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Defining the Barrier of Split Tolerance in Allogeneic Mixed Chimerism

by

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ABSTRACT

Establishing mixed hematopoietic chimerism is a promising approach to develop donor-specific tolerance to transplanted organs. Establishing tolerance may eliminate the need for long-term immunosuppressive therapy, prevent chronic rejection and in the case of Type 1 Diabetes (T1DM), reverse autoimmunity. However, even in the long-lasting presence of a donor organ or donor hematopoietic cells, some allogeneic tissues from the same donor can be rejected; a phenomenon known as split tolerance. With the current goal of creating mixed chimeras using clinically feasible amounts of donor bone marrow and with minimal conditioning, split tolerance may become more prevalent and its mechanisms need to be explored.

The work in this thesis can be broadly divided into four components. First, we discuss chimerism and its potential as an adjuvant for islet transplantation for the treatment of T1DM. Second, using the relevant autoimmune non-obese diabetic (NOD) mouse model, we demonstrate that NOD NK cells are a substantial barrier to allogeneic chimerism in the presence or absence of adaptive immunity. Third, we use radiation chimeras to show that the split tolerance NOD mice develop has contributing components from both radiation sensitive and radiation resistant cellular compartments. Furthermore, we have identified T cells, but not NK or B cells, as cells that both resist chimerism induction and mediate split tolerance. We then developed a successful non-myeloablative chimerism induction protocol based on recipient NOD T cell depletion. Finally, we examined the role of MHC class I expression on recipient

vascular endothelial cells in CD8 T cell mediated indirect allograft rejection. Our results suggest that the commonly held notion that recipient MHC class I expression on recipient vasculature ingrowth into non-vascularized allografts cannot be the primary explanation for indirect rejection by CD8 T cells and that additional mechanisms of indirect recognition by CD8 T cells must be involved.

This work has identified cells that resist chimerism induction and cells that mediate split tolerance in NOD mice. This has allowed the generation of a successful chimerism induction protocol that produces tolerance towards fully allogeneic islets. In addition, we have challenged the previously accepted mechanisms of indirect rejection by CD8 T cells. Combined, this work has highlighted some of the mechanisms of split tolerance and has developed means to mitigate its occurrence.

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LIST OF SYMBOLS, NOMENCLATURE OR ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
ABO	Carbohydrate blood group system
Ag	Antigen
AIRE	Autoimmune regulatory
Anti-CD40L	Anti-CD40 Ligand
APC(s)	Antigen presenting cell(s)
B6	C57BL/6
BCR	B cell receptor
BM	Bone marrow
BMC	Bone marrow cell
BMT	Bone marrow transplant
BUS	Busulfan
CTLA-4-Ig	Cytotoxic T-lymphocyte-associated protein immunoglobulin
DC(s)	Dendritic cell(s)
DN	Double negative
DNA	Deoxyribose nucleic acid
DP	Double positive

FcγR	Fc-gamma receptor
FITC	Fluorescein isothiocyanate
FL	Fetal liver
FLC(s)	Fetal liver cell(s)
g	Gram
GFP	Green Fluorescent Protein
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
H&E	Hematoxylin and eosin
HLA	Human leukocyte antigen
HSC(s)	Hematopoietic stem cell(s)
IFN	Interferon
Ig	Immunoglobulin
Il-2R γ	Interleukin-2 receptor gamma
i.p.	Intraperitoneal
i.v.	Intravenous
K	Kilo
KO	Knockout
MHC	Major histocompatibility complex
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NIMA	Non-inherited maternal antigen
NCI-Frederick	National Cancer Institute at Frederick

NK	Natural killer cell
NOD	Non-obese diabetic
NIMA	Non-inherited maternal antigens
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
RAG1	Recombination activating gene 1
SEM	Standard error of the mean
SP	Single Positive
T1DM	Type 1 diabetes mellitus
TCR	T cell receptor
TSA	Tissue-specific antigen
TLR	Toll like receptor
TBI	Total body irradiation
Treg(s)	T regulatory cell(s)
WT	Wild type

Chapter 1: Introduction to Type 1 Diabetes Mellitus and Islet Transplantation - A Role for Tolerance Induction

1.1. TRANSPLANTATION FOR TYPE 1 DIABETES MELLITUS

Type 1 diabetes mellitus (T1DM) is a common disorder that affects approximately 250 000 Canadians (1), almost two million Americans (2), and for unclear reasons, its incidence is increasing (3,4) . T1DM is a chronic disease that decreases the life expectancy of young children and adolescents that predominantly manifest this disorder (5). Complications of T1DM include acute events, such as diabetic ketoacidosis, as well as chronic conditions, such as atherosclerosis, retinopathy, neuropathy and nephropathy (6,7). T1DM is an autoimmune disease that results from autoreactive T cells mediating the destruction of β cells in pancreatic islets (8). The pathogenesis of the development of T1DM is multifaceted, involving many genes, signaling pathways, and immunological cells. Although the underlying mechanisms of developing autoimmunity are complex, the overall immunological consequence is a breakdown of self-tolerance (9). Tolerance is the absence of an immune response towards what would otherwise be an immunogenic antigen challenge (10) and is necessary to prohibit reactive immune cells from inducing immunity to normal self-antigens and thereby damaging self-tissues. T1DM develops with the right combination of genetic susceptibility and exposure to environmental triggers that leads to a breakdown in self-tolerance. This failure of tolerance results in the development of an autoreactive immune response against islet β cells (11).

