B6 islet transplant rejection (bottom). >30% of both T cell populations produce either cytokine upon ex vivo re-stimulation, with significant proportions of both T cell populations producing both cytokines. Figure 3-3d: Quantification of representative data across recapitulates approximate proportions of CD45.1+ cells which are CD4+, CD8+, CD19+ in the spleen of NOD mice at baseline and quantification of single cytokine-producing and dual cytokine-producing CD4 and CD8 T cells isolating from disease recurrence lesions (n=4) or B6 islet transplant rejection lesions (n=5) following PMA/ionomycin re-stimulation. Gray tracings represent graft infiltrate CD4+ or CD8+ T cells in the absence of polyclonal restimulation, whereas black tracings represent graft-infiltrating T cells in the presence of polyclonal ex vivo restimulation. No statistically significant differences in graft infiltrate composition or response to polyclonal re-stimulation were observed using 2-way ANOVA tests.
Figure 3-4: Auto antigen-specific CD4 and CD8 T cells infiltrate both disease recurrence lesions and B6 islet transplant rejection lesions in NOD mice. Figure 3-4a: Percentages of spleen, disease recurrence infiltrate, or B6 islet transplant rejection infiltrate CD45.1+CD4+ T cells which bound either Insulin B10:23 or Chromagranin A peptide tetramers (which are I-Ag7-restricted). Figure 3-4b: Percentages of CD45.1+CD4+ NOD allograft rejection infiltrate, CD4+ that bound either Insulin B10:23 or Chromagranin A peptide tetramers. Figure 3-4c: Percentage of disease recurrence and allograft rejection infiltrate CD45+CD8+ T cells which bound Kd-IGRP206-214 tetramer. Figure 3-4d: Summary data: graphical representation of the percentage data presented in figure 3a, 3b, and 3c, pooled over 4 mice for MHC II-restricted tetramers for both disease recurrence and allograft rejection and 3 mice for IGRP tetramer for both disease recurrence and allograft rejection. Statistical significance tested via 2-way ANOVA, * indicates P<0.05 fold enrichment relative to NOD spleen. Spleen auto antigen tetramer data collected from the mice analyzed for tetramer-binding cells within transplant rejection lesions. All three autoantigen-specific T cell populations were significantly enriched in disease recurrence lesions compared to both NOD spleen and allograft rejection lesions.
Figure 3-5: MHC class I expression is required for autoimmune disease recurrence, but is dispensable for B6 islet transplant rejection. Figure 3-5a: NOD islets lacking B2M expression, and therefore expressing low levels of MHC class I, are not acutely rejected in autoimmune NOD recipients, p<0.0001. Figure 3-5b: However, B6 islets lacking B2M and expressing low levels of MHC class I are acutely rejected in autoimmune NOD recipients (p<0.0001) in a CD4 T cell-dependent manner. Figure 3-5c: Representative flow cytometric data from T cell infiltrate of rejected B6.B2M transplants showing strong CD4 T cell and CD8 T cell cytokine production responses immediately following rejection of MHC class I-deficient allografts. Gray tracings represent graft infiltrate CD4+ or CD8+ T cells in the absence of polyclonal restimulation, whereas black tracings represent graft-infiltrating T cells in the presence of polyclonal ex vivo restimulation. Statistical comparisons between transplant recipient groups are log-rank tests.

Autoimmune disease recurrence but not allograft rejection, whereas MHC class II expression on the transplanted islets is not required for either transplant rejection process.

MHC-bald NOD-background islets are not acutely rejected in autoimmune recipients

To investigate the mechanism of CD4 T cell-dependent rejection of MHC class II-deficient islet transplants, we took the MHC-deficient islet donor system to its logical conclusion, and bred MHC-bald islet donors on the B6 background (B6.B2M x B6.C2D) and NOD
(NOD.B2M x NOD.CIITA) backgrounds. Tellingly, figure 3-7a demonstrates that B6.MHC-bald islets are not rejected in STZ-induced diabetic BALB/c mice. In agreement, figure 3-7b shows that NOD.MHC-bald islets are not acutely rejected (within 20 days post-transplant) in autoimmune NOD recipients. These data suggest that TCR-MHC-mediated contact is required for effector T cells to physically interact with, and mediate rejection of, islet transplants – including in the setting of autoimmune diabetes. Results shown in figure 3-8 illustrate studies in which we tested whether lack of acute rejection of NOD.MHC-bald islets in autoimmune recipients were due to peri-transplant protection mediated by regulatory T cells. Previous work suggests that conventional CD4+CD25+Foxp3+ regulatory T cells delay diabetes onset in NOD mice, since islet-antigen-specific regulatory CD4 T cells express high levels of CD25 (160) and insulin-specific tolerance in NOD mice can be broken by PD1 blockade (161). In our hands, antibody-mediated inhibition of neither CD25 nor PD1 promoted acute rejection of NOD.MHC-bald islets. In addition, PD1 inhibition at days 100-120 post-transplant did not promote islet transplant rejection (data not shown). These data led us to conclude that this non-rejection phenotype is due to lack of ability of autoimmune effector T cells to physically interact with MHC-bald NOD islets, and not due to protection afforded NOD.MHC-bald islets by regulatory T cells.

**MHC-bald B6 background islets are acutely rejected in a CD4 T cell-dependent manner in autoimmune NOD mice**

Next, in figure 3-9, we transplanted B6.MHC-bald islets into autoimmune NOD recipients, and found that these islets are acutely rejected (MST 12 days), albeit delayed relative to MHC-bearing allografts (MST 6 days). In addition, and in sharp contrast with figure 3-8a, STZ-induced diabetic NOD mice eventually reject B6.MHC-bald islets (p=0.0002), which suggests an unusually strong minor-antigen-directed T cell response in NOD mice. Lastly, to determine the
Figure 3-6: Syngeneic or allogeneic MHC II knockdown islets are rejected in autoimmune NOD transplant recipients. Figure 3-6a: Acute NOD.CIITA islet transplant rejection in autoimmune NOD recipients requires CD8 T cell, p=0.0124. Figure 3-6b: In contrast, acute rejection of B6.C2D islets in autoimmune NOD recipients requires CD4 T cells, p=0.0013 Figure 3-6c: Representative data from T cell infiltrate following rejection of B6.C2D islets in autoimmune NOD recipient demonstrates both CD4 T cells and CD8 T cells infiltrate the transplant and demonstrate recent activation upon ex vivo re-stimulation. Gray tracings represent graft infiltrate CD4+ or CD8+ T cells in the absence of polyclonal restimulation, whereas black tracings represent graft-infiltrating T cells in the presence of polyclonal ex vivo restimulation.
Figure 3-7: STZ-treated diabetic mice do not reject MHC-bald islets and autoimmune NOD mice do not reject MHC-bald syngeneic islets. Figure 3-7a: STZ-induced diabetic BALB/c mice reject B6 islets bearing either MHC class I (B6.C2D) or MHC class II (B6.B2M), but not B6.MHC-bald islets lacking expression of either MHC on transplanted islet cells, p<0.0001. Figure 3-7b: autoimmune NOD recipients do not acutely reject NOD.MHC-bald islets, p<0.0001. Eventual rejection is, we think, immune-mediated. STZ-induced NOD mice do not reject NOD.MHC-‘bald’ islets (see Figure 3-8a).

cellular requirement and potential for participation of autoreactive T cells in rejection of
B6.MHC-bald islets, we undertook depletion experiments and utilized the auto antigen tetramer reagents utilized in figure 3-3.

Our results demonstrate that rejection of B6.MHC-bald islets in autoimmune NOD recipients is CD4 T cell-dependent, CD8 T cell-independent, and NK cell-independent (figure 3-10a), as are all the other allograft rejection processes characterized in this report. In addition, we detected MHC II-restricted, auto antigen-specific CD4 T cells within rejected B6.MHC-bald
islet grafts, suggesting participation of autoreactive CD4 T cells as at least a partial explanation for the difference in time to rejection between autoimmune and STZ-induced diabetic NOD mice. However, autoreactive CD4 T cells were enriched to a lesser degree in B6.MHC-bald grafts relative to NOD.RAG grafts, suggesting participation of other T cell responses to yield equivalent time to rejection (MST 11-13 days for both rejection processes).

Figure 3-8: Autoimmune recipients reject some NOD.MHC-bald transplants, and non-rejection does not require regulatory T cells. Figure 3-8a: Zero of eight STZ-treated male NOD recipients rejected NOD.MHC-bald islets in the 100 days following transplantation, whereas six of nine autoimmune NOD recipients of MHC-bald syngeneic islets eventually rejected transplants (reproduced from Figure 3-7b). Figure 3-8b: Peri-transplant treatment with neither anti-CD25 (PC61) or anti-PD1 (J43) antibodies promoted accelerated rejection in autoimmune NOD recipients of NOD.MHC-bald islets. Untreated recipients from Figure 3-7b shown. Not shown: treatment with PD1 blockade antibody at days 100-120 post-transplant did not promote rejection of NOD.MHC-bald transplants (n=3). Untreated recipients of NOD.MHC-bald’ islets are the same in figures 3-8a and 3-8b.
**Discussion**

Clinically, autoimmune diabetic recipients of whole pancreas or isolated islet transplants demonstrate resistance to chronic immune suppression therapies (153, 154). For >20 years, a conundrum in the autoimmunity and transplantation fields has been which T cell response – autoimmune or alloimmune – predominates in this setting and mediates resistance to therapeutic intervention. The experiments in this report were designed to test whether MHC expression on transplanted islets was required for transplant rejection – implicitly testing the hypothesis that autoimmune T cells accelerate alloimmune responses.

Surprisingly, we found that MHC expression on transplanted islets is *not* required for rejection of B6-background islets in autoimmune recipients. Thus, the presence of non-MHC differences between islet donor and autoimmune diabetic recipient are sufficient to promote islet transplant rejection. This is in stark contrast to results in chemically-induced, non-autoimmune diabetic recipients of MHC-bald islet transplants, 100% of whom remained euglycemic >100 days post-transplant. Intriguingly, even in chemically-induced young male NOD mice, B6-background MHC-bald islet transplants were eventually rejected. Rejection in strep-induced male NOD recipients was significantly delayed compared to autoimmune female NOD recipients (MST 58 days compared to MST 12 days, respectively). These data strongly suggest the presence of a third category of T cell response in NOD mice – directed against non-MHC antigens – which can mediate islet transplant rejection independent of *either* autoimmune disease status or allogeneic MHC molecules, but is accelerated in the presence of autoimmune disease status. Importantly, B6.MHC-bald islets are not rejected in SZ-induced diabetic BALB/c mice, which differ in both minor MHC antigens from the B6 strain, as does the NOD. However,
Figure 3-9: NOD recipients reject B6.MHC-bald islets: Autoimmune NOD mice acutely reject B6.MHC-bald islet transplants (MST 12 days), whereas STZ-induced diabetic NOD female mice rejected B6.MHC-bald islet transplants eventually (MST 58 days) compared to lack of rejection in SZ.BALB/c male mice (reproduced from Figure 3-7a). Significant differences between rejection of B6.MHC-‘bald’ islets in autoimmune (MST 12d) and SZ-induced diabetic female NOD mice (MST 58d), p<0.0001.
Figure 3-10: Autoimmune NOD rejection of B6.MHC-bald islets requires CD4 T cells and is associated with intragraft accumulation of autoantigen-specific CD4 T cells. Figure 3-10a: Rejection of B6.MHC-bald islets in autoimmune NOD recipients requires CD4 T cells and does not require CD8 T cells or NK cells, p=0.003. Figure 3-10b: Rejection of B6.MHC-bald islets in autoimmune NOD mice is associated with accumulation of autoreactive CD4 T cells specific for known auto-antigens. Plots shown are either spleen or graft infiltrate CD45.1+CD4+ cells which bind MHC II-restricted auto antigen tetramers. Figure 3-10c: Quantification of autoreactive CD4 T cell accumulation across disease recurrence (NOD.RAG islets), allograft rejection (B6 islets), and B6.MHC-bald islets demonstrates less enrichment of autoreactive insulin B chain-specific CD4 T cells in B6.MHC-bald grafts compared to syngeneic grafts, p<0.05, as well as enhanced enrichment of autoreactive ChgA-specific CD4 T cells from B6 islets rejection lesion to B6.MHC-bald islet rejection lesion. Data for NOD spleen, disease recurrence lesion, and allograft rejection lesion are reproduced from Figure 3-4 for direct comparison to B6.MHC-bald rejection lesions.

B6.MHC-bald islets are rejected in SZ-induced NOD mice (MST 58 days) suggesting the presence of a non-MHC-peptide-derived response mediated by NOD CD4 T cells and restricted to recipient MHC class II molecules which can eventually destroy an islet transplant in the absence of graft cell MHC as well as the absence of autoimmune diabetes disease status.

However, our data demonstrate that MHC class I expression is an absolute requirement for rejection of syngeneic (NOD-background) islets in the autoimmune recipient. From these data, it appears that autoimmune CD8 T cells follow the canonical contact-mediated rules of engagement – as long as the transplanted islets are from the identical genetic background. It
appears that non-MHC-reactive CD4 T cells in NOD mice do not operate by the canonical TCR-MHC contact-mediated mechanisms. Both CD4 and CD8 T cells, as well as B cells, appear to participate in rejection of MHC-bald islet transplants in autoimmune recipients based on their presence within rejection lesion, but only CD4 T cells are required. Furthermore, a detectable fraction of these CD4 T cells are specific for well-characterized MHC II-restricted auto antigens. Importantly, NOD-background MHC-bald islet transplants are not acutely rejected in autoimmune NOD recipients, despite overt diabetic disease status. These data suggest that minor-antigen-directed responses by CD4 T cells in NOD mice ‘unleash’ or ‘license’ autoreactive CD4 T cells to enter the graft and mediate damage via the cytokines IFN-γ and TNF-α. This difference in rejection phenotype of MHC-bald non-NOD islets with MHC-bald NOD islets correlates with a difference in the roles of CD25+ cells – a proxy for CD4+ regulatory T cells – in disease recurrence and allograft rejection in the autoimmune recipient (see chapter VI). We observed that inhibition of regulatory T cells via PC61 antibody treatment accelerated rejection of syngeneic islets, but slightly delayed rejection of allogeneic islets in autoimmune recipients. These data are consistent with a model in which self-MHC-restricted islet-specific regulatory T cells delay rejection of syngeneic islet transplants, but no role for self-MHC-restricted regulatory CD25+ cells in protection of MHC-disparate, or by extension, MHC-bald, islet transplants in autoimmune recipients.