Currently, there is no cure for T1DM, and therapy involves dietary modifications, blood glucose monitoring and the administration of exogenous insulin (12). To this end, novel glucose sensors and insulin delivery devices are being developed to maintain normoglycemia and avoid the complications associated with T1DM (13). A proportion of T1DM patients experience glucose lability, where glucose levels are difficult to control despite the best attempts at monitoring and insulin delivery. With aggressive attempts to control blood glucose levels within the targets outlined in the Diabetes Control and Complications Trial (12), these T1DM patients may also develop hypoglycemia unawareness. For "brittle" T1DM patients, those with labile glucose levels and/or those who have reduced hypoglycemia awareness, pancreas transplantation is an established therapeutic option (14,15). Since these patients usually also have some degree of kidney dysfunction, pancreas transplantation is commonly combined with a kidney transplant and is rarely done alone. Recipients of a pancreas transplant obtain the benefits of sustained normoglycemia and are less prone to secondary diabetic complications, such as nephropathy (16). However, pancreas transplantation requires a major surgical intervention and subsequent immunosuppressive therapy.

Clinical islet transplantation is an attempt to restore functional β cell mass and obtain the benefits of normoglycemia, similar to pancreas transplantation, by the substituting a radiological procedure for an operation (17-19). Islet transplantation is an experimental procedure that obtains levels of insulin independence that are increasingly close to those achieved in whole pancreas

transplantation. In addition, regardless of insulin independence, islet transplantation can stabilize some secondary complications of diabetes when compared to patients receiving optimal medical therapy (20,21). Although beneficial and less invasive than pancreas transplantation, islet transplantation is not without its drawbacks. Some of these drawbacks associated with islet transplantation include its availability only in centers with Good Manufacturing Practice islet isolation facilities and loss of functional islet mass after transplant (which usually necessitates islet donations from more than one donor) (22). Downsides that islet and whole pancreas transplantation have in common are allograft rejection, and complications secondary to immunosuppression. In the case of islet transplantation, initial high-dose immunosuppression is often difficult to wean because there are no reliable clinical markers of islet accommodation or injury if dosing is reduced (23). Furthermore, there is a complication of restoring islet mass in autoimmune patients; despite immunosuppressive medications, the recurrence of autoimmunity after pancreas transplant is 5-6% (24). Since islet transplantation is still a relatively rare procedure (571 islet transplants have been performed worldwide as of January 2010 (25)), data regarding the recurrence of autoimmunity after islet transplant has not been published. However, as more islet transplants are performed, the recurrence of autoimmunity may become more apparent. Given the benefits of beta cell replacement and the consequences of alloimmunity or recurrent autoimmunity, transplantation (either whole pancreas or islets) for T1DM would benefit from the induction of central and/or peripheral tolerance.

1.2. IMMUNOLOGICAL TOLERANCE

The mammalian immune system has evolved into a sophisticated orchestra that functions primarily to protect the host from pathogenic agents (26). In addition, the immune system eradicates cells that show signs of malignant transformation (27) and can facilitate healing through the removal of damaged tissues (28). However, despite its complexities, autoimmunity is an example signifying that the immune system is not flawless. With many diverse functions, understanding the intricacies and failures of the immune system is a difficult undertaking. Therefore, in order to facilitate its study, researchers have compartmentalized the immune system. As such, the immune system can be functionally divided into innate and adaptive arms (with some overlap), immune responses can either be tolerance versus immunity, and the type of immune response invoked can be divided into classes. However, it is important not to focus solely on a specific niche interest and instead to interpret individual results into a broader understanding. Therefore, as the main focus of this thesis is tolerance, a brief description of tolerance theories is provided.

One of the hallmarks of the immune system is the discrimination of the target of immune reactivity. This discrimination is the method by which the immune system avoids mounting an attack against self-tissues while reacting strongly to pathogenic agents. Although the mechanisms and fine specificity differ, both the innate and adaptive arms of the immune system possess the ability to distinguish immunological targets (29). However, the classification of recognizing ‘self’ and ‘non-self’ does not do the immune system justice. This is

because ‘self’ cells can be legitimately attacked by the immune system if they show signs of stress, infection or malignant transformation (27,30,31). As such, the immune system develops tolerance towards normal self-cells and tissues, while responding appropriately to abnormal cells and pathogenic microbes. Tolerance is necessary to prohibit reactive immune cells from inducing immunity to normal self-antigens and damaging host tissues. Immunological tolerance was recognized over a century ago when Paul Ehrlich recognized “the organism possesses certain contrivances by means of which the immunity reaction...is prevented from working against the organism’s own elements” (32). The exact mechanisms by which the immune system achieves tolerance has been under investigation ever since.

Investigating the mechanisms of immunological tolerance is clinically relevant because delineation of the processes involved can lead to therapeutic interventions. Of particular interest are interventions related to the synthetic induction of tolerance that could be beneficial in reversing autoimmune diseases and the avoidance of immunosuppression in allogeneic organ transplantation. In order to facilitate the understanding of adaptive immunological tolerance, it can be broadly divided into central and peripheral mechanisms. Focusing on the adaptive immune system, and specifically on the T cell repertoire, central and peripheral mechanisms occur in the thymus and the peripheral tissues, respectively.

1.2.1. T cell Development and Central Tolerance

T cells are lymphocytes of the adaptive immune system that express a unique antigen-specific receptor: the T cell receptor (TCR). Clonal selection theory postulates that each T cell will only express one type of TCR (33), and therefore, strictly capable of interacting with one type of antigen. However, the TCR does not recognize its specific antigen alone; antigen recognition occurs between the TCR and processed peptides presented in the context of major histocompatibility complex (MHC) proteins (34). The TCR protein is generated through the transcription and translation of gene segments that are randomly rearranged (35). Due to this random generation, the TCRs are capable of recognizing a diverse array of antigens, thereby giving the immune system an incredible ability to identify foreign pathogens. However, due to its random nature, TCR gene segment rearrangements could generate a TCR protein that can recognize self-antigens. Therefore, in order to avoid autoimmunity, the T cell repertoire must undergo a selection process to purge self-reactive clones and ensure tolerance of normal self-antigens. The initial selection process has two arms (positive and negative) and occurs in the thymus (36-39).

The thymus is responsible for the development and maturation of T cells and is also the initial site for the development of T cell tolerance (40,41). Immature thymocytes differentiate from a lymphoid progenitor cell in the bone marrow, but true development does not occur until the progenitor reaches the thymus (42). Through interactions with thymic stromal cells, immature T cells slowly mature and the expression of cell-surface proteins change. Thymocytes

enter the thymus not expressing a TCR. Similarly, at this early time, thymocytes do not express either of the co-receptors, CD4 and CD8, and are termed "double negative" cells. During the double negative (DN) phase, TCR gene rearrangements are occurring and a functional TCR is eventually expressed on the cell surface (38). At this time, both CD4 and CD8 are also expressed, generating the "double positive" (DP) cell. Before leaving the thymus, one of either CD4 or CD8 is lost and the mature T cell becomes "single positive" and has only one co-receptor molecule (43). Positive selection is a maturation process that acts upon the DP cells. At this stage, thymic epithelial cells provide survival signals solely to those T cells that express a TCR that can interact with self-MHC (37). This interaction between DP thymocytes and thymic epithelial cells must be of sufficient affinity for the thymocyte to receive survival signals, but not strong enough to induce death signals (see next section on negative selection). If thymocytes do not have a TCR capable of interacting with self-MHC, they die by neglect (44). After positive selection on self-MHC, the T cell repertoire becomes restricted in identifying future antigens only when presented in complex with self-MHC (30,45). However, a notable exception to the MHC restriction of T cells is the case of alloreactivity (46,47). The frequency of alloreactive T cells is up to 1,000-fold higher than the frequency of T cells specific for any single anigen-MHC complex. This high frequency of alloreactivity is likely a by-product of positive selection that chooses only T cells are able to interact with MHC proteins. A substantial barrier to allogenic organ transplantation is, therefore, due to these T cells interacting with and becoming activated by foreign MHC

displaying surface components that allow for the formation of thousands of "interaction antigens" (46).

Negative selection of the immature T cell repertoire also occurs in the thymus. Negative selection of thymocytes is mediated by thymic epithelial cells in the thymic medulla and cortex and by bone marrow derived antigen presenting cells (39) and T cells (48). These cells display self-antigens in the context of self MHC to SP and DP thymocytes (49,50). In contrast to what happens to mature T cells interacting with their cognate antigen in the periphery, immature SP and DP thymocytes that recognize and strongly interact with self-antigen-MHC in the thymus undergo clonal deletion, receptor editing or anergy (38). The interaction leading to negative selection between cells expressing self-antigens and maturing T cells must be of higher affinity than the interaction required positive selection. In this manner, thymocytes are selected based on their ability to interact with self-MHC (positive selection leading to MHC restriction), but not interact with too high of an affinity for self-peptide-self-MHC (negative selection avoiding autoimmunity). Elimination of these self-reactive T cells at an early point avoids the potentially detrimental effects of exporting a mature T cell that recognizes a self-antigen from the thymus.

Central tolerance is necessary for self-tolerance; however, there are three potential situations whereby autoreactive thymocytes can exit the thymus. First, during negative selection, is it possible for T cells to interact with all self-peptides presented in thymus and undergo apoptosis if the interaction is of sufficient affinity? Second, is it possible for antigen presenting cells to express all self-

antigens in the thymus, including tissue specific antigens (TSA)? The comprehensive nature of antigen display in the thymus received significant support with the discovery of the autoimmune regulatory (*Aire*) gene (51-54). With Aire promoting promiscuous gene expression in the thymic medullary epithelial cells, it may "facilitate tolerance induction to self-antigens that would otherwise be temporally or spatially secluded from the immune system" (51,55). However, it is not clear that Aire can actually lead to the expression of all self-antigens in the thymus (56). Therefore, the regulation of promiscuous gene expression not under Aire control may implicate the existence of other transcription regulators that drive tissue specific gene expression (57). Alternatively, antigens that are not expressed in the thymus under Aire (or any other transcription regulator) control may be the preferred targets for autoreactive T cells. Third, central tolerance may not be complete because the interaction of the TCR with peptide-MHC is a not binary survival/deletion response; there are different affinities for the interactions. Therefore, all interactions will have a minimum affinity threshold required to trigger a sufficient signal for deletion. Since only developing T cells with strong affinity to self-antigen-self MHC undergo negative selection, a population of low avidity self-reactive T cells may escape negative selection and be able to respond to autoantigens in the periphery (58). Despite the potential shortcomings of central tolerance, it is indeed necessary for tolerance to self-antigens. For example, patients with defective central tolerance (*Aire* gene mutation) manifest multiple autoimmune phenotypes

(59). However, despite its necessity, central tolerance mechanisms are insufficient to eliminate all potentially self-reactive T cells.