Overall, our data suggests that auto antigen-specific responses and anti-allogeneic MHC responses are not the only T cell responses in the autoimmune recipient capable of promoting islet transplant rejection. A critical piece of data in this chapter is the eventual rejection of MHC-bald B6-background islets in STZ-treated diabetic NOD mice (MST 58 days), which indicates non-MHC, minor antigen-directed T cell responses in NOD mice – in the absence of autoimmune diabetes – are sufficient to eventually reject islet transplants. Interestingly, this transplant
rejection response is slightly but not significantly faster than rejection of NOD MHC-bald islets in autoimmune recipients (MST 87 days) and is in stark contrast with indefinite survival of B6.MHC-bald islets in chemically-induced diabetic BALB/c mice (MST >100 days). These results highlight minor antigen-specific T cell responses as a previously under-estimated contributor to islet transplant rejection in the autoimmune recipient. Whether this phenomenon in the NOD mouse extends to human patients with autoimmune diabetes is an important clinical follow-up, which could potentially aid in explaining the profound resistance to immune suppression and tolerance-promoting therapies in this patient population following pancreas or islet transplantation.
CHAPTER IV

NON-MHC NOD GENES DRIVE TOLERANCE-RESISTANT TRANSPLANT REJECTION IN NOD MICE

Introduction

As shown in chapter III, NOD mice reject pancreatic islet transplants that are MHC-bald but differ in non-MHC antigens. In addition, SZ-treated NOD mice reject MHC-matched islets (B6.g7 islets → SZ-treated NOD) more rapidly than non-NOD strains reject MHC-matched islets (B6.g7 islets → SZ-treated BALB.g7), in spite of reported immune suppressive effects of streptozotocin (162). These data support a model in which NOD mice exhibit strong CD4 T cell responses to processed antigens presented derived from either donor MHC or donor non-MHC molecules and presented by recipient class II MHC on antigen presenting cells to recipient CD4 T cells. Indeed, others have generated data which support the contention of an unusually strong CD4 T cell response to processed antigens in NOD mice (112), in particular in the case of targeting transplanted islet allografts for destruction (113). From these data, it is unclear whether autoimmune disease status in NOD mice drives this transplant rejection phenotype, whether diabetes-associated MHC, I-Ag7, drives altered peptide presentation promoting accelerated anti-transplant responses in general, or whether diabetes status and I-Ag7 synergize to promote rapid non-MHC antigen responses.

In this chapter we interrogated non-MHC or minor antigen-directed T cell responses in NOD mice. Data shown in figure 3-9b demonstrates that minor antigen differences are sufficient to promote islet transplant rejection in the NOD mouse strain in the absence of autoimmune diabetes disease state. This result differs from those using non-autoimmune-prone mouse strains (eg, BALB/c) which do not reject MHC-bald islet transplants. Based on our results in chapter III, we hypothesized that MHC-matching may engage islet-specific regulatory T cells in NOD mice. We further predicted this engagement would lead to a degree of islet graft
protection in autoimmune NOD mice. We (118, 144) and others (125) have shown that anti-CD154 therapy promotes development of transplant-specific regulatory T cells. Based on the above, we tested whether MHC matching islet transplants in overtly diabetic NOD mice would benefit islet transplant survival, and if not, whether treatment with anti-CD154 antibody therapy would enhance survival of MHC-matched transplants. For these studies, we used B6.H-2g7 mice, which do not develop autoimmune diabetes (165) and which have recently been shown to have very little invasive insulitis (166), despite diabetes-associated MHC. In non diabetes-prone mouse strains, MHC-matching transplants on congenic backgrounds leads to delayed rejection due to minor antigenic differences between strains (MST approximately 25-30 days). To determine whether autoimmune disease status or NOD background genes were more responsible for rejection of B6.H-2g7 islets, and how the tempo of this rejection process would compare to non-autoimmune-prone strains, we transplanted MHC-matched islet transplants in SZ-induced NOD male mice.

Lastly, we sought to determine whether MHC-directed and minor antigen-directed T cell responses in MHC-congenic mice were amenable to co-stimulation blockade-based therapy. Earlier results indicated that strep-induced diabetic NOD mice were not amenable to transplant tolerance to MHC-mismatched (B6) or minor-antigen mismatched (B6.g7) transplants. We hypothesized that I-Ag7 was not intrinsically resistant to islet transplant tolerance induction. Therefore, we predicted that when removed from the context of NOD background genes, these congenic mice would be amenable to islet transplant tolerance mediated by anti-CD154 antibody. We tested whether I-Ag7, removed from NOD background genes to the autoimmunity-resistant B6 or BALB/c background, would mediate accelerated rejection due to minor MHC antigen differences alone, and if so, whether this rejection phenotype was sensitive to treatment with anti-CD154 antibody treatment. These experiments provide mechanistic
insight into the role of MHC-mediated antigen presentation in islet transplant rejection in NOD mice, demonstrating that while STZ-treated pre-diabetic NOD mice are resistant to islet allograft tolerance induced by anti-CD154 therapy, MHC-congenic B6.g7 or BALB.g7 mice are amenable to co-stimulation perturbation-induced islet transplant tolerance across MHC or minor antigen barriers.

**Results**

**NOD CD4 T cells proliferate in response to co-culture with B6.MHC-bald spleen cells**

Intrigued by the observation shown in Figure 3-10, I conducted a conventional *in vitro* MLR experiment comparing NOD spleen T cell proliferation (efluor proliferation dye) and cytokine production (IFN-γ and TNF-α) responses to 4-day co-culture with irradiated spleen cells from B6 (positive control), B6.B2M+/−, B6.C2D, and B6.MHC-bald mice. Results demonstrate weak CD8 T cell response against B6.B2M+/− spleen cells and weak CD4 T cell responses against B6.C2D spleen cells. However, and in agreement with graft rejection data, I observed relatively strong CD4 T cell proliferation in response to co-culture with B6.MHC-bald spleen cells (figure 4-1). These data suggest that NOD CD4 T cells, in particular, respond to non-MHC, minor antigen genetic differences.

**MHC-matched Islet Transplants are Acutely Rejected in NOD mice**

Based on our observations in chapter III demonstrating rejection of B6.MHC-bald islets in STZ-treated NOD male mice, we tested whether MHC matching would promote islet transplant survival in autoimmune NOD mice. Data in figure 4-2a shows that MHC-matched islet transplants from either BALB.g7 or B6.g7 donors are rejected in the autoimmune recipient as if they were fully allogeneic (MST 6-7 days). This MHC-matched allograft rejection is more rapid than rejection of syngeneic islets (MST 13 days), suggesting that the addition of minor-antigen-specific responses outweighs any benefit to the graft derived from interactions with self-MHC-
Figure 4-1: NOD CD4 T cells proliferate in vitro in response to co-culture with B6.MHC-bald spleen cells. Figure 4-1a: 4-day in vitro MLR experiment as described in materials and methods. NOD spleens were isolated and co-cultured for 4 days in the presence of irradiated (2,000 rads) B6 MHC-bearing or MHC-deficient cells, as shown. Proliferation was quantified by dilution of efluor proliferation dye. Figure 4-1b: Quantification of differences in CD4 and CD8 T cell proliferation, summated over T cells isolated from n=5 responding NOD mice. # = p<0.0001, % = p<0.01, * = p<0.05. Significant differences are relative to NOD anti B6 T cell proliferation.

restricted regulatory T cells and emphasizes the strength of the ‘minor antigen’ response mediated by I-Ag7. To directly test any beneficial role of CD25+ regulatory T cells in rejection of MHC-matched transplants, we could have depleted these cells using the PC61 antibody. In addition, figure 4-2b shows that MHC-matched islet transplants are rejected in 9-10 days in SZ-treated NOD mice, a statistically indistinguishable time frame from rejection of MHC-mismatched B6 islets in SZ-induced diabetic NOD mice. This result is in contrast to expectations:
Figure 4-2: MHC-matched islets are rejected more quickly than expected in NOD mice. Figure 4-2a: MHC-matched islets generated on either the B6 background (B6.g7) or BALB/c background (BALB.g7) are rejected more quickly than NOD.RAG islets (p<0.05). Control NOD.RAG and B6 islet rejection reproduced from Figure 3-2. Figure 4-2b: SZ-treated NOD male mice reject MHC-matched islets in 9-11 days, an indistinguishable time frame from rejection of MHC-disparate islets in female NOD mice. Control disease recurrence transplants (MST 13 days) are reproduced from Figure 3-2.

MHC is named partly because of the rapid recipient T cell responses promoted by host-donor differences. We therefore did not pursue CD25+ cell depletion experiments.

Anti-CD154 therapy is not efficacious in NOD mice

In figure 4-3, SZ-induced diabetic NOD male mice reject allogeneic (B6) islets in a standard time frame of 11 days (4-3a), compared to accelerated rejection of 6 days in autoimmune recipients shown in figure 4-3b (p<0.05). In agreement with arguments made in chapter III, this suggests that autoimmunity accelerates islet allograft rejection in NOD mice. Further, NOD mice demonstrate strong resistance to islet allograft tolerance-promoting therapies (127). Figure 4-3b demonstrates that anti-CD154 treatment prolongs B6 islet survival in SZ-induced diabetic NOD mice, but does not prevent eventual rejection, in stark contrast to the B6 or BALB/c strains, in which this therapy leads to >100 day allograft survival in >70% of

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Figure 4-3: Both minor mismatched islet rejection in autoimmune recipients and MHC mismatched rejection in SZ-induced NOD mice resist anti-CD154 therapy. Autoimmune diabetic NOD mice reject MHC-matched islets (B6.g7 or BALB.g7) in the same time frame (MST 6-7 days) as parental strains B6 or BALB/c (Figure 4-2a) and peri-transplant anti-CD154 only moderately prolongs survival of B6.g7 islets (untreated compared to treated: p=0.0141). In addition, anti-CD154 therapy does not prolong survival of BALB.g7 islets in autoimmune NOD recipients. Also: peri-transplant anti-CD154 treatment does not promote long-term B6 islet transplant tolerance in streptozotocin-induced diabetic NOD male mice, but does prolong survival relative to untreated controls, p<0.0001. Importantly, 0 of 9 anti-CD154-treated SZ.NOD recipients of B6 islets reached 100 days with physiologic normal blood glucose levels.
Figure 4-4: MHC congenic mice are amenable to islet transplant tolerance using anti-CD154 therapy. Figure 4-4a: peri-transplant anti-CD154 therapy promotes long-term B6 islet transplant tolerance in streptozotocin-induced diabetic BALB.H-2g7, p<0.05. Figure 4-4b: MHC congenic mice reject minor mismatched islets with delayed kinetic (B6.g7 → BALB.g7) and transplant rejection due to this minor-antigen-directed T cell response can be prevented by anti-CD154 treatment (BALB.H-2g7 → B6.H-2g7), p<0.001.

recipients (118, 125). These data strongly suggest that resistance to islet allograft tolerance induction is an intrinsic characteristic of NOD mice independent of autoimmune disease status.

Diabetes-associated MHC does not confer resistance to anti-CD154-induced islet transplant tolerance in congenic mice

Based on our result in figure 4-3b, we wanted to determine whether diabetes-associated MHC (H-2g7) was sufficient to confer resistance to islet allograft tolerance, independent from NOD background genes. Therefore, in figure 4-4a, I transplanted allogeneic B6 islets into STZ-induced diabetic BALB.g7 mice with or without anti-CD154 treatment. Results
demonstrate that BALB.g7 mice can be made tolerant to islet allografts using this therapy (p<0.05), suggesting that H-2g7 is not intrinsically resistant to co-stimulation blockade-based therapies. Figure 4-3b tests a related question relevant because of the results in figure 3-10 in which minor antigen-disparate but MHC-bald B6 islets were rejected in STZ-induced NOD mice. We tested the speed of rejection due to minor antigen differences in H-2g7-matched recipients in the absence of autoimmune disease status and whether H-2g7 is amenable to islet transplant tolerance across minor antigen differences. We observed delayed rejection (MST 20 days) due to minor antigen differences, which are amenable to long-term tolerance induction by CD154 perturbation (p<0.05). These results demonstrate that H-2g7-bearing mice are not resistant to transplant tolerance induced across either MHC barriers or minor antigen differences, further highlighting the role of NOD background genes in this resistance phenotype. Importantly, survival nephrectomy of long-term euglycemic recipients in either of these cases (n=2 each at day 100-110 post-transplant) demonstrated graft-dependent euglycemia: within 4 days of nephrectomy, blood glucose levels had risen >300 mg/dl (data not shown).

**NOD T cells proliferate in response to co-culture with MHC-matched cells**

Intrigued by the observations in Figure 3-10 and Figure 4-1, I conducted a conventional MLR experiment comparing NOD spleen T cell proliferation (efluor proliferation dye) responses to 4-day co-culture with irradiated spleen cells from B6 (positive control), B6.g7 (MHC-matched, minor mismatched) and NOD.B10 (MHC mismatched, minor antigen-matched) mice (figure 4-5). Results demonstrate equivalent strength of CD4 and CD8 T cell responses between the anti-B6 response (MHC mismatch and minor mismatch), anti-B6.g7 response (minor mismatch only) and anti-NOD.B10 response (major mismatch only). These data indicate that NOD CD4 T cells respond to exposure to MHC-matched spleen cells by proliferating *in vitro*. 

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Figure 4-5: Ex vivo NOD T cells respond equally strongly against B6 islets, MHC-matched B6 congenic islets, and MHC-disparate, NOD-background islets. Figure 4-5a: 4-day in vitro MLR experiment as described in materials and methods. NOD spleens were isolated and co-cultured for 4 days in the presence of irradiated (2000 rads) B6, B6.H-2g7, or NOD.B10 spleen cells. Proliferation was quantified by dilution of efluor proliferation dye. Figure 4-5b: Quantification of differences in CD4 and CD8 T cell proliferation, summated over T cells isolated from n=5 responding NOD mice, using 2-way ANOVA tests. # = p<0.0001, % = p<0.01, * = p<0.05. Significant differences are relative to NOD anti B6 T cell proliferation.

Discussion

Diabetes risk correlates with MHC haplotype in mouse (I-Ag7) and man (DQ-8, DR-4). However, mice from non-NOD backgrounds engineered to express disease-associated I-Ag7 do not develop autoimmune diabetes (166) suggesting that MHC does not determine diabetes risk in and of itself. Combined with genome-wide association study data demonstrating multiple correlations between T1D development and polymorphisms in T cell activation and antigen
presentation genes, in particular Idd9/11 (95, 167), a model develops in which diabetes-associated MHC is required but not sufficient for diabetes onset. These data suggest that MHC haplotype is not determinative of diabetes development and suggest important roles for environmental factors – including the pro-inflammatory environment in which T cells develop in diabetes-prone human patients and animal models – in the development of pancreatic islet-specific autoimmunity.

Intriguingly, NOD mice demonstrate enhanced CD4 T cell responses to non-MHC derived ‘minor’ antigens, which manifests in unexpectedly rapid rejection of MHC matched islet transplants (figures 4-2 and 4-3) and unexpectedly robust in vitro responsiveness to MHC-matched cells (figure 4-5). We observed indistinguishable time to allograft rejection (B6 or BALB/c islets) or MHC-matched islet transplant rejection (B6.g7 or BALB.g7) in autoimmune NOD mice. In addition, SZ-treated diabetic NOD male mice demonstrate faster-than-expected rejection of B6.g7 or BALB.g7 islets, indicating that contrary to non-diabetes-prone strains, MHC-matching in the presence of I-Ag7 and the absence of autoimmune diabetes does not promote islet transplant survival in NOD mice.

Removing I-Ag7 from the context of NOD background genes appears to ameliorate this phenotype: SZ-treated B6.g7 or SZ-treated BALB.g7 islet transplant recipient mice demonstrate islet transplant rejection times closer to those of the parental strains (10-12 days MST) than the NOD strain (6-7 days MST). Furthermore, anti-CD154 therapy is sufficient to promote long-term tolerance to both minor and major MHC mis-matched islet transplants in non-NOD background, I-Ag7-bearing mice, but not in strep-treated or autoimmune diabetic NOD mice. For example, in this chapter we demonstrate that 5/11 SZ-treated BALB.g7 mice transplanted with B6 islets (MHC mismatched) and treated with anti-CD154 remained euglycemic for >100 days. We also demonstrate in this chapter that 5/6 SZ-treated B6.g7 mice remain euglycemic for >100 days.
following transplantation with BALB.g7 islets (MHC-matched, minor mismatched) in the presence of peri-transplant anti-CD154 treatment. In addition, we demonstrated that euglycemia was graft-dependent for both MHC mismatch and minor mismatch transplants in H-2g7-bearing recipients made tolerant with anti-CD154 therapy.

An experiment to test the sufficiency of NOD background genes in the promotion of rapid islet transplant rejection would be to use SZ-treated NOD.B10 mice, which do not develop autoimmune diabetes, and transplantation of B10.BR (MHC-matched) or BALB/c (MHC mismatched) islets. If NOD background genes are sufficient to promote rapid rejection of MHC-matched islet transplants, SZ-treated NOD.B10 mice would reject B10.BR islets in 10-12 days, not 25-30 days as was observed in rejection of BALB.g7 islets in untreated SZ-induced diabetic B6.g7 mice. If NOD background genes are sufficient to promote resistant to transplantation tolerance, NOD.B10 recipients of either B10.BR or BALB/c islets would not remain euglycemic for 100 days post-transplant. Based on the results presented in this chapter, I predict that NOD.B10 mice would rapidly reject both MHC-disparate and MHC-matched islet transplants, and further that both of these rejection responses would resist long-term tolerance induction by anti-CD154 therapy. I further speculate that Idd9/11 may play a role in this resistance (115).

The data presented in this chapter complement results from the Greiner group at UMass (168, 169). Briefly, this group has shown using genetic means that (a) the Idd3 locus confers resistance to islet allograft tolerance induced by CD154 blockade when knocked into B6 mice, and (b) the B6 Idd3 locus confers susceptibility to islet allograft tolerance induced by CD154 blockade when knocked into NOD mice. Further, this group has shown that IL-2 production and the ability of anti-CD154 treatment to promote deletion of alloreactive CD8 T cells track with the Idd3 locus (169). In a related study, the Wicker group has demonstrated multiple diabetes-relevant differences between the (diabetes-prone) NOD strain and the
(diabetes resistant) C57Bl/10 strain at Idd9.1 and Idd9.2, including the signaling molecule Lck, which is downstream from TCR engagement and mTOR, which is the target of the immune suppressive drug rapamycin (170).

An additional possibility is that impairments in NOD NK cells may inhibit the ability to induce islet allograft tolerance with anti-CD154 treatment. Even though NK cells are not required for diabetes onset in NOD mice (71), NK cells play a critical non-redundant role in CD154 blockade-induced transplant tolerance (126). A method to address this possibility would be to induce diabetes with SZ in male NOD.NK1.1 mice (171), which harbor the NK cell activating ligands from the B6 strain, which is highly amenable to CD154 blockade-induced islet allograft tolerance. Experimentally we would transplant SZ-treated NOD.NK1.1 recipients with allogeneic islets (C3H for full MHC mismatch) and treat with anti-CD154 therapy. One potential explanation for the relative ineffectiveness of CD154-mediated transplant tolerance in NOD mice is lack of effect on NK cells. Since NK cells are required for islet allograft induced by anti-CD154 in the B6 strain, I speculate that NOD.NK1.1 mice would be more amenable to CD154 blockade-induced islet allograft tolerance than SZ-treated NOD.BDC male recipients.

Overall, these results strongly suggest that I-Ag7 does not intrinsically promote rapid or therapy-resistant islet transplant rejection in the absence of NOD background genes and the pro-inflammatory environment characteristic of NOD mice. Furthermore, our results imply that NOD background genes, in particular Idd3 and/or Idd9, confer the observed acceleration in minor-antigen responsiveness and the observed resistance to islet transplant tolerance-promoting therapy in the absence of autoimmune disease status.
CHAPTER V

AUTOIMMUNITY AS AN ENDOGENOUS SOURCE OF HETEROLOGOUS ALLOIMMUNITY

IN COLLABORATION WITH THE NAKAYAMA LAB AT THE BARBARA DAVIS CENTER FOR

CHILDHOOD DIABETES AND DEPARTMENT OF PEDIATRICS

Introduction

Despite data from the NOD mouse and from clinical islet and pancreas transplantation experience demonstrating strong anti-MHC-directed T cell responses in autoimmune diabetic recipients of islet allografts, it is perplexing how autoreactive self-MHC-restricted effector T cells could directly contribute to rejection of MHC-disparate islet allografts. Autoimmune T cells in the periphery are selected during thymic development based on ability to interact with self-MHC. How these self-MHC-restricted, beta cell peptide-specific T cells participate in islet allograft rejection is unclear. One potential explanation for these empirical observations is heterologous alloimmunity. Foundational results described by the Emory group indicated that anti-viral memory leads to T cell expansion and participation in rejection of skin transplants (172), and resistance to tolerance induction. These results highlighted the concept of ‘heterologous’ immunity, involving T cells which are memory/effector phenotype specific for one antigen mediate productive immune responses against structurally unrelated peptides. In mouse models of viral infection, this concept has been extensively studied by the Welsh group (173–177). Briefly, these studies have shown that there are some predictable scenarios in which heterologous protection by viral-specific CD8 T cells is due to similarity in antigenic peptides between viruses. In other settings, the reasons appear stochastic, but remain observable.

The concept of cross-reactive memory T cells appears on the surface to disagree with canonical understanding of T cell selection and antigenic specificity. Heterologous alloimmunity implies a degree of ‘sloppiness’ in TCR-mediated recognition, which could be both useful (in the
case of clearing viral infections) and dangerous (in the case of autoimmunity or transplantation). However, there are examples of structural similarity that could facilitate the same TCR responding to multiple, presumed unrelated, antigens derived from flu peptides (178–181). In other words, lack of evidence need not be interpreted as lack of possibility.

Recently, the Fairchild group at the Cleveland Clinic has demonstrated that pre-existing endogenous memory CD8 T cells can mediate heart allograft rejection following exposure to cold ischemia in a mouse model (182), providing further validation of the relevance of MHC cross-reactive memory T cells in transplant rejection. In the setting of autoimmune diabetes, heterologous T cells may arise if T cells which are specific for islet-derived auto antigens cross-react, by chance, with graft cell-derived MHC molecules (direct alloreactivity) or respond to graft cell MHC loaded with beta cell-derived peptides (cross-restriction). In either case, autoreactive T cells would then have a ‘second reason’, or route, for responding – proliferating and producing beta cell-toxic cytokines including IFN-γ and TNF-α – after exposure to peptides derived from transplanted beta cells, and through this activation mediating the rejection of transplanted islets.

The clinical observation of accelerated rejection of non-islet transplants in autoimmune diabetic recipients could be better understood in the context of an autoreactive T cell receptor being competent to confer T cell responsiveness against allogeneic MHC molecules. The phenomenon of heterologous alloimmunity could, therefore, provide at least a partial explanation of how, mechanistically, islet allografts are unexpectedly rejected more rapidly than syngeneic islet transplants in autoimmune diabetic recipients. In addition, heterologous alloimmunity could provide a potential explanation for rapid kidney transplant rejection in autoimmune recipients (24, 183–185). We set out to test whether we could detect enrichment of autoreactive T cells within rejected islet allografts in NOD, which, when recapitulated ex vivo,
would demonstrate alloreactivity against graft cell-derived MHC molecules, as shown in figure 5-1. In other words, we intended to search for heterologous T cells within rejected islet allograft lesions in NOD mice – an anatomic location in which we would expect strong enrichment of this category of T cell. We used 454 sequencing to detect enrichment of known autoreactive TCR sequences from endogenous pancreas to transplant rejection lesion in autoimmune diabetic NOD mice, which were transplanted with NOD.RAG\(^{+/−}\) (syngeneic) islets or C3H (allogeneic) islets. We then recapitulated the most highly allograft-enriched TCR alpha/beta pairs \textit{ex vivo} in 5KC cells and tested the resulting cell lines for both islet reactivity and anti-C3H spleen cell reactivity.

### Are autoreactive T cells in the allograft?


\textbf{Figure 5-1: Dual-reactive TCR characterization workflow.} C3H mice express different MHC alleles at both class I and class II loci from the recipient NOD strain, and are therefore considered to be fully allogeneic for transplantation purposes. Transplants normalized blood glucose levels of frankly diabetic female NOD mice (BG>300 mg/dl for 3-4 consecutive days prior to transplantation) for 6-7 days before return to hyperglycemia (BG>300 mg/dl), at which time both the endogenous pancreas and transplant lesion were excised and RNA was isolated for TCR sequencing via 454 methodology.
in standard *in vitro* tests measured by IL-2 ELISAs. In this report we describe several TCR alpha/beta combinations which were (1) highly enriched in allograft rejection lesions relative to spleens of diabetic NOD mice, (2) demonstrated *ex vivo* IL-2 production when co-cultured with NOD islets and candidate autoantigen peptides, and (3) demonstrated *ex vivo* IL-2 production when co-cultured with C3H spleen cells. These data provide formal evidence for both the (1) presence of, and (2) enrichment within islet allograft lesions in autoimmune NOD mice of both CD4+ and CD8+ T cells that demonstrate heterologous alloimmunity as described above. To our knowledge these data represent the first formal demonstration of this category of dual-reactive T cells isolated from autoimmune recurrence lesions.

**Results**

*Disease onset-associated T cell receptors are found within both disease recurrence lesions and allograft rejection lesions in NOD mice*

We hypothesized that disease onset-associated TCR sequences would be present within disease recurrence lesions in NOD mice. Data shown in figure 5-2 demonstrate that fully MHC mismatched C3H islets are rapidly rejected in autoimmune NOD recipients. Results depicted in figure 5-3 demonstrates presence of disease onset-associated TCR alpha sequences within rejection lesions in NOD following rejection of either NOD.RAG islets (isografts, A, B, and C in figure 5-3) or allografts (figure 5-3 D, E, and F). MHC-disparate islet allografts – in this case pancreas donors were C3H mice – are rapidly destroyed in the autoimmune recipient (6-7 days) whereas disease recurrence occurs in a median of 11 days (Figure 5-2). Previous data has shown that islet allograft rejection in NOD mice is CD4 T cell-dependent and CD8 T cell-independent. Therefore, we were particularly interested in dual-reactive CD4 T cells isolated from islet allograft rejection lesions in autoimmune NOD mice. Results in figure 5-3 suggest a more linear relationship between TCR α sequences found in both the endogenous pancreas and disease
Figure 5-2: Fully allogeneic islet transplants are acutely rejected in autoimmune NOD recipients. Disease recurrence in NOD mice reproduced from Figure 3-2. When compared with rejection kinetics of fully MHC mismatched C3H islets, disease recurrence is significantly prolonged (p=0.0006). In the following figures, we isolated pancreas-associated and rejection lesion-associated T cells from the rejection lesions of these two groups of autoimmune transplant recipients.

Recurrence lesion compared to the relationship between TCR α sequences detected in both the endogenous pancreas and the allograft rejection lesion, indicating either less enrichment for pancreas-associated TCR α sequences or the additional allograft-reactive TCR α sequences detected in the allograft lesions. Results depicted in figure 5-4 and figure 5-5 schematically represents the degree of overlap between the endogenous islets and the allograft rejection lesions for all three allograft recipient mice analyzed. For example, mouse 9805 59% of allograft TCR α chains were derived from ~7% of pancreas-associated α chains and 62% of graft-derived β chains were derived from ~5% of pancreas-derived β chains. TCR α chain analysis appears across the top of figure 5-4 and the left of figure 5-5. TCR β chain analysis appears on the bottom of figure 5-4 and on the right of figure 5-5. Overall, we observed similar levels of TCR α and TCR β...
Figure 5-3: Degree of TCR sequence overlap between NOD pancreas and allograft rejection lesions. TCR alpha sequences were compared between endogenous islets (x-axis) and graft rejection lesions (y-axis) in autoimmune NOD recipients of pancreatic islet transplants. Figure 5-3A, B, and C represent autoimmune disease recurrence lesions, in which NOD.RAG\(^{-/-}\) islets were rejected. Figures 5-3D, E, and F represent allograft rejection lesions in which C3H islets were destroyed. The difference in R-squared values (embedded within each graph above) suggests less correlation with TCR α sequences found in the pancreas and the allograft lesions, compared between pancreas and disease recurrence lesions. This may be the rest of dilution of the frequency of autoreactive TCR sequences by ‘naïve alloreactive’ TCR sequences within the allograft rejection lesions. Data generated and analyzed by Maki Nakayama and Laurie Landry.

chain migration from pancreas to allograft lesion within mice, but a surprisingly large range of TCR migration from the endogenous islets between mice (12% up to 59%), suggesting that either autoimmune disease recurrence or allograft rejection processes can precipitate rapid islet allograft rejection in NOD mice. In particular, we cannot conclude that islet allograft rejection in
Figure 5-4: Pancreas-associated TCR sequences are detected within allograft rejection lesions in diabetic NOD mice. Figure 5-4 depicts unique TCR α and TCR β sequence reads from the endogenous pancreas (left), allograft lesion (right) and sequences which appeared in both anatomical locations for the three allograft lesions analyzed (red, middle overlap area). Data generated and analyzed by Maki Nakayama and Laurie Landry.
autoimmune recipients enriches TCR α sequences in the graft to a lesser degree than autoimmune disease recurrence in NOD mice: figure 5-4 and figure 5-5 demonstrate overlap between these percentages of sequences shared (p=0.11). Interestingly, we counted the TRAV16*02/TRAJ42*01 TCR alpha among the top-5 most frequent sequences in all three disease recurrence lesions. This TCR is known to respond strongly to the NRP-V7 mimotope of the IGRP206-214 peptide (described below), and has previously been characterized by multiple other groups. We therefore felt confident in our general approach to TCR sequence analysis. Next, to determine pathways of autoantigen and alloantigen recognition, we selected TCR α and β chains frequently detected within allograft rejection lesions in NOD mice, shown in table 5-1, for
Upon initial analysis of frequent TCR sequences, we detected multiple TCR α and TCR β sequences which were highly enriched within rejected allograft lesions in NOD mice. We chose a subset of these TCR sequences to conduct a matching study in 5KC hybridoma cells, as described in methods.