1.2.2. Peripheral Tolerance

Self-reactive T cells that avoid negative selection in the thymus must be controlled or eliminated in the peripheral tissues. Evidence that peripheral tolerance mechanisms exist comes in part from experimental temporal transplantation studies. In these studies, tissues that were surgically removed and re-introduced at a later time in the organisms' life were shown to be immunologically rejected (60,61). If central mechanisms were sufficient, the re-introduction of tissue to the same recipient should have led to graft acceptance. In addition, TCR transgenic mice models have demonstrated the presence of self-reactive T cells in the periphery (62) and other T cells that are unable to cause an immune response against their target antigen (63). Described methods of peripheral tolerance are not necessarily mutually exclusive and include ignorance, anergy, regulatory cells, clonal deletion, and cytokine deviation (64). Tolerance theories have been developed and subsequently modified to explain constantly emerging experimental data. In broad terms, it was thought that tolerance was related to the timing of antigen exposure; first in the life of the organism (65,66), and later in the life of the lymphocyte (67). Other theories take into account the amount of antigen and timing of exposure (68) and the environment surrounding antigen exposure (68-70).

1.2.3. Immunological Tolerance Theories

1.2.3.1. Tolerance and the Timing of Antigen Exposure

Although hypothesized by Ehrlich in the early 1900's (32), a theory to explain immunological discrimination was only formally created by Burnet in 1957 (66,71). Said researcher predicted early life antigen exposure would eliminate immune reactivity, explaining how self-tolerance is established. In support of Burnet's theory, tolerance had been previously observed by the *in vivo* experiments of Billingham, Brent and Medawar in 1953 (65). In their pioneering experiments, testes, kidney, and splenic tissues injected intra-embryonically into a fetal allogeneic mouse would allow that mouse (once mature) to accept a skin graft from the donor strain. These experiments showed that tolerance is strain specific and the induction of tolerance requires the transfer of foreign antigens early in the life of the organism (into the immature immune system). Re-discovered observations by Owen in 1945 (72) and contributions by Hasek in 1953 (73) provided further support for the conclusion that tolerance could be induced early in the life of the organism. As the immature immune system develops, all of the processes that tolerize self-reactive T cells are potentially available for donor-reactive T cells (10). However, there were problems with the model that early life antigen exposure would eliminate immune reactivity. First, in contrast to the elimination of self-reactive lymphocytes predicted by Burnet's clonal selection theory (71), other non-deletional mechanisms of tolerance have been identified (64). Second, lymphocytes are continually produced throughout the life of the organism and the process regarding how a newly produced

lymphocyte develops tolerance in a mature immune system required additional explanation. Third, if tolerance could be created in the immature immune system, the recipient should accept any transplant permitting it is given in the early stages of life. This prediction was challenged with experimental observations that transplanted tissues, although given in the fetus, become the target of rejection as the organism matures (74). Moreover, the tolerance or rejection of transplanted tissues given in the setting of an immature immune system can be explained by the ability of the graft to establish microchimerism (10,48).

In 1959, Lederberg contributed the alternative idea that tolerance was the property of immature lymphocytes rather than immature animals (67). In this manner, developing lymphocytes that interact with self-antigen can lead to tolerance; the first notion of central tolerance. However, this model is incomplete as it does not take into account any tolerance mechanisms acting on mature lymphocytes. For example, how are lymphocytes that have low antigen-receptor avidity (thereby escaping central deletion) tolerized? Or, what happens to self-reactive lymphocytes whose antigen is not expressed in the central lymphoid organs (despite Aire expression)? In addition, tolerance mechanisms must control B cells undergoing somatic hypermutatation, where a potential problem exists due to a lymphocyte mutating into a self-reactive clone outside of central lymphoid organs (75). Despite their shortcomings, the first tolerance theories include at least two aspects that are still in use today. First, tolerance involves the interaction of a foreign antigen and the host immune system; a concept that has subsequently been redefined as ‘signal one’. Specifically, this interaction is

between an antigen or epitope and the corresponding T cell receptor or B cell antigen receptor. Second, the concept of central tolerance acting early in the life of the lymphocyte is thought to be a primary mechanism of self-tolerance.

1.2.3.2. Tolerance and Help

In addition to signal one, the introduction of a second 'helper' signal to explain the development of tolerance came in a 1970 theory by Bretscher and Cohn (76). If an effector helper T cell can provide a second signal to the naïve T cell immunity is generated, while in the absence of the second signal, tolerance occurs. This model solves the problem of tolerance development in lymphocytes that are continually being produced, as any self-reactive T cell produced in the mature immune system will not receive 'help' in an environment lacking self-specific help. Additionally, in this model, there is a tolerance window early in life when the immune system is developing. Therefore, there is an absence of helper T cells in the immature immune system which leads to robust tolerance to all self-tissues. Another important aspect of this two signal 'helper' model is the notion of linked recognition. Linked recognition implies that helper T cells are only able to provide the necessary stimulation for an immune response to effector B and T cells if their T cell receptor recognizes an epitope of the same antigen. However, the commonly held criticism of the helper model, as described by Cohn, is how do the first helper T cells develop if there is no help provided for their own development? This is a difficult question to answer using Cohns' helper model. Although it can be explained through the antigen dependent or antigen

independent generation of helper T cells from immature T cells (77,78), this explanation would also require that potentially self-reactive spontaneously generated helper T cells have time to be deleted before becoming full-scale helpers (78,79). In addition, knowing that antigen presenting cells display many different antigens at any given time, it is difficult to explain how self-reactive T cells encountering self-antigen on an antigen presenting cell (APC) do not receive a second signal from an adjacent helper T cell recognizing a foreign antigen.