TCR α sequence A4 from mouse 9812 was not tested because it is derived from MAIT cells. Data generated and analyzed by Maki Nakayama and Laurie Landry.

Importantly, all of the TCRs characterized were highly enriched from the endogenous pancreas to the allograft rejection lesion.

**TCRs highly enriched in allograft rejection lesions confer responsiveness to both NOD islets and C3H spleen cells in vitro**

Figure 5-6 depicts several TCR α/β combinations that confer responses to both autoantigens (left) and C3H spleen cells (right), derived from two different allograft rejection lesions in NOD mice (top represents mouse 9860, bottom represents mouse 9812). Part A demonstrates cell lines 9860A1B1 and 9860A3B3 respond to both an autoantigen among the panel tested, as well as irradiated C3H spleen cells. As such, these cell lines were our candidate...
Figure 5-6: Recapitulated TCRs from allograft lesions confer both autoreactive and alloimmune IL-2 production. A-B depict heat maps representing IL-2 production by 5KC hybridoma cells transfected with TCR combinations shown. White represents 0-30 pg/ml, light gray represents 30-60 pg/ml, medium gray represents 60-90 pg/ml, dark gray represents 90-120 pg/ml, and black represents >120 pg/ml IL-2 production. IL-2 was measured by ELISA following overnight culture as described in methods. Responses shown are by 100,000 5KC cells stimulated by 400,000 NIT-1 or C3H spleen cells or 200 µg/ml of peptides in the presence of 100,000 NODCa or NODCb spleen cells. A represents TCR α and TCR β chains derived from mouse 9860, whereas B represents TCR α and TCR β chains derived from mouse 9812. Data shown in C demonstrates that the IL-2 response against irradiated C3H cells mediated by cell line 9860A1B1 could be blocked using anti-Dk antibody. Data shown in D demonstrates that the IL-2 response from cell line 9860A3B3 against irradiated C3H cells could be blocked by addition of anti-I-Ak antibody. Data generated and analyzed by Maki Nakayama and Laurie Landry.

'heterologous' cells. Of note, cell line 9860A2B2 appears to be alloreactive but not autoreactive, a specificity which we expected to find in these rejected allografts.

In figure 5-7 we further determined that cell line 9860A1B1 is truly islet-reactive (responds to co-culture with NIT-1 insulinoma cells) following reconstitution with CD8. In
Figure 5-7: Antigen specificity of dual-reactive TCR sequences derived from allograft lesions. Figure A represents the IL-2 response of several cell lines to co-culture with insulinoma (NIT1) cells following reconstitution with CD8. Figure B represents the IL-2 response of cell line 9860A3B3 to co-culture with pS3 mimotope of the WE14 peptide derived from chromagranin A. Peptide re-stimulation used 100,000 5KC-T cells per well, 96-well plates, with 100 µg/ml peptide concentrations. Data generated and analyzed by Maki Nakayama and Laurie Landry.

In contrast, cell line 9860A3B3 responds to the pS3 peptide mimic of the chromagranin A-derived WE14 peptide following reconstitution with CD4. Importantly, figure 5-7 also demonstrates that IL-2 production by both of these cell lines can be blocked through inclusion of anti-MHC antibodies in the restimulation conditions. The insulin-reactive cell line can be blocked by anti-Dk antibody, confirming its MHC class I restriction, whereas responsiveness of the pS3-reactive cell line can be blunted with inclusion of anti-I-Ag7 antibodies, confirmed its MHC class II restriction. Lastly, figure 5-8 confirms that dual reactive (peptide or NIT1-reactive and
To verify that reconstituted TCRs maintained the ability to confer IL-2 production in response to overnight co-culture with 6-9 x 10^5 NOD.RAG^/- islet cells treated with 1000 U/ml IFN-γ overnight prior to stimulation. Presumptive dual-reactive cell line 9860A1B1 produced IL-2 in response to co-culture with whole NOD.RAG islets. Cell line 9860A2B2 is, we believe, alloreactive and not autoreactive. Cell lines 9812A3B1, and 9812 A3B2 produced modest amounts of IL-2, whereas cell line 9812A5B1 produced the largest amounts of IL-2 in response to co-culture with NOD.RAG islets. Data generated and analyzed by Maki Nakayama and Laurie Landry.

alloreactive) cell lines remain responsiveness to whole intact NOD.RAG^/- islets in vitro, with the exception of cell line 9860A2B2, which appears to be alloreactive only, as depicted in figure 5-6.

**Discussion**

The potential for autoreactive T cells to spontaneously cross-react with allogeneic MHC molecules or MHC-derived peptides is an intriguing potential explanation for the empirical observation of significantly accelerated allograft rejection in autoimmune recipients compared
Recent studies have demonstrated the presence of viral memory CD8 T cells in rejected skin allografts in mice (172) as well as the presence of memory CD8 T cells in rejected cardiac allografts in mice (182). To our knowledge this principle of dual-reactive autoreactive T cells had not previously been demonstrated in the NOD mouse following islet allograft rejection. In this study we harvested allograft-infiltrating T cells at the time of transplant rejection in NOD mice and took an unbiased TCR sequencing approach. The comparison T cell population was pancreas-resident, presumed disease onset-associated, T cells from the same diabetic mice. We observed moderate enrichment of disease onset-associated TCRs from the pancreas to the graft rejection lesion in NOD mice transplanted with NOD.RAG\(^{\text{1/2}}\) islets, a process which takes a median of 13 days. Autoimmune NOD mice reject allogeneic significantly more rapidly (MST 6 days for C3H islets). However, disease onset-associated TCRs still heavily infiltrate these transplants, suggesting a large autoimmune disease recurrence component. In addition, TCRs below the limit of detection in the pancreas heavily infiltrated the allograft rejection lesions, diluting out the disease onset-associated TCRs. These TCRs are presumably the ‘naïve alloreactive’ component of the overall anti-transplant response in autoimmune NOD recipients.

In this report we demonstrate several \(\alpha\beta\) TCR pairs isolated from allograft rejection lesions, which when recapitulated \textit{ex vivo} and stimulated with NOD islets and C3H spleen cells \textit{in vitro}, mediated hybridoma cell responses to \textit{both} categories of antigens. One of these cell lines required CD4 co-receptor, was pS3 (chromagranin A)-specific and was blocked by anti-I-Ag7 antibodies. The other cell line required CD8 co-receptor and was blocked by anti-Dk antibodies. In summary, we report for the first time evidence on a per cell basis that TCRs derived from autoreactive T cells are (1) enriched in allograft rejection lesions in NOD mice, and (2) mediate anti-MHC responses as well as anti-islet responses when recapitulated \textit{in vitro}. To our knowledge these data represent the first formal demonstration of this category of dual-reactive
T cells derived from an autoimmune T cell population. Following this discovery, a next logical step is to translate these findings into therapeutic strategies to decrease the responsiveness of this category of T cells. For example, as a general approach, recent reports have indicated that memory T cells may be amenable to IL-7 receptor blockade therapy, in particular in mouse models of T1D (141, 142). In addition, the Santamaria group has demonstrated the ability to blunt autoreactive IGRP-specific CD8 T cells using an immunization strategy based on expanding low-affinity peptide-specific CD8 T cells (111). These therapies may prove beneficial to inhibit the activation of heterologous alloreactive T cells in autoimmune recipients. Of particular importance in T1D are kidney transplant recipients and simultaneous pancreas-kidney transplant recipients (24, 183, 184, 186). Historically, T1D patients have represented a significant proportion of kidney transplant recipients due to end-stage renal failure consequent to long-term hyperglycemia. Immune suppression therapies put the diabetic kidney transplant recipient at great risk of developing infections which could necessitate cessation of immune suppression, potentially leading to T cell-mediated damage of kidney transplants. Therefore, developing alternatives to global immune suppression therapies which could slow or prevent memory T cell – in particular islet-specific memory T cell – targeting of non-pancreatic islet transplants in T1D individuals is a worthy clinical goal.
CHAPTER VI

EMPIRICALLY DERIVED COMBINATORIAL T CELL-DIRECTED THERAPY

DELAYS AUTOIMMUNE DISEASE RECURRENCE

Introduction

In individuals with poorly-controlled type 1 diabetes (T1D), diabetes-related disease complications can develop over time which severely impact quality of life (133-136). In contrast to treatment with insulin-replacement therapy via injections or insulin pump-based continuous infusion, whole pancreas or isolated pancreatic islet transplantation is a potentially curative therapy for T1D (152–154). However, autoimmune diabetic pancreatic islet transplant recipients – in particular the NOD mouse – are strikingly resistant to islet transplant tolerance-promoting therapies which are effective in non-autoimmune, chemically-induced diabetic mouse models (118, 127). Because results in autoimmune recipients (ie. NOD mice) do not agree with proof-of-concept studies in non-autoimmune-prone mouse strains (ie. chemically-induced diabetic B6 mice), the development of T cell-specific transplant tolerance-promoting therapies in the clinic for the T1D patient receiving an islet or whole pancreas transplant is challenging. Therefore, clinical protocols couple with islet or whole pancreas transplantation currently focus on global immune suppression as the strategy of choice (122, 154, 187).

A potential reason for discordant results between autoimmune and non-autoimmune recipients is that autoimmune recipients treated with tolerance-promoting therapies harbor memory T cell populations which will specifically target the transplanted insulin-producing cells. The effects of tolerance-promoting therapies on autoimmune memory T cells are challenging to interpret from validation studies in which naïve alloreactive T cells are inhibited in their responses to pancreatic islets. It is well-established that autoreactive T cells traffic to the pancreas due to specific signaling and activation events intrinsic to T cells (188). Recent results
have demonstrated that autoreactive CD4 T cells are activated, in part, through CD154-mediated signaling (189), that T cell-T cell interaction through CD40-CD154 signaling promotes autoimmunity in NOD mice (190), and that perturbation of CD154-mediated signaling can modify autoreactive CD8 T cell responses (191). In addition, allograft rejection mediated by memory T cells – which would be the case in diabetic NOD mice – appears to resist regulation (192). We therefore tested how a therapy validated in non-autoimmune recipients, anti-CD154 antibody treatment (118, 144), fails to prevent autoimmune disease recurrence in the NOD mouse model of T1D. Surprisingly, this therapy significantly delayed autoimmune disease recurrence in NOD mice. To determine both (1) how anti-CD154 mediated this unexpected graft prolongation, and (2) learn how autoimmune T cells circumvented this otherwise effective transplant tolerance-promoting therapy, we conducted ex vivo analyses on graft-infiltrating T cells at the time of transplant rejection. We chose to separately analyze affects of anti-CD154 on autoimmune CD4 and CD8 T cells. Our results suggested moderate effects on CD4 T cells and minimal effects on CD8 T cells. Therefore, we added transient CD8 T cell depletion as a second therapeutic component. Encouragingly, this combination therapy – both co-stimulation perturbation via anti-CD154 and transient CD8 T cell depletion – increased MST to 54 days in autoimmune recipients. This result with our novel combination therapy in autoimmune recipients represents a significant step forward in the delay of autoimmune diabetes in NOD mice (127).

We then applied this combination therapy to the clinically relevant model of NOD mice receiving islet allografts (B6 islets), with the goal of learning from the failure of this promising therapeutic combination in the clinically relevant setting. Results demonstrated much less affect of the combination therapy on intra-graft CD4 T cell responsiveness in the allograft setting, suggesting anti-MHC-reactive CD4 T cells as a major barrier to islet allograft tolerance in
autoimmune recipients. In addition, we observed disparate results between autoimmune NOD recipients of B6 islets (MHC mismatch, non-MHC-mismatch), congenic B6.g7 islets (MHC match, non-MHC mismatch), and NOD.B10 islets (MHC mismatch, non-MHC match) in autoimmune recipients. Briefly, we observed greater resistance to our combination therapy from CD4 T cells in recipients of B6.g7 congenic islets than in recipients of MHC-mismatched NOD.B10 islets. These data suggest the minor antigen-directed T cell responses we studied in chapter IV contribute to the profound resistance to therapeutic intervention we observe in diabetic NOD mice. These data further suggest that the combination of autoreactive T cell responses with either MHC-directed or minor antigen-directed T cell responses synergize to strongly resist T cell co-stimulation blockade-based therapies, even though each T cell response is individually amenable to this therapy. Overall, our results highlight important differences in the mechanisms of autoimmune disease recurrence and islet allograft rejection, which will need to be taken into account in future clinical protocols aimed at replacing lost beta cell mass and re-establishing self-tolerance to beta cells in diabetic patients.

Results

Anti-CD154 therapy delays autoimmune disease recurrence through effects on CD4 T cells

Autoimmune diabetic recipients are resistant to transplant tolerance-promoting therapies in the case of pancreatic islets, but also, strangely, multiple other organ/tissue types including cardiac allografts and skin allografts (57, 127, 163, 193). We aimed to determine the degree of resistance to islet allograft tolerance-promoting therapies characteristic of autoimmune (islet-specific) T cells in NOD mice. We first demonstrate in figure 6-1a that STZ-induced diabetic NOD male mice do not reject syngeneic (NOD.RAG-/-) islets, whereas autoimmune NOD female mice reject syngeneic islets in MST 13 days (p=0.0046). Next, in results shown in figure 6-1a, we treated autoimmune NOD mice with CD154 blockade therapy and
demonstrate that autoimmune disease recurrence in the NOD mouse is resistant to anti-CD154 treatment, though MST is nearly doubled compared to untreated recipients (MST 13 days improved to 22 days). In results shown in figure 6-1b we separate the effects of anti-CD154 therapy on autoreactive T cell subsets in NOD mice grated with syngeneic islets. Graft Infiltrate CD4 and CD8 T cells – isolated at the time of rejection and re-stimulated ex vivo – demonstrate a trend toward reduced cytokine production in the presence of peri-transplant anti-CD154 treatment compared to untreated recipients. Based on statistically non-significant reduction in proportion of IFN-γ-TNF-α co-producing CD4 T cells in the presence of peri-transplant anti-CD154 treatment, and our T cell depletion results from figure 3-1c demonstrating a critical role for autoimmune CD8 T cell in disease recurrence, as well as our desire to avoid depleting regulatory CD4 T cells (the depletion of which appears to accelerated disease recurrence in NOD mice, as shown in figure 6-2a), we added peri-transplant CD8 T cell depletion as a second component to our therapy in NOD disease recurrence.

The combination of anti-CD154 with peri-transplant CD8 T cell depletion significantly delays autoimmune disease recurrence

Data shown in figure 6-3 illustrate the efficacy of our dual therapy (peri-transplant anti-CD154 and anti-CD8 treatment) in autoimmune NOD recipients of syngeneic islets. This combination extends MST to 54 days, an unheard-of result in autoimmune recipients. Two of ten recipients in the dual therapy group reached 100 days post-transplant euglycemic. In addition, graft infiltrate ex vivo re-stimulation at the time of eventual transplant rejection in these recipients demonstrated an on-going effect on the CD4 T cell autoreactive CD4 T cell response, demonstrated by a significant reduction in IFN-γ-TNF-α co-producing CD4 T cells isolated from dual therapy-treated recipients. Importantly, recent articles (141, 142) have
Figure 6-1: anti-CD154 therapy moderately delays autoimmune disease recurrence in NOD mice. Figure 6-1a: Autoimmune diabetic mice (defined as 3 consecutive days with blood glucose reading >250 mg/dl) reject 500 hand-picked NOD.RAG islets, transplanted under the right kidney capsule in MST of 13 days (rejection defined as 2 consecutive days with blood glucose reading >300 mg/dl, retroactively defining day 1 > 300 mg/dl as the day of rejection), whereas streptozotocin-induced diabetic NOD male mice do not reject NOD.RAG islets (MST > 100 days), p=0.0046 and anti-CD154 antibody treatment prolongs NOD.RAG islet survival in autoimmune recipients to MST 22 days, p=0.0293 compared to untreated recipients. Anti-CD154 treatments were 125 µg on days -1, 2, 7, and 9 relative to islet transplantation. Figure 6-1b: Cells gated on CD45.1+CD4+ cells following ex vivo PMA/ionomycin re-stimulation, as described in methods. Representative data showing moderately decreased ex vivo responsiveness by transplant-infiltrating CD4 T cells in the presence of anti-CD154 therapy (top row, for reference compare to untreated recipients shown in figure 3-2b). Bottom row: cells gated on CD45.1+CD8+ cells following ex vivo PMA/ionomycin re-stimulation, as described in materials and methods. We did not observed parallel reductions in ex vivo CD8 T cell responsiveness in the presence of anti-CD154 therapy compared to untreated recipients (shown in figure 3-2c). Figure 6-1c: Quantification of results across n=3 CD154-treated mice and n=5 untreated NOD recipients of NOD.RAG islets. No significant differences were noted, but a trend toward fewer IFN-γ/TNF-α co-producing CD4 T cells (top) and CD8 T cells (bottom) was noted. Gray tracings represent graft-infiltrating T cells cultured in the absence of polyclonal restimulation, whereas black tracings represent cytokine production by graft-infiltrating T cells in the presence of polyclonal restimulation.
Figure 6-2: CD25+ T cells delay autoimmune disease recurrence but not allograft rejection in NOD mice. Figure 6-2a: Treatment with anti-CD25 antibody days -1 and +2 relative to transplantation with NOD.RAG islets accelerates autoimmune disease recurrence in NOD mice, p=0.0027. Figure 6-2b: Treatment with anti-CD25 antibody days -1 and +2 relative to transplantation with B6 islets moderately delays islet allograft rejection, p=0.0344. Untreated controls are the same as shown in Figure 3-2.
Figure 6-3: the combination of anti-CD154 and transient CD8 T cell depletion promotes significantly prolonged islet transplant survival in autoimmune NOD mice. Figure 6-3a: Peri-transplant CD8 T cell depletion significantly delays rejection of NOD.RAG islets in autoimmune diabetic NOD recipients, p=0.0020. CD8 T cell depletion therapy was 20 mg/kg of antibody clone 53-6.72 administered ip days -2, 0, 2, 7, and 14 relative to islet transplantation. CD8 T cell depletion therapy effectively depleted >90% of the relevant T cell populations (data not shown). Untreated control group the same as shown in Figure 3-2. Figure 6-3b: Rationale behind development of anti-CD154/anti-CD8 dual therapy to delay autoimmune disease recurrence in NOD mice. Figure 6-3c: The addition of peri-transplant CD8 T cell depletion to anti-CD154 therapy synergistically increased survival of NOD.RAG islets in autoimmune NOD recipient, p=0.0008 compared to untreated recipients. In addition to greatly prolonged graft survival time, we observed a significant reduction in CD4 T cells which produced both IFN and TNF upon ex vivo re-stimulation in dual therapy recipient mice (n=3), p=0.029 relative to untreated mice (n=5).

shown promising results in diabetes onset reversion using blockade antibodies specific for the IL-7 receptor. We applied one of these reagents (A7R34) to the autoimmune disease recurrence setting, and observed superior effect with our empirically-derived dual therapy (figure 6-3).
**Dual therapy in less effective in delaying rejection of MHC mismatched or MHC-matched islet transplants in autoimmune recipients**

We therefore extended this promising therapeutic combination which inhibited autoreactive CD4 T cells and transiently depleted autoreactive CD8 T cells to the clinically relevant allograft scenario ([figure 6-4a]). Unfortunately this combination therapy demonstrated less significant survival benefit for allograft recipients (MST 13 days). To determine whether MHC-matching would enhance the efficacy of this therapeutic approach in autoimmune recipients, as shown in [figure 6-4b] I transplanted autoimmune recipients with MHC-matched islets and dual therapy. Interestingly, and in agreement with the overall conclusion of chapter V, the survival of MHC-matched islets in autoimmune recipients was not enhanced beyond the level seen with allogeneic islets (MST 14 days), but was improved beyond untreated NOD recipients of MHC-matched transplants (MST 7 days, p<0.05).

We then took an analytical approach to determine the effects of anti-CD154 therapy and dual therapy on graft-infiltrating T cells in autoimmune NOD mice grafted with B6 islets ([figure 6-5]). In contrast to graft-infiltrating T cells which precipitated rejection of NOD.RAG^-/- islets in the presence of dual therapy, in B6 islet rejection lesions in the presence of either CD154 single therapy or dual therapy we did not observe trends toward decreased percentages of T cells producing either IFN-γ or TNF-α individually or both cytokines simultaneously. These data suggest that islet allograft rejection in autoimmune recipients strongly resists this therapeutic combination that significantly delayed autoimmune disease recurrence. Our next step was to investigate at the effects of dual therapy on CD4 T cell-mediated rejection of MHC-matched (B6.g7) islets in autoimmune NOD recipients. Results in [figure 6-6] show the potential for a trend toward decreased percentage of co-cytokine-producing CD4 T cells in the presence of dual therapy compared to untreated or anti-CD154-treated recipients. However, no
statistically significant reductions in CD4 T cell cytokine production were noted, despite doubling of graft survival (MST 7 days untreated compared to MST 14 days dual therapy-treated). This lack of demonstrable decrease in CD4 T cell cytokine production suggests the existence of CD4 T cells that respond to additional non-MHC-derived antigens and which potentiate rejection of MHC-matched islet transplants in NOD mice.

**MHC mismatched islet transplants demonstrate a trend toward improved survival in autoimmune recipients in the presence of dual therapy, compared to MHC-matched islets**

We then applied our dual therapy to the MHC mismatched, but minor antigen-matched NOD.B10 islet donor in autoimmune NOD recipients (figure 6-7). In this experiment we aimed to address whether the MHC-directed T cell response in NOD mice was as resistant to this combination therapy as the minor antigen-directed T cell response we studied above. In the presence of dual therapy, MHC-mismatched NOD.B10 islets survive a shorter time than syngeneic NOD.RAG\(^{-}\) islets (MST 54 days compared to MST 23 days, p=0.0199). In addition, we observed a non-significant increase of median transplant survival in the presence of dual therapy in autoimmune recipients compared to MHC matched B6.g7 or mismatched B6 islets (MST 23 days compared to 16 days or 13 days, respectively, p-values 0.7244 and 0.7031). These data suggest, but do not prove, that MHC-directed T cell responses may be more amenable to therapeutic intervention than minor-antigen-directed T cell responses in NOD mice. Results in figure 6-7b illustrate *ex vivo* re-stimulation experiments using NOD.B10 rejection lesion T cells from autoimmune NOD mice suggest a non-significant trend toward reduced responsiveness compared to dual therapy-treated NOD recipients of either B6 islets or B6.g7 islets. The transplant survival comparison in the presence of dual therapy shown in **figure 6-8 and figure 6-9** suggests that while autoreactive and alloreactive CD4 T cell responses may be amenable to
Figure 6-4: Dual therapy does not provide the same degree of protection to either MHC-matched or MHC-mismatched islet transplants in autoimmune recipients. 

Figure 6-4a: anti-CD154 modestly prolongs survival of allogeneic B6 islets in autoimmune NOD recipients (MST 6 days to 9 days), whereas the combination with peri-transplant CD8 T cell depletion significantly prolongs transplant survival to MST 13 days (p<0.05)

Figure 6-4b: anti-CD154 modestly prolongs survival of MHC-matched B6.g7 islets in autoimmune NOD recipients (MST 7 days to 9 days), whereas the combination with peri-transplant CD8 T cell depletion significantly prolongs transplant survival to MST 14 days (p=0.0057).
Figure 6-5: Anti-CD154 therapy does not significantly decrease graft infiltrate CD4 or CD8 T cell responses against B6 islets. Figure 6-5: Representative transplant-infiltrate ex vivo restimulation cytokine production, demonstrating very little affect of anti-CD154 therapy on graft infiltrate T cell cytokine production (CD4 T cells top (A), CD8 T cells bottom (B)). Gray tracings represent graft-infiltrating T cells cultured in the absence of polyclonal restimulation, whereas black tracings represent cytokine production by graft-infiltrating T cells in the presence of polyclonal restimulation.
Figure 6-6: Responsiveness against MHC-matched islet transplants is not significantly reduced in NOD graft infiltrate CD4 T cells. Figure 6-6a: TOP: in the absence of treatment, graft infiltrate CD4 T cells following NOD rejection of MHC-matched (B6.g7) islets respond strongly to PMA/I re-stimulation. Gray tracings represent graft-infiltrating T cells cultured in the absence of polyclonal restimulation, whereas black tracings represent cytokine production by graft-infiltrating T cells in the presence of polyclonal restimulation. Bottom: in the presence of dual therapy, we observed a non-significant trend toward decreased proportion of dual cytokine-producing CD4 T cells within rejected MHC-matched islet transplants in autoimmune NOD recipients.

anti-CD154 therapy, the minor-antigen-directed CD4 T cell response may, in fact, be the T cell response most responsible for therapy resistance in the diabetic NOD mouse.

Discussion

Our results demonstrate that autoimmune CD4 T cell responses to their cognate antigens can be delayed by anti-CD154-directed therapy. Analysis of rejected islet grafts indicate that autoreactive CD8 T cell responses are less affected by this therapy, in agreement with literature describing decreased dependence of anti-viral CD8 T cells upon co-stimulation for...
secondary activation (124, 194). Therefore, in our autoimmune recipients, we added peri-transplant CD8 T cell depletion as a second therapeutic component. This addition synergistically improved MST of syngeneic islets to 54 days in autoimmune recipients and is associated with decreased \textit{ex vivo} cytokine production by graft-infiltrating CD4 T cells at the time of transplant rejection (figure 6-4). These results suggest that the autoimmune response to pancreatic islets in NOD mice, in isolation, is amenable to transplant tolerance-promoting therapies.

Figure 6-7: Rejection of MHC-mismatched islet transplants in autoimmune recipients can be significantly delayed by dual therapy. Figure 6-7a: In untreated autoimmune NOD recipients, NOD.B10 islets are rejected in MST 7 days, which can be significantly delayed by dual therapy to MST 23 days (p=0.0024). Figure 6-7b: Following eventual rejection of NOD.B10 islets in autoimmune NOD recipients, PMA/I re-stimulation reveals a trend toward increased TNF-α single-producing and dual cytokine-producing CD4 T cells compared to rejection lesions of MHC-matched B6.g7 transplants in the presence of dual therapy in autoimmune NOD recipients.
Encouraged by these results, we applied this dual therapy to the clinically relevant scenario of pancreatic islet allografts (B6 islets) transplanted into autoimmune diabetic NOD mice. Unfortunately, this combination had much less dramatic graft prolongation effects in this scenario.

Figure 6-8: Comparison between T cell resistance to dual therapy across islet transplantation rejection scenarios in autoimmune diabetic NOD mice. Figure 6-8a: Despite a doubling of graft survival time in recipients of NOD.RAG islets, when compared across n=3 mice for each group, we detected no significant differences between graft-infiltrating CD4 T cell responses in dual therapy-treated recipients of NOD.RAG and NOD.B10 islets. Figure 6-8b: We observed a significant difference in the proportion of graft-infiltrating CD4 T cells which produced TNF-α only in NOD.B10 islet rejection lesions compared to B6 islet rejection lesions, despite doubled graft survival in NOD.B10 recipients. Figure 6-8c: Similar to figure 6-8b, we observed a significant difference in the proportion of TNF-α single-producing CD4 T cells isolated from NOD.B10 islet rejection lesions compared to B6.g7 islet rejection lesions in autoimmune recipients under the cover of dual therapy. We also observed a significantly higher proportion of dual cytokine-producing CD4+ T cells in rejected B6.g7 islet lesions. Figure 6-8d: comparison of islet transplant survival times in autoimmune NOD recipients across donor strains and treatment combinations: *** = p<0.001, ** =p<0.01, * = p<0.05 compared to untreated recipients.
setting (MST 13 days for B6 islets, figure 6-5). This decreased transplant survival benefit for allografts in the presence of dual therapy correlated with less affect on ex vivo graft infiltrate CD4 T cell cytokine compared to disease recurrence-derived CD4 T cell cytokine production. These data suggest that alloreactive CD4 T cells are a major barrier to islet transplant tolerance induction in NOD mice, consistent with our findings in chapter III that depletion of CD4 T cells, but not depletion of CD8 T cells, significantly delayed rejection of B6 islets in NOD mice (figure 3-1). Intriguingly, and in agreement with chapter III, MHC-matched islet transplants from genetically disparate mouse strains (B6.g7) benefited from this combination therapy to the degree seen with allografts (MST 16 days), not to the degree seen with syngeneic islet transplants (MST 54 days). These results highlight the presence of a third category of T cell response to islet transplants in the autoimmune recipient that is directed against non-MHC derived antigens. In addition to and in agreement with the graft rejection phenotypic data, ex vivo re-stimulation of CD4 T cells isolated from dual therapy-treated, rejected B6.g7 islet transplants in NOD mice contained higher percentages of cytokine-producing cells than CD4 T cells isolated from rejection lesions of syngeneic islets in the presence of dual therapy (figure 6-8). These results suggest that the combination of autoreactive and minor antigen-directed CD4 T cell responses resists therapeutic intervention with this combinatorial treatment, in contrast to the therapeutic affect we observed in conventional disease recurrence. Furthermore, the transplant survival data suggest the combination of minor antigen-directed and autoimmune T cells (B6.g7, MST 16 days) resists therapeutically intervention as strongly as the combination of all three T cell responses (minor antigen-directed, MHC-directed, and autoimmune), as seen in rejection of B6 islets (MST 13 days). In sum, the minor antigen-directed CD4 T cell response in autoimmune NOD mice is both unexpectedly robust and remarkably resistant to therapeutic intervention.
To test whether alloreactive CD4 T cells were as resistant to dual therapy as minor antigen-directed CD4 T cells, we transplanted minor match/MHC mismatch (NOD.B10) islets into autoimmune NOD mice in the presence of our dual therapy. Results demonstrated a trend toward improved graft survival for minor match/MHC mismatch islets (MST 23 days) compared to MHC-matched islets (MST 16 days) in the presence of our dual therapy. Following eventual transplant rejection, ex vivo graft infiltrate CD4 T cell re-stimulation experiments suggested trends toward higher percentages of TNF-γ single-producing CD4 T cells and lower percentages of IFN-γ-TNF-α co-producing CD4 T cells upon re-stimulation in the NOD.B10 recipients compared to B6.g7 recipients. These data suggest, contrary to our expectation, that the combination of autoimmune T cell responses with anti-MHC-directed CD4 T cell responses in NOD mice may be more amenable to our dual therapy than is the combination of autoimmune T cell responses and minor antigen-directed T cells responses. In summation, these results suggest the minor antigen-directed CD4 T cell response in NOD mice is a significant and therapy-resistant component of transplant rejection, and may in fact represent a more stringent barrier to islet transplant survival in the NOD mouse than are MHC-directed T cell responses. In addition, these data demonstrate that the autoimmune T cell response, if isolated through transplantation of islets genetically identical to the recipient, is amenable to transplant tolerance-promoting, T cell-directed therapy (figure 6-4). Future tasks include improving the efficacy of therapeutic combinations in the autoimmune NOD mouse transplanted with NOD islets, potentially through memory T cell inhibition (141, 142) and/or concurrent adoptive transfer of ex vivo-generated islet-specific regulatory T cells (195–197).
CHAPTER VII
DISCUSSION