Co-stimulation is another two signal mechanism of tolerance development. The co-stimulation model originally proposed by Lafferty and Cunningham in 1975 was designed to explain why immune reactivity could be greater for alloreactivity than xenoreactivity (80). It has subsequently been amended as a model for invoking tolerance. The original idea of Lafferty was that a T cell interaction with its specific antigen on an APC was not enough to elicit an immune response, and a second stimulatory signal was required. If only signal one is provided to the T cell by the APC, the T cell becomes inactive. However, before the idea of co-stimulation could be applied to tolerance models, the ability of an APC to selectively co-stimulate one T cell and not another needed to be defined. In 1989, Janeway proposed that the selective presentation of co-stimulatory molecules to T cells by the APC was due to the APC itself recognizing bacteria (69,81). Pattern recognition receptors (PRR) have since been discovered and are able to identify evolutionarily conserved bacterial products such as peptidoglycans, lipopolysaccharides and mannose-rich oligosaccharides. Once recognizing the foreign bacteria, the APC becomes stimulated and expresses

the co-stimulatory molecules required by T cells for activation. The idea of the APC making the decision to stimulate an immune response was taken forward by the danger model hypothesized by Matzinger in 1994 (70). The danger model predicts that APCs will become stimulated and offer co-stimulation to T cells only in the presence of "danger signals" released by endogenous cells that are damaged, stressed or infected. A common theme with co-stimulation models is that signal one is a negative signal, and in the absence of signal two, leads to T cell tolerance either by deletion or anergy (tolerance is recessive). In this manner, self-reactive T cells would be tolerized in the periphery because the recognition of their cognate self-peptide should not be associated with a second signal.

1.2.3.3. Tolerance and Co-Inhibition

Co-inhibition was proposed by Sinclair in 1971 to describe the mechanism by which the Fc receptor portion of an antibody inhibits the B cell (82). Subsequently applied to T cell tolerance (83,84), the co-inhibition model utilizes signal one through the TCR for antigen specificity and also includes second signals. In contrast to the co-stimulation models, signal one is positive, and second signals can be either stimulatory or inhibitory. The result of a T cell encountering its antigen in the presence of co-inhibition is that it becomes functionally unresponsive, deleted or anergic, whereas co-stimulation can serve to enhance the immune response. Therefore, instead of coming from a single interaction, signal two in this model is derived from the culmination of stimulatory and inhibitory signals. Cytotoxic T lymphocyte antigen (CTLA)-4

and FAS are two such inhibitory proteins that down-regulate the activity of both T cells and APCs. However, similar to the problem that faced co-stimulation mechanisms: how are the signals for co-inhibition controlled? One suggestion has been that chronic antigen receptor signalling leads to enhanced co-inhibitory signalling (84). Another approach to solve the problem of co-inhibitory control is that these signals are antigen independent. Therefore, during normal physiological conditions co-inhibitory signals dominate to promote tolerance and prevent the undesired activation of the immune system. However, under stressful conditions or in the presence of foreign antigens, co-stimulation overtakes and leads to immune activation.

1.2.3.4. Tolerance and Regulation

The identification of a specific subset of regulatory T cells (T regs), that are capable of suppressing an immune response, offers an antigen specific mechanism to the co-inhibition model of peripheral tolerance. T regs are generally identified as CD4 CD25 FoxP3 positive cells and play a crucial role in controlling immunological responses (85,86). Specifically, T regs are able to suppress T cell, NK cell, macrophage, and dendritic cell responses through direct cellular or cytokine mediated interactions (87). Interactions through the TCR allows for an antigen specific mechanism of immune suppression. T regs can out-compete other potentially self-reactive T cells that are interacting with the same antigen on the dendritic cell surface. This is achieved because natural T regs are thought to be produced in the thymus from T cells with strong TCR interactions to

thymic epithelium, but not strong enough to become negatively selected (deleted) in the thymus (87). In addition, through TCR interaction, T regs can down-regulate dendritic cell functions, thereby hindering the activation of other T cells (87). T regs can also express granzyme A and kill activated T cells through a perforin dependant mechanism (88). Aside from effects that involve TCR interactions, T regs have an effect on the local environment through the release of the immunosuppressive cytokines and the constitutive display of co-inhibitory receptors/ligands (86). Some of the immunosuppressive cytokines released by T regs are transforming growth factor β, interleukin-35 and interleukin-10 (89). A co-inhibitor associated with T regs is the constitutively expressed protein CTLA-4 (90). CTLA-4 mediates T cell suppression through interactions with CD80 and CD86 surface proteins, which are expressed on APCs. Thus, T regs can suppress an immune response in both an antigen specific and antigen non-specific fashion. During the course of an immune response, T regs may also play a role protecting bystander cells and limiting the overall magnitude of the response (91).