Context of this Dissertation

Type 1 diabetes (T1D) affects approximately 1.5 million Americans and represents at least a $17 billion burden to the US health care system. Clinical management currently focuses on insulin-replacement therapy, due to destruction of >70% of insulin-producing beta cells at the time of clinical diagnosis (198). Beta cell destruction results from insufficient negative selection of T cells in the thymus and inappropriate peripheral activation T autoimmune-prone T cells (199, 200). T1D is ‘predictable’ in that HLA DQ8/DR4 confer significant risk for developing the disease (98): however, there are environmental factors which promote disease that are poorly understood, but may include an increasingly sterile early-life environment in the months following birth in developed countries, known as the hygiene hypothesis (201). The mouse model of T1D, the NOD mouse, has been utilized to demonstrate that T1D is a T cell-mediated autoimmune disease. Many of the relevant auto antigens have been identified, a significant proportion of which are associated with insulin secretory granules (53, 202). While MHC type in the mouse (HLA in humans) correlates with T1D risk, it does not transfer risk to non-autoimmune-prone strains: the B6.H-2g7 mouse does not develop autoimmune diabetes (165). An interesting recent hypothesis in T1D pathogenesis is that diabetes-related MHC/HLA promotes neo-antigen expression of modified self-peptides in the context of post-translational modifications due to inflammatory environments (203). This hypothesis could partially explain why NOD mice, but not MHC-congenic mice, develop islet-directed autoimmunity.

Despite insulin replacement therapy following diagnosis with T1D, some patients experience labile blood glucose level management (5), defined by both HbA1c >8.0% (which correlates to an average BG reading of 180 mg/dl over the preceding 90-120 days) and frequent
hypoglycemic episodes (multiple episodes per week <50 mg/dl during which assistance is required). Labile blood glucose level management – as well as duration of diabetes in years – is associated with development of diabetes-related complications primarily affecting the microvasculature. Complications of diabetes can affect several organ systems, including the eyes (18), the kidneys (19), and peripheral nerves (20). Perhaps the most debilitating diabetic complication is hypoglycemic unawareness – the loss of the ability to detect when one’s blood glucose level has dropped to dangerously low levels (<50 mg/dl), which can lead to seizures, comas, or in the most severe cases, death.

In attempts to ameliorate the development of diabetes-related complications in long-standing T1D patients, clinicians and insulin pump manufacturing companies continuously work to develop features to limit ‘excursions’ (<50 mg/dl or >250 mg/dl) in blood glucose levels. Toward this goal, insulin pump-based diabetes management is ever-improving, including recent additions of continuous glucose monitors (CGM), which test interstitial glucose level at least once every 5 minutes, and which can communicate this information via radio frequency to the insulin pump. An exciting recent addition to insulin pump therapy is the low threshold-suspend feature to the newest models of insulin pumps (204–208). This feature requires communication between a CGM device and the insulin pump, and ceases delivery of insulin when interstitial glucose levels reach a pre-set threshold (eg. 65 mg/dl) to avoid development of hypoglycemia, especially overnight.

Insulin replacement therapy is a treatment for T1D, not a biologic cure. Whole pancreas or isolated pancreatic islet transplantation for patients with T1D represents a curative therapy for this chronic autoimmune condition, meaning transplant recipients whose grafts functioned would no longer be required to inject exogenous insulin to maintain blood glucose levels (151). Unfortunately, insulin independence following either whole pancreas or isolated islet
transplantation has been an elusive goal (153). This is due to targeting and responses by both islet-specific autoreactive T cells (4, 24, 25) and conventional allograft MHC-reactive T cells (209) following transplantation of insulin-producing cells. In addition, as one might expect islet transplant rejection in autoimmune diabetic recipients strongly resists therapeutic intervention (122). The Edmonton transplantation group made a significant step forward in 2000 by introducing strong immune suppression protocols, promoting transplant survival longer than previous clinical protocols and giving hope to the field that transplantation may provide a path toward a cure (151). Over time, however, islet transplants in autoimmune recipients in the presence of strong immune suppression still lose function (153). Therefore, due to both relative lack of efficacy and the increased risk for severe infections from immune suppression, the islet transplantation field needs to shift away from global immune suppression and toward more focused T cell-directed therapies.

This dissertation project focused on the study of T cell-mediated pancreatic islet transplant rejection in autoimmune diabetic NOD mice. My work specifically addressed the following four hypotheses; in chapter III, we determined whether islet graft cell MHC expression is required for transplant rejection in autoimmune NOD recipients. In chapter IV, we determined if, in the absence of overt autoimmune disease status, T cells in NOD mice rapidly respond to non-MHC graft cell differences. In chapter V, we determined if individual TCRs isolated from islet allograft rejection lesions in autoimmune diabetic recipients would exhibit enriched ability to cross-react with allogeneic MHC molecules. Finally, in chapter VI, we determined if autoimmune T cells, similar to anti-viral memory T cells, have decreased requirements for co-stimulation signals for secondary activation. I address each chapter individually below.
Chapter III – Islet Transplant Rejection Independent of Graft MHC Expression in NOD mice

It had been shown by others (128, 163, 164), and we confirmed, that islet allografts are more rapidly rejected than syngeneic islets in autoimmune diabetic transplant recipient. It was also known at the outset of these studies that NOD islets lacking B2M expression (NOD.B2M−/−) are not acutely rejected in autoimmune recipients (133), which suggests that CD8 T cell contact with MHC class I on transplanted islets is required for rejection in autoimmune recipients. Since current clinically relevant islet transplant donor is derived from genetically unrelated cadavers, we wished to study whether MHC expression on transplanted non-self islets is required for islet rejection in autoimmune recipients. Toward this end, we obtained B6.B2M−/− and B6.C2D mice from Jackson labs. When islets from either of these strains are transplanted into autoimmune NOD mice, the islets were rapidly rejected in a CD4 T cell-dependent, CD8 T cell-independent, manner (figures 3-1, 3-4, and 3-5). These data suggest that CD4 T cells are the necessary T cell population for islet allograft rejection, not CD8 T cells. My data in the islet allograft rejection model are strikingly similar to those of the Unanue group, who found that diabetes transfer by CD4 T cell clones depends on macrophages as the final effector cell which mediates beta cell death (42). My data are consistent with a model in which autoreactive CD4 T cell activation, upon recognition of autoantigens derived from islet allografts, is required to set in motion processes leading to beta cell death. In other words my data suggest that autoreactive CD4 T cells can specifically direct macrophages to kill transplanted beta cells independent of CD8 T cells and NK cells.

Therefore, to directly test whether MHC expression on non-self islets is required for islet transplant rejection in autoimmune recipients, we generated B6-background mice deficient in both MHC class I and MHC class II (B6.MHC-bald). I found these islets are acutely rejected in autoimmune NOD recipients (MST 12 days), though with delayed kinetics relative to MHC-
bearing allografts \((p<0.0001)\). Rejection of MHC-bald allografts is CD4 T cell-dependent, and CD8 T cell- and NK cell-independent (figure 3-8). These data demonstrate that CD4 T cells are the critical participant in islet graft rejection in autoimmune NOD mice, regardless of MHC expression. Furthermore, rejection of B6.MHC-bald islets is associated with graft infiltration by autoreactive CD4 T cells specific for either insulinB10:23-derived peptides or chromogranin A-derived peptides (figure 3-9). These two antigenic specificities are key CD4 T cell responses in T cell-mediated diabetes onset in mouse and man (202).

In this dissertation, I present data demonstrating significant enrichment of these two populations of CD4 T cells in autoimmune disease recurrence lesions compared to the spleens of the same mice or allograft rejection lesions in NOD mice \((p<0.05)\). When compared to the proportion of autoreactive graft-infiltrating CD4 T cells from MHC-sufficient allografts at the time of rejection, graft-infiltrating CD4 T cells isolated from B6.MHC-bald transplants demonstrate a trend toward enrichment of autoreactive CD4 T cells (figure 3-9). These data suggest that autoreactive CD4 T cells are activated and able to accumulate within – and presumably participate in rejection of – B6.MHC-bald islet transplants, despite the lack of MHC class II to physically interact with. These results beg the question: what T cell response allows autoreactive CD4 T cells to accumulate in MHC-bald transplants?

In sharp contrast, we confirmed that NOD.B2M\(^{-/-}\) islets are not acutely rejected in autoimmune recipients (MST >100 days), and further that NOD-background islet deficient in both MHC class I and MHC class II (NOD.B2M\(^{-/-}\) x NOD.CIITA\(^{-/-}\); hereafter termed NOD.MHC-bald) are not acutely rejected in autoimmune recipients (MST 87 days), despite the presence of pre-existing autoreactive T cells. Interestingly, islets isolated from NOD.CIITA\(^{-/-}\) mice, which have greatly reduced MHC class II expression and are deficient in CD4+ T cells, and do not develop autoimmune diabetes (210), are acutely rejected in autoimmune NOD mice in a CD8 T cell-
dependent manner (figure 3-4). In addition, in the infiltrate of rejected NOD.CIITA^/- islets in NOD mice, we observed increased percentages of CD8+ T cells and elevated CD44 expression on graft-infiltrating CD8 T cells (data not shown), emphasizing the central importance of CD8 T cell/MHC class I interactions to autoimmune disease recurrence.

The lack of rejection of NOD.B2M^/- or NOD.MHC-bald islets in autoimmune recipients does not appear to be mediated by regulatory T cells. We treated autoimmune recipients of the above islets peri-transplant with either CD25 blockade antibody (PC61) or PD1 blockade antibody (J43), neither of which promoted acute rejection (figure 3-7). However, rejection of NOD.RAG^/-, MHC-bearing, islet transplants is accelerated by CD25 inhibition (figure 6-3), indicating that regulatory T cells may act in protection of MHC-matched transplants in autoimmune recipients. In contrast, CD25+ cell inhibition moderately prolongs B6 islet survival in autoimmune NOD recipients (figure 6-3). However, this protection does not appear to extend to MHC-bald transplants or to MHC-mismatched transplants. Overall, these data suggest that CD25+ regulatory CD4 T cells may delay rejection of MHC-bearing NOD-background islets, but do not protect MHC-disparate islets or NOD.MHC-bald islets. Lastly, in an in vitro MLR experimental set-up, NOD CD4 T cells proliferate extensively in response to B6. B2M^/- spleen cells, but not against B6.C2D spleen cells (figure 4-1). As one might expect, the converse is true for B6.C2D stimulating cells: CD8 T cells proliferate extensively, whereas CD4 T cells do not. However, and consistent with the islet transplant rejection data, NOD CD4 T cells proliferate extensively in response to 4-day co-culture with B6.MHC-bald spleen cells (figure 4-1). These data demonstrate an unexpected and strong CD4 T cell response against B6.MHC-bald cells, in agreement with rejection of B6.MHC-bald islet transplants in autoimmune NOD mice being a CD4 T cell-dependent process.
From these data, I conclude that the primary ‘accelerator’ of B6.MHC-bald islet transplant rejection in NOD mice are non-MHC ‘minor antigen’ responses mediated by CD4 T cells. Based on this supposition, I speculate that the genetically engineered islet transplant that would stand the best chance of long-term function in the autoimmune diabetic recipient in the clinic are MHC I-deficient self-derived beta cells. NOD islets genetically deficient in the B2M molecule survived slightly longer than (MST >100 days), but not statistically different from NOD.MHC-bald islets (MST 87 days) in my hands. Current trends in directed differentiation of induced pluripotent cells (iPS cells) give hope to the cellular transplant research community that in the not-too-distant future there will be scalable methods to produce patient-derived, insulin-responsive beta cells in quantities sufficient for transplantation (211–214). The above results suggest one genetic modification to these self-derived beta cells, namely lack of MHC class I expression, which will greatly enhance transplant survival. Importantly for potential clinical application, I observed >100 day survival of MHC class I-deficient NOD islets in autoimmune recipients in the absence of T cell-directed therapies or general immune suppressive treatments.