It is controversial as to whether T regs are actually mediators of self-tolerance or general inhibitors of any immune response. If T regs have a dominant role in tolerance, and assuming the widely held view that T regs operate through linked recognition, it is difficult to reconcile how a single minor-H mismatched skin graft becomes rejected (48). Another argument against T regs having a dominant role in the maintenance of tolerance comes from an *in vitro* experiment where T regs could not suppress naive T cell proliferation in the presence of APC's activated through their lipopolysaccharide toll-like receptors

(92). If this phenomenon occurred *in vivo* there would be a breakdown in self-tolerance in the presence of bacterial infection. Although the precise mechanisms of action, targets, and function in self-tolerance are still being elucidated it is known that T regs play an important role in immune homeostasis. This critical role is demonstrated by the severe lymphoproliferative and autoimmune conditions that result under natural or experimental conditions where T regs are absent (93,94).

1.2.3.5. Tolerance and Ignorance

Ignorance is a mechanism of peripheral tolerance that is characterised by a T cell that recognizes self-antigen, but does not mount a response because the antigen is either sequestered or present in low concentrations in the secondary lymphoid tissues. The ignorance model of Zinkernagel (68) predicts that all antigens that are normally found in the peripheral lymphoid tissues will induce tolerance. Furthermore, tissue specific antigens will not induce an immune response by self-reactive T cells because these antigens are only transiently present in the lymphoid tissues and at low concentrations. Therefore, self-reactive T cells that have avoided central deletion will reside in secondary lymphoid organs and ignore their self-antigen. Immunity only results against antigens not normally found in the lymphatic system if they are present in sufficient concentrations for a sufficient length of time. Importantly, ignorant T cells are not truly tolerant as they are capable of mounting an immune response if they recognize their target antigen under the proper circumstances. The potential for

an immune response under the 'right conditions' creates an argument against the widespread use of ignorance as a primary means of avoiding damage to self-tissues; because the 'right conditions' may not be uncommon. For example, the presence of any infection would lead to the trafficking of APCs carrying foreign antigens to lymphoid tissues which initiate an immune response against those foreign antigens. However, there is also the potential to initiate an immune response against all other self and tissue specific antigens being presented by that APC at the time of lymph node entry, leading to autoimmunity.

1.2.3.6. Accommodation

Tolerance, by definition, implies that an immune response is not initiated against a certain antigen, and therefore the antigen remains intact. After allogeneic organ transplantation, a situation can arise where an immune response (mainly B cell and complement meditated) occurs yet the allograft remains relatively intact. The absence of graft injury in the face of immunity is termed accommodation (95). Therefore, although not tolerance, accommodation may have a role in allograft survival. The process of accommodation was first described in blood carbohydrate antigen (ABO) incompatible renal transplant recipients (96,97). These recipients were pre-conditioned to decrease the amount of pre-formed antibody prior to the ABO incompatible renal allograft, however, blood carbohydrate antibody returned to the recipient circulation. Surprisingly, the renal allografts were able to survive and function without hyper-acute or acute vascular rejection. Accommodation is a property of the allograft, and

mechanisms are thought to include the up regulation of protective or immunomodulatory genes that may include *heme oxygenase-1, A20, Bcl-2 and Bcl-xL* (98). However, the overall contribution of accommodation on allograft acceptance is controversial. First, accommodation is difficult to measure and quantify as there are no specific markers for its occurrence (99). Second, although accommodation may be present in ABO-incompatible transplants, its effects are less clear in the setting of Human Leukocyte Antigen (HLA) mismatched grafts (100). This may be due the type of antigen (carbohydrate or protein) which has an influence on the antibody class produced and the dependence on T cell help.

1.2.4. Tolerance Conclusion

Under physiological conditions, there is not yet a unifying theory by which T lymphocyte tolerance to self-antigens is initiated and maintained. However, the above models have enabled the understanding of some of the mechanisms involved in immunological self-tolerance. Multiple tolerance mechanisms act upon the T cell from the moment it is able to recognize an antigen through its TCR. Tolerance models must also take into account the timing of antigen exposure, the environment in which the antigen is exposed and the interactions between the innate and adaptive immune systems. In addition to these considerations, it has been recently proposed that the current definition of the immune system is too narrow and that all tissues contribute to both tolerance and immunity (101).

1.3. HEMATOPOIETIC CHIMERISM FOR THE TREATMENT OF T1DM

In the case of T1DM, allograft tolerance would prohibit rejection (acute and chronic and allo- and auto-) while avoiding the long-term use of immunosuppressive medications. Current immunosuppressive medications have made organ transplantation possible; however, both pancreas and islet transplantation expose recipients to a lifetime of immunosuppression, thereby increasing infection and malignancy rates (102). Furthermore, many immunosuppressives are nephrotoxic and islet toxic (103). Given the many side-effects of immunosuppression, the beneficial effects of avoiding, or only short-term use of them would be profound. Although allograft tolerance is the "holy grail" of transplantation, in T1D patients this achievement may not be enough as autoimmunity towards β cells may reoccur (24,104). Therefore, treatment of T1D patients via transplantation offers an exceptional challenge for tolerance induction as it must overcome both allo- and auto-immunity. Generating mixed allogeneic chimerism (the presence of two genetically distinct cells occupying a single organism) has long been recognized as a method of inducing operational tolerance towards allografts (105). Chimerism is effective at generating tolerance because it takes advantage of central tolerance (discussed further in chapter 2). Mixed chimerism is also clinically applicable, with numerous trials attempting to take advantage of the robust tolerance associated with chimerism (106,107). Additionally, in the case of transplantation for an autoimmune disease, chimerism may be able to "re-educate" the immune system to avoid recurrent autoimmunity (108,109).

Before chimerism can become an established method of inducing tolerance for clinical transplant at least three areas require further investigation. First, experimental induction protocols must be continually refined to be as minimalistic and effective as possible. In addition, concerns regarding tolerance resistance in autoimmune recipients will need to be further investigated. Second, the safety of chimerism must be established. The safety profile must include the negative side effects of the induction protocol and the inherent risks of bone marrow transplantation such as graft-versus-host disease (GVHD). Third, the exact mechanisms of tolerance that are operating in the presence of hematopoietic chimerism must be investigated. This area of investigation should include identification of the roles of central and peripheral tolerance mechanisms (discussed further in chapter 2). This section briefly summaries the rationale, clinical applicability and recent advances relating to chimerism induction and the concerns of GVHD.

1.3.1. Mixed Chimerism Induction Protocols

The induction of mixed chimerism requires transplanted hematopoietic stem cells (HSC) to migrate to their bone marrow niche and engraft without being destroyed by the host immune system. This requirement highlights two separate criteria that host conditioning must accomplish: overcoming the barrier of alloreactive T cells and NK cells that are capable of destroying donor bone marrow cells, and creating vacant niches for donor bone marrow cells to occupy. Both criteria can be accomplished by lethal radiation (105), and overcoming host

alloreactive cells can be accomplished by the transplantation of mega-doses of bone marrow under the cover of co-stimulatory blockade (110). However, the goal to create mixed chimeras using clinically feasible amounts of donor bone marrow and with minimal conditioning has led to the development of milder regimes.

1.3.1.1. Creation of Stem Cell Niche 'Space'

HSCs are thought to firmly reside in physically discrete locations (niches) in the bone marrow (111). Only when residing in their specific niche are HSC's able to survive and retain their stem cell identity (112). Therefore, in order create a mixed chimera, donor HSCs must migrate to and replace some of the host bone marrow HSC niches. The requirement for the creation of niche 'space' is demonstrated in immune reconstitution experiments where HSCs engraft to a lesser extent if conditioning is not preformed (113,114). Therefore, for efficient chimerism induction, some degree of myelo-ablation is employed. Total body irradiation (TBI) kills some of the recipient HSC population, making it easier for donor HSCs to engraft. Similarly, transplantation of large numbers of bone marrow cells allows easier donor HSC engraftment (115,116). However, for clinical applications, it is un-feasible to give mega-dose bone marrow transplants and large doses of TBI outside of the setting of HSC transplant for malignancy.

Busulfan is a DNA alkylating chemotherapuetic agent that exerts its major effects on HSCs (117). As such, busulfan has been used in human bone marrow transplantation induction protocols instead of TBI to create space for allogeneic

marrow engraftment (118). Busulfan has also been successful at creating mixed chimerism in mice using irradiation-free induction protocols (119,120). However, chemotherapeutic agents are not without side effects (117). Therefore, omission of chemotherapeutic agents from chimerism induction protocols would enhance their safety. The administration of an antibody that inhibits c-kit function has been shown to be effective at clearing endogenous HSC niches, thereby allowing for donor HSC engraftment (121). Alternatively, it has been recently shown that a physiological cell division-independent egress of HSCs leaves behind empty niches that can be occupied by donor HSCs (122). In light of this evidence, without host myelo-reductive therapy, repetitive transplantation of smaller doses of bone marrow may allow for greater engraftment.

1.3.1.2. Conventional Immunosuppression

The success of immunosuppressive medications at preventing acute graft rejection has led to impressive allograft survival rates at one year (123). However, despite the continual use of these medications, chronic rejection can still occur and contributes to progressively worse allograft survival at five and ten years post-transplant (123). This trend indicates that chronic T and B cell mediated rejection are not controlled well by conventional immunosuppression. Immunosuppression is also not without its side effects. Acute side effects can include mouth ulceration, dyslipidemia and thrombocytopenia (124), while chronic side effects such as increased malignancy (125) and infection rates (126) have also been observed. Additionally, during rejection episodes,

immunosuppression dosing is usually increased or combined with steroids which can intensify short term side effects (127). Ideally, with the generation of allograft tolerance, long-term use of immunosuppressives can be avoided.

Mixed chimerism approaches to allograft tolerance currently require the use of induction immunodepletion or modulation in order for efficient HSC engraftment. Administering immunosuppressive medications only during induction and after organ transplantation would mitigate the short and long term complications of their use. Immunodepletion of T cells with thymoglobulin or anti-CD2 are already a part of many solid organ transplant induction protocols (127); and anti-CD3 can be a valuable addition in clinical islet transplantation (128). In addition to induction therapy, short-term administration of immunosuppressive medications may increase chimerism levels. Although the goal of inducing donor specific tolerance through the creation of mixed chimerism might obviate the need for the continual use of immunosuppressive medications, short term use of such immunosuppressive medications are required in the initial establishment of chimerism (106).