It is formally possible that differences in endogenous retroviral superantigens between the B6 islet donor strain and the recipient NOD strain precipitated a portion of the CD4 T cell response responsible for B6.MHC-bald islet transplant rejection in SZ-induced NOD mice (215). If true, this suggests a similar time to rejection for autoimmune diabetic and SZ-induced diabetic NOD recipient of B6.MHC-bald islets. However, we observed MST of 12 days for B6.MHC-bald islets in autoimmune NOD recipients and MST 58 days in SZ-induced recipients (p<0.001), suggesting, to us, a CD4 T cell-dependent ‘chronic rejection’ phenotype reminiscent of clinical allograft rejection in the presence of strong immune suppression treatments. I consider it more likely, in the autoimmune recipient, that the presence of autoreactive T cells specific for peptides produced by the transplanted cells acted as an ‘adjuvant’ to accelerate the nascent
CD4 T cell response against non-MHC-derived peptide differences between the donor and the recipient. Importantly, these non-MHC peptide differences – which were not identified as part of this dissertation project – are sufficient to promote MHC-bald transplant rejection in the absence of autoimmune diabetes status in the NOD mouse and are not sufficient to promote rejection of B6.MHC-bald islets in BALB/c mice (which also differ from the B6 donor strain with respect to endogenous retroviral superantigens). We interpret the acceleration of this response in autoimmune recipients to be attributable directly to the presence of a large number of graft-derived peptide-specific T cells. That the MHC-bald transplants are rejected in the non-autoimmune recipient with delayed kinetics (MST 58 days) indicates, to us, the presence of a strong CD4 T cell response – restricted by recipient MHC class II molecules – against non-MHC-derived transplantation antigens. This response, if present, is not sufficient to promote rejection of MHC-bald islets in other mouse strains, including the BALB/c strain, and suggests that a portion of the resistance to therapy-induced islet allograft tolerance in NOD mice is due to this strong CD4 T cell response directed against non-MHC transplantation antigens. Table 7-1 summarizes islet transplants and results presented in chapter III.

**Chapter IV – Unexpected strength of minor antigen-directed T cell responses in NOD mice**

I investigated the role of non-MHC, non-auto antigen-driven graft rejection in the NOD mouse. This line of experimental inquiry began with the observation that MHC-matched islet transplants from non-NOD background mice (B6.H-2g7 or BALB.H-2g7) are rejected more rapidly in autoimmune NOD recipients than syngeneic (NOD background) islets (figure 4-2). These results indicated strong T cell responses against a third category of antigen: non-MHC, non-islet-derived antigens that differ between strains of mice, which we call ‘minor’ antigens. Based on
Transplants Performed for Chapter III

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Table 7.1: Transplants performed for chapter III. From these data we conclude that MHC class I is required for autoimmune disease recurrence, whereas recipient CD4 T cells are required for islet allograft rejection in autoimmune diabetic NOD mice.

The results presented in chapter III, we hypothesized that MHC-matched transplants may benefit from T cell-directed therapies which promote or enhance regulatory T cell number and/or function. We therefore treated autoimmune recipients of MHC-matched islet transplants with anti-CD154 therapy (figure 4-3), as others have shown this therapy to promote development of graft-specific regulatory T cells (125). Unfortunately, anti-CD154 therapy did not have as significant an impact as intended, which suggests that minor antigen-reactive T cells may resist...
co-stimulation blockade-based therapy. Results from the Greiner group at UMass suggest that this resistance is characteristic of the NOD strain (57) and may map to Idd3 or Idd9 (168, 169).

Accelerated T cell responses against non-MHC-derived, non-auto antigens in the autoimmune recipient may provide at least a partial explanation of the long-observed resistance to induced transplant tolerance to any organ type in diabetic recipients (24). This clinically relevant question has not been previously addressed in mouse models in which diabetes-associated MHC is removed from the disease state, experiments which we pursued in this chapter. In addition, these data suggest that the summation of the autoreactive T cell response with the minor antigen-directed response (B6.g7 islets transplanted into autoimmune NOD mice) yields equivalent time to islet transplant rejection as the summation of these two T cell responses and the anti-MHC response (B6 islets transplanted into autoimmune NOD mice). To determine the speed of rejection of MHC-mismatched but minor antigen-matched islet transplant rejection in autoimmune recipients, we transplanted diabetic NOD recipients with NOD.B10 islets. We observed MST of 7 days, which is not significantly delayed relative to rejection due to the summation of autoimmune and alloreactive T cell responses described above for B6.g7 islets or the summation of three T cell responses (B6 islets). These data demonstrate that the combination of either MHC or minor antigen differences with autoimmune T cell responses synergize to promote acute transplant rejection which is more rapid than disease recurrence. To isolate effects of the minor antigen response mediated by I-Ag7 in the absence of autoimmune disease status, we utilized the streptozotocin (SZ) model of chemically-induced diabetes. SZ-treated pre-diabetic NOD mice reject MHC-matched islets (BALB.g7 or B6.g7) in the time frame typically seen when non-autoimmune-prone mice reject MHC-disparate grafts (10-12 days), in contrast with SZ-treated B6.g7 mice, which rejected MHC-matched BALB.g7 islets in MST 25 days (figure 4-4). These data suggest that in the absence of
overt autoimmune disease status, mice bearing diabetes-associated MHC and the presence of NOD background genes are driven to rapidly reject minor antigen-disparate transplants.

We then determined whether diabetes-associated MHC, I-Ag7, promotes therapy-resistant transplant rejection due to minor antigen differences separate from the autoimmune response characteristic of NOD mice. Toward this end, we utilized B6.g7 and BALB.g7 mice as transplant recipients. When we induced diabetes in MHC congenic mice (BALB.g7) with SZ and transplanted with allogeneic (B6) islets, we observed normal time to allograft rejection (MST 11 days). Further, approximately 50% of these recipients could be induced to become long-term tolerant to transplants via anti-CD154 therapy and euglycemia was shown to be graft-dependent via return to hyperglycemia following survival nephrectomy (n=2). To directly test whether diabetes-associated MHC mediates rapid and therapy-resistant islet transplant rejection due to minor antigen differences alone, we induced diabetes with SZ in B6.g7 mice followed by transplantation with BALB.g7 islets. Untreated recipients exhibited expected delayed rejection due to minor antigen differences – MST 22 days – and importantly 5 of 6 recipients were made long-term tolerant to these transplants through anti-CD154 therapy and euglycemia was shown to be graft-dependent via return to hyperglycemia following survival nephrectomy (n=2). These data demonstrate that diabetes-associated MHC is not intrinsically resistant to islet allograft tolerance or tolerance against ‘minor’ transplantation antigens induced by anti-CD154 therapy and suggest that NOD background (non-MHC) genes drive resistance to induced transplantation tolerance in both of these settings.

In agreement with rapid rejection of MHC-matched transplants, NOD-derived CD4 T cells exhibited robust proliferative responses in a conventional MLR experiment when MHC-matched B6.g7 spleen cells were used as the stimulating population (figure 4-5). In fact, the degree of CD4 T cell proliferation was roughly equivalent between MHC-matched, minor-
antigen-matched, or minor-and-MHC-mismatched stimulating cells. These data corroborate the
in vivo transplant rejection data, suggesting that NOD CD4 T cells display unexpectedly robust
responses to minor antigen mismatched cells in addition to strong responses against allogeneic
MHC molecules. In summary, our data from the MHC congenic pancreatic islet transplantation
experiments demonstrate that – outside the context of NOD background genes – diabetes-
associated MHC (1) does not promote rapid islet rejection due to minor antigen differences
only, and (2) does not mediate therapy-resistant rejection of either islet allografts or minor
antigen mismatched islet transplants. Therefore, I speculate that NOD background genes – in
particular Idd3 and Idd9 – synergize with disease-associated MHC, driving the unexpected minor
antigen responses in non-autoimmune NOD mice. Table 7-2 summarizes the islet transplants
performed for chapter VI.

Transplants Performed for Chapter IV

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Table 7-2: Transplants performed for chapter IV. From these data we conclude that MHC-
matched islet transplants are rapidly rejected in NOD mice in the absence of autoimmune
disease status, and that allograft rejection in NOD mice resists tolerance induction by anti-
CD154 therapy.
Chapter V – Autoimmunity as an Endogenous Source of Heterologous Alloreactivity

An emerging concept in the anti-viral T cell response literature, as well as the transplantation rejection literature, has focused on the issue of ‘heterologous’ immunity as a barrier to transplantation. In the anti-viral T cell response literature, this term describes effector/memory T cells specific for one virus which, when the host is challenged with subsequent viral pathogens, mediate responses to the second – structurally unrelated – pathogen (216, 217). In the transplantation literature, this term describes memory T cells specific for an antigen unrelated to the transplant (for example, viral-specific memory T cells) which cross-react, by chance, with graft-derived MHC molecules and mediate transplant rejection, which is in some instances resistant to therapeutic intervention (172, 218).

This apparent cross-reactivity of memory T cells for new challenges to the host immune system appears to be a general phenomenon of memory T cells, and may be a major factor behind the long-term protection resulting from vaccination (173, 177). The concept of heterologous immunity has recently has validated in the basic transplantation literature. For example, viral memory T cells have been shown to rapidly accumulate in rejected skin allografts in mice (172, 219), and memory T cells accumulate in rejecting cardiac allografts in aged mice (182). The autoimmune setting represented by the NOD mouse model is an additional scenario in which there is a large burden of memory T cells, in this case memory T cells which are specific for graft-derived autoantigens. We sought to determine whether this concept of heterologous immunity might also apply to autoimmune T cells – to the detriment of the host – in the case of allogeneic MHC on transplanted islets.

We hypothesized that autoreactive T cells which cross-react, by chance, with allogeneic MHC molecules would both (1) recruit naïve alloreactive T cells which could accelerate graft rejection, and (2) disarm regulatory T cells which likely delay rejection of syngeneic islets in
autoimmune recipients. In addition, we speculated that alloreactive T cells which are also autoreactive could accelerate overall graft rejection through increasing necrotic death of alpha cells and other non-beta cells, creating a more inflammatory micro-environment in which beta cells would have to continue to produce insulin in response to local glucose concentration. Based on the potential for autoimmune memory T cells to cross-react with graft cell MHC, we predicted that we would observe enrichment of alloreactive T cells within rejected islet allografts in diabetic NOD mice. We further predicted that the most-enriched of these TCRs, when compared to the pancreas-resident TCRs isolated from transplant-rejecting NOD mice, would demonstrate responsiveness to both NOD islets and spleen cells from the allogeneic strain the transplants were isolated from, C3H (H-2k).

To test these suppositions, and in collaboration with the Nakayama lab at the Barbara Davis Center, we isolated total RNA from graft rejection lesions and endogenous pancreatic islets at the time of transplant rejection (figure 5-3). We utilized Roche 454 sequencing to determine the following in autoimmune diabetic NOD mice: (a) disease recurrence-associated TCR α sequences, as well as (b) islet allograft rejection-associated TCR α and TCR β sequences. In both scenarios above, we also sequenced TCRs found within the native pancreas of graft-rejecting NOD mice at the time of graft rejection as a proxy for disease onset-associated TCR sequences. We observed 50-70% overlap (n=3) between native pancreas and disease recurrence lesion in autoimmune NOD mice. We detected multiple TCRs known to mediate disease onset, including insulin-reactive, IGRP-reactive, and chromagranin A-reactive TCR sequences. Importantly, we observed enrichment of these TCR sequences – and others – from the pancreas to the disease recurrence lesion in autoimmune NOD mice. One limitation of this sequencing approach is that we sequenced in the TCR α and TCR β chains separately, so that to determine pairing of α and β chains we were required to conduct in vitro matching studies, described
below. These data demonstrated the utility of this experimental approach, and suggested this was a viable strategy to search for ‘new’, and potentially heterologous, TCR sequences in allograft rejection lesions in NOD mice. We believe a similar approach could be applied to allograft rejection lesions in human transplantation settings to determine the potential of this strategy to identify dual-reactive TCRs associated with clinical islet allograft rejection.

Allograft rejection lesions in NOD mice demonstrated a range of TCR overlap with the native pancreas from 15% to 59% (n=3). This was not a significant difference with the degree of overlap seen in disease recurrence lesions (p=0.11). An overlap of 15% between the native pancreas and the allograft rejection lesion indicates that this recipient rejected their allograft rapidly due primarily to anti-MHC and anti-minor-antigen-directed T cells responses, not to autoimmune disease recurrence. The lack of down-regulation of both of the above responses probably played a significant role in the rapidity of this rejection response. In contrast, 59% TCR sequence overlap between native pancreas and allograft rejection lesion indicates that this recipient displayed a large degree of disease recurrence, within our range of detected disease recurrence-associated TCR sequences in syngeneic transplant recipients, indicating that the lack of down-regulation of additional T cell responses may also have been a ‘tipping point’ favoring rapid rejection. Interestingly, the range of 15% to 59% disease overlap in allograft rejection recipients was generated from genetically identical NOD recipients who rejected genetically identical C3H islets in an identical time frame of 6-7 days. These data demonstrate a larger degree of heterogeneity in synchronous outcomes in genetically identical recipients than previously appreciated. Furthermore, this level of heterogeneity when genetic differences are fixed is a potential major confounding factor for more clinically relevant studies (eg. out bred islet donors and out bred transplant recipients) of asynchronous phenomena.
We reasoned that TCRs that mediated anti-MHC responses would be highly enriched in the allograft, and absent or very rare in the pancreas. We also reasoned that, on a sequence level, these TCRs would be detected in disease recurrence lesions, but more highly enriched in allograft rejection lesions. Therefore, our next experimental step was to conduct an in vitro matching study of allograft-enriched TCR alpha and TCR beta chains. We selected allograft rejection-associated TCRs which were highly enriched relative to the pancreas in diabetic NOD mice. We used TCR constructs loaded into MSCV vector to infect Phoenix cells, and then collected the supernatant of infected cells to transduce 5KC hybridoma cells, which lack endogenous TCR sequences. We used IL-2 production by transfected 5KC cells in response to co-culture with allogeneic spleen cells or NOD islets as a read-out of alloreactivity. The Nakayama lab generated four T cell lines which reproducibly expressed IL-2 when co-cultured with C3H spleen cells. Importantly, all four of these cell lines responded to co-culture with NOD.RAG\(^{-/-}\) islets with IL-2 production. This formally demonstrated the presence of at least four dual-reactive TCRs isolated from an islet allograft in a diabetic recipient – validating our experimental approach. Interestingly, one of these cell lines responded strongly to the ps3 peptide derived from chromagranin A, which indicated we may have re-isolated the BDC2.5 cell line. However, we did not: this TCR sequence differed from the BDC2.5 TCR sequence, which has been studied for >20 years is not alloreactive against B6 or C3H.

In conclusion, the presence of heterologous alloimmunity may provide at least a partial explanation as to why islet allografts are so rapidly rejected in autoimmune recipients: the presence of a parallel 'memory' anti-allograft response which may both (1) potentiate the autoimmune response and (2) disable islet-specific regulatory T cells. Heterologous alloimmunity may also partially explain why this combined auto/allo response is resistant to therapy – the dual-reactive T cells may in fact be those resistant to therapy. Lastly, heterologous
alloimmunity may partially explain why transplants other than islets are both rapidly rejected and this rejection is resistant to therapy in autoimmune diabetic recipients. In autoimmune recipients, autoreactive T cells represent a seed population of memory T cells, of which 1-10% would be expected to cross-react with any given allogeneic MHC type, in essence meaning autoimmune recipients are pre-sensitized against any transplanted tissue of any allogeneic haplotype. Theoretically, this principle may apply to other populations of autoimmune T cells, so may represent an extremely relevant pathway of accelerated transplant rejection in recipients with underlying autoimmune conditions. This unfortunate out-come of T cell cross-reactivity may have particularly dire consequences for T1D recipients of kidney allografts (24).

Chapter VI – Empirical Development of a Combination Therapy Which Significantly Delays Autoimmune Disease Recurrence

In small animal models, Islet allograft tolerance-promoting therapies are usually tested and validated in STZ-induced, non-autoimmune prone diabetic mouse strains. Since the extension of these therapies to the NOD mouse – and by extension to the human recipient who is autoimmune diabetic – has been problematic, I studied how an efficacious and extensively characterized T cell-directed therapy fails to prevent autoimmune disease recurrence in NOD mice. We hypothesized that autoimmune T cells would strongly resist therapeutic intervention with anti-CD154 antibody treatment. This therapy was of interest to us because of previous work in our lab (118, 144), and others has demonstrated that anti-CD154 can mediate CD4 T cell-dependent, transferrable, islet allograft tolerance in non-autoimmune diabetic recipients.

Unexpectedly, MST for syngeneic transplants in autoimmune recipients was 22 days, significantly prolonged compared to untreated mice (p=0.0293). When we analyzed graft infiltrate T cells from these mice, we observed a trend toward decreased graft infiltration by autoreactive CD4 T cells and significantly reduced activation status and cytokine production by
graft-infiltrating CD4 T cells in anti-CD154-treated recipients. Importantly, we did not observe parallel decreases in graft infiltrate CD8 T cell responses. Based on this finding that CD8 T cells appeared to resist tolerance-promoting therapies, we applied adjunct anti-CD8 therapy to NOD islet graft recipients in an attempt to improve graft outcome. Autoimmune mice treated with peri-transplant anti-CD8 therapy demonstrated MST of 23 days following islet transplant (figure 6-4), approximately the same result as anti-CD154 therapy. However, when combining these therapies, NOD mice exhibited significantly delayed autoimmune disease recurrence (MST 54 days, figure 6-4). Importantly, NOD.RAG^-/- islets function indefinitely in STZ-treated NOD male mice (MST >100 days), indicating that eventual rejection of NOD.RAG^-/- islets in dual therapy mice is indeed T cell-mediated. Ex vivo re-stimulation of graft infiltrate T cells following rejection in the presence of dual therapy demonstrated significantly decreased percentage of graft-infiltrating CD4 T cells co-producing both IFN-γ and TNF-α in response to PMA/I re-stimulation (p=0.029), suggesting that the additional component of removing autoreactive CD8 T cells further inhibited the ability of autoreactive CD4 T cells to respond to transplanted beta cells. Encouragingly, 2 of 10 dual therapy-treated autoimmune recipients of NOD.RAG^-/- islets remained euglycemic >100 days post-transplant. These results suggest that the autoimmune T cell response to pancreatic islets is amenable to T cell-directed intervention – in particular co-stimulation blockade – and raises the question of whether the anti-allograft component of the T cell response may be T cell compartment in which resistance to therapy resides.

Therefore, we applied our combination therapy to the clinical scenario of islet allografts in autoimmune recipients. Firstly, single therapy of anti-CD154 with islet allografts in autoimmune recipients provides more modest graft survival benefit (MST extended from 6 days to 10 days) than in rejection of syngeneic islets and ex vivo re-stimulation of graft infiltrate CD4 T cells indicated that anti-CD154 therapy had no effect on decreasing cytokine production by
graft-infiltrating CD4 T cells. In addition, peri-transplant CD8 T cell depletion has virtually zero benefit in allograft recipients (MST unchanged from untreated allograft recipients). In the autoimmune recipient transplanted with allogeneic (B6) islets, our dual therapy demonstrated much less benefit (MST 13 days) and had very little measurable effect on ex vivo cytokine production by graft-infiltrating CD4 T cells following transplant rejection (figure 6-7). This phenotypic difference may be due to many factors, including (1) the additional component of alloreactive CD4 T cells – shown to be a major factor in allograft rejection in chapter III – as well as minor antigen-reactive CD4 T cells – shown to be a major component of transplant rejection in NOD mice in chapter IV – and heterologous auto/allo-reactive CD4 T cells and CD8 T cells – shown to infiltrate islet allografts in chapter V. From these data I conclude that the combination of allograft rejection with disease recurrence in autoimmune recipients represents a more stringent barrier to therapeutic intervention than does autoimmune disease recurrence.

To determine whether autoimmune NOD mouse resistance to this therapy was primarily due to T cell responses directed against MHC-derived antigens (described in chapter III) or against minor antigenic targets (described in chapter IV), I transplanted autoimmune NOD mice with either MHC-mismatched but minor-matched islets (NOD.B10) or MHC-matched but minor mismatched (B6.g7) islets. Results demonstrated equivalent resistance to dual therapy in autoimmune recipients transplanted with MHC-matched/minor-mismatched islets (MST 14 days), but prolonged transplant survival in recipients grafted with MHC-mismatched/minor-matched islets (MST 23 days). Ex vivo re-stimulation showed a trend toward increased percentage of TNF-α single-producing CD4 T cells and a trend toward decreased % of IFN-γ-producing-TNF-α-producing CD4 T cells in graft infiltrate of rejected NOD.B10 islets compared to graft infiltrate of rejected B6.g7 islets. These data suggest, unexpectedly, a more robust CD4 T cell response against minor antigens than against MHC-derived antigens in autoimmune NOD
mice, measured on both the whole-animal (transplant outcome) level and the ex vivo re-stimulation (T cell cytokine production) level.

To reiterate, in the presence of dual therapy graft survival was non-significantly prolonged for MHC-mismatched (NOD.B10) grafts compared to MHC-matched (B6.g7) grafts in autoimmune NOD mice. These data suggest that the minor antigen-directed T cell response against transplants in autoimmune mice is more therapy-resistant than MHC-directed T cell responses in NOD mice, an unexpected and potentially clinically relevant finding. In addition, the combination of either MHC-directed T cell responses or minor antigen-directed T cell responses with autoimmune disease recurrence are more resistant to therapeutic intervention than is disease recurrence in isolation. Table 7-3 summarizes the islet transplants performed for chapter VI.

**Transplants Performed for Chapter VI**

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<td>6-3a</td>
</tr>
<tr>
<td>NOD.RAG</td>
<td>aNOD</td>
<td>dual tx</td>
<td>10</td>
<td>54</td>
<td>6-3a</td>
</tr>
<tr>
<td>B6</td>
<td>aNOD</td>
<td>αCD154</td>
<td>13</td>
<td>9</td>
<td>6-4a</td>
</tr>
<tr>
<td>B6</td>
<td>aNOD</td>
<td>dual tx</td>
<td>5</td>
<td>13</td>
<td>6-4a</td>
</tr>
<tr>
<td>B6.g7</td>
<td>aNOD</td>
<td>αCD154</td>
<td>6</td>
<td>10</td>
<td>6-4b</td>
</tr>
<tr>
<td>B6.B10</td>
<td>aNOD</td>
<td>dual tx</td>
<td>5</td>
<td>16</td>
<td>6-4b</td>
</tr>
<tr>
<td>NOD.B10</td>
<td>aNOD</td>
<td>--</td>
<td>7</td>
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<td>6-7a</td>
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<td>NOD.B10</td>
<td>aNOD</td>
<td>dual tx</td>
<td>6</td>
<td>23</td>
<td>6-7a</td>
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</table>

**Table 7-3:** Transplants performed for chapter VI. From these data we conclude that dual therapy derived from analyzing autoimmune disease recurrence-associated T cells does not as strongly inhibit rejection of MHC mismatched or minor antigen mismatched islet transplants in the NOD recipient.
Overall Conclusion of Dissertation and Presentation of Model

Our data, summarized in table 7-4, indicate that there are three concurrent, partially overlapping, T cell responses directed against islet transplants in autoimmune recipients, and that the combination of any two of these T cell responses results in rapid transplant rejection which strongly resists therapeutic intervention. Chapter III demonstrated that MHC expression on non-self islets is not required for acute islet transplant rejection in autoimmune recipients. In contrast, and in agreement with others, I demonstrated that TCR-MHC-mediated interactions between autoreactive CD8 T cells and islet-expressed MHC class I are required for autoimmune disease recurrence in NOD mice. I speculate that autoreactive CD4 T cells do not require TCR-mediated contact with allograft cells to mediate rejection through macrophages as an intermediary cell type. Chapter IV demonstrated that CD4 T cell responses in NOD mice to minor transplantation antigens are more resistant to anti-CD154 therapy than are MHC-directed CD4 T cell responses in autoimmune diabetic NOD mice. Chapter V demonstrated the presence of autoreactive T cells within rejected allograft lesions which cross-react with graft cell MHC, and which are highly enriched in rejected allograft lesions. In vitro studies in chapter V went on to characterize at least two allograft-derived TCRs which mediated both autoreactive and alloreactive responses. Lastly, the combination therapy developed in chapter VI may be a promising adjunct therapy to transplantation with self-derived MHC-bearing islet transplant in the diabetic human islet transplant recipient. Results in chapter VI highlighted the therapy-resistant nature of the combination of autoimmune disease recurrence with either MHC-directed or minor antigen-directed T cell responses, even though all three of these T cell responses can be delayed in isolation in I-Ag7-bearing mice.

These data indicate that the most appropriate material for islet replacement therapy in autoimmune recipients may be self-derived beta cells. This suggests patient-specific iPS-derived
beta cells generated and expanded in vitro. Our data further suggests MHC I-deficient self-derived islets would persist more effectively than MHC-bearing self-derived islets. In the presence of MHC on grafted islet cells, it is better to be self-derived than MHC-matched. The therapeutic combination developed in chapter VI of this dissertation demonstrated that minor-antigen-directed CD4 T cell responses in autoimmune NOD mice strongly resist therapeutic intervention, an unanticipated and potentially clinically relevant finding. These data suggest a

### Differences between autoimmune disease recurrence and allograft rejection in the NOD islet transplant recipient

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Isograft</th>
<th>Allograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tempo of Rejection</td>
<td>MST 11 days</td>
<td>MST 6 days</td>
</tr>
<tr>
<td>Roles of T cell sub-sets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD25+ cell depletion accelerates tempo of rejection?</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>CD4+ T cells required for rapid rejection?</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>CD8+ T cells required for rapid rejection?</td>
<td>Yes</td>
<td>no</td>
</tr>
<tr>
<td>Role of MHC on the graft in rejection process</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHC class I on transplanted islets required?</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>If no, are CD4+ T cells required for rejection?</td>
<td>---</td>
<td>yes</td>
</tr>
<tr>
<td>If no, are CD8+ T cells required for rejection?</td>
<td>---</td>
<td>no</td>
</tr>
<tr>
<td>MHC class II on transplanted islets required?</td>
<td>No</td>
<td>no</td>
</tr>
<tr>
<td>Are CD4+ T cells required for rejection?</td>
<td>No</td>
<td>yes</td>
</tr>
<tr>
<td>Are CD8+ T cells required for rejection?</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>MHC I/II double-KO islets survive long-term?</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Effect of Treg inhibition?</td>
<td>none</td>
<td>.....</td>
</tr>
<tr>
<td>Are CD4+ T cells required for rejection?</td>
<td>---</td>
<td>yes</td>
</tr>
<tr>
<td>Are CD8+ T cells required for rejection?</td>
<td>---</td>
<td>no</td>
</tr>
<tr>
<td>Rejected islet infiltrate (aim 1 and 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-graft Th1/Th17 cellular response following PMA/I restimulation?</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Detectable autoantigen CD4+ T cell response?</td>
<td>yes, insulinB10:23, ChgA</td>
<td>Yes, insulinB10:23, ChgA</td>
</tr>
<tr>
<td>Detectable autoantigen CD8+ T cell response?</td>
<td>Yes, IGRP</td>
<td>yes, IGRP</td>
</tr>
<tr>
<td>Effect of tolerance-promoting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival benefit to CD154 blockade therapy?</td>
<td>yes – MST 22 days</td>
<td>yes – MST 9 days</td>
</tr>
<tr>
<td>Decrease in intra-graft CD4+ T cell PMA/I response?</td>
<td>yes – 10-fold decrease</td>
<td>no decrease</td>
</tr>
<tr>
<td>Decrease in intra-graft CD8+ T cell PMA/I response?</td>
<td>no decrease</td>
<td>no decrease</td>
</tr>
<tr>
<td>Additive effect of CD8+ cell depletion to anti-CD154 treatment?</td>
<td>yes – MST 54 days</td>
<td>yes – MST 13 d</td>
</tr>
</tbody>
</table>

**Table 7-4: Differences between autoimmune disease recurrence and allograft rejection in the NOD islet transplant recipient.** Results of data chapter 1, 2, and 4 summarized with respect to major hypotheses addressed in the introduction chapter.
model whereby minor-antigen-reactive or MHC-reactive T cells ‘unleash’ autoreactive T cells to attack, and in parallel ‘disarm’ islet-specific regulatory T cells from protecting pancreatic islet transplants in autoimmune diabetic recipients. As mentioned above, these results have implications for clinical strategies of beta cell replacement in poorly managed T1D patients.

**Rapid rejection of allografts requires CD4 T cells**

**Rapid rejection of self islets requires CD8 T cells**

**Therapy Resistant**

*Allograft rejection*
- CD4-dependent
- Islet MHC-independent
- CD25+ depletion delays

**Therapy Amenable**

*Disease recurrence*
- Largely CD8-dependent
- Islet Class I-dependent
- CD25+ depletion accelerates

**Aim 3**: subset of autoreactive T cells spontaneously respond to allogeneic MHC – and appear to be enriched within rejected allografts

**Figure 7-1**: Model of pancreatic islet transplant rejection in autoimmune recipients. Our data suggest a model whereby CD4 T cells in NOD mice do not require TCR-MHC mediated contacts to facilitate islet transplant destruction (chapter III). Furthermore, our data suggest that CD4 T cells in NOD mice mediate islet transplant rejection due to minor antigen differences alone, in the absence of autoimmune diabetes (chapter IV). However, in the MHC-sufficient islet allograft scenario, CD4 T cells – as well as CD8 T cells – may cross-react with graft cell MHC, accelerating transplant rejection (chapter V). Finally, we empirically determined a T cell-directed combination therapy which significantly delays autoimmune disease recurrence, but has much less effect on rejection of either MHC-matched (B6.g7) or MHC mismatched (NOD.B10) islets (chapter VI).
REFERENCES