1.3.1.4. Co-stimulatory Blockade

As discussed in the previous section, signalling through co-stimulatory molecules is required for a full T cell response. Of the many co-stimulatory proteins, the best characterized ones are the CD40-CD40L and CD80/86-CD28 co-stimulatory pathways. CD40 is a co-stimulatory protein expressed on APCs and B cells that interact with CD40L on T cells (129). In the presence of a TCR-

MHC-peptide interaction, signaling through CD40-CD40L leads to activation of the APC and subsequent upregulation of the cell surface receptors CD80 and CD86. CD80 and CD86 can interact with the co-stimulatory molecule CD28 that is constitutively expressed on T cells, leading to T cell activation and proliferation (129,130). Cytotoxic T lymphocyte antigen (CTLA) -4 is a receptor expressed on activated T cells, and upon interaction with its ligands (CD80 and CD86), delivers signals that attenuate T cell activation and proliferation (131). In this manner, there is control over the intensity and duration of the immune response.

The discovery of co-stimulatory molecules has led to the development of agents that interrupt these co-stimulatory pathways in order to decrease T cell activation after organ transplant. Blocking these pathways specifically interrupts signalling between T cells and APCs, with the goal of inducing T cell tolerance. For example, blocking the CD40-CD40L interaction using monoclonal antibodies against CD40L can induce T cell anergy and prolong allograft survival (132). Understanding the structure of CTLA-4 has led the development of the recombinant immunoglobulin fusion protein, CTLA-4-Ig. This protein can interrupt the signalling between CD28 and CD80/86 (133) by saturated binding to CD80 and CD86 (134), thus inhibiting T cell activation. In support of an important role for CTLA-4 for the induction of peripheral T cell tolerance, a recent study demonstrates signalling through donor CD80/86 and recipient CTLA-4 on alloreactive T cells is required for the maintenance of tolerance in chimerism induced through CD154 co-stimulatory blockade (135).

Combination therapy with conventional immunosuppressives and co-stimulatory blockade may enhance the generation of mixed chimerism. Sirolimus is an immunosuppressive agent that blocks interleukin-2 signal transduction and inhibits cell-cycle progression (136) and has shown promise in chimerism induction (137). Although some medications, such as tacrolimus, have been shown to interfere with tolerance development in mixed chimeras, sirolimus has had beneficial effects on tolerance (138,139). One study showed that when used alongside co-stimulatory blockade, sirolimus decreases the dose of radiation required to achieve mixed chimerism, and promotes tolerance induction (116). Even in the absence of radiation, sirolimus and co-stimulatory blockade as post-transplant treatment have been shown to promote bone marrow engraftment (139). Once chimerism is established and its mechanisms of tolerance take over (see chapter 2), withdrawal of immunosuppression can be attempted.

1.3.2. Barriers to the Application of Mixed Chimerism for T1DM

1.3.2.1. Toxic conditioning protocols

Efforts to reduce the toxicity of recipient conditioning have paved the way for bone marrow transplantation (BMT) to be used outside of the setting of malignancy. BMT has been used extensively in the treatment of hematological malignancies (140,141). In these cases, recipient conditioning is usually intense, consisting of near-complete myeloablation or lymphoablation in order to destroy cancerous cells (140). However, the toxicities and side effects associated with myeloablation or lymphoablation are difficult to justify if not being used for the

treatment of cancer. Therefore, milder non-myeloablative conditioning protocols have been developed to expand the realm of HSC based therapies. As such, HSC therapy has been successfully introduced in the clinical setting for the treatment of severe sickle cell (142) and autoimmune diseases (108). In addition, non-myeloablative conditioning protocols have been used successfully in combined kidney and bone marrow transplant for the generation of tolerance (106,107). However, BMT conditioning protocols will require further refinement in order to be applied in clinical islet transplant for T1DM. Given the risks associated with chronic T1DM and islet transplant with chronic immunosuppressive use, the side effect profile of chimerism induction must be reduced to at least match current medical therapies.

In an attempt to further decrease the toxicities of chimerism induction, protocols incorporating co-stimulatory blockade have been used. For example, minimal conditioning and non-myeloablative protocols incorporating anti-CD40L has led to the successful establishment of chimerism in non-human primate models (143). Unfortunately, translation of these agents to the clinic for use in transplantation was unsuccessful. When anti-CD40L was used in the treatment of patients with systemic lupus erythematous, it led to thromboembolic complications (144). Although CTLA-4-Ig (abatacept) showed encouraging results in islet and kidney transplantation in non-human primates, prolonged graft survival only occurred in a minority of animals (132,145). However, despite these setbacks, the success of co-stimulatory blockade in animal models of transplantation has been recognized. As such, new agents that interfere with

CD40-CD40L or CD80/86-CD28 interactions are being explored for use in transplantation. For example, a second generation human CTLA-4-Ig (belatacept) with higher affinity for CD28 was developed in order to increase the efficiency of blocking the CD28 co-stimulation signal. Belatacept significantly prolonged kidney graft survival in non-human primates, and is currently being evaluated in clinical transplant trials (133). Furthermore, the combination of belatacept, thymoglobulin and sirolimus have been used successfully in achieving single donor islet engraftment (146). Targeting the CD40 side of the CD40-CD40L with monoclonal antibody has shown some success in liver and islet xenograft transplantation in non-human primates (147,148). Although co-stimulation blockade is a relatively new treatment modality, and still undergoing clinical trials, the potential for its application in chimerism may lead to induction protocols that require only minimal conditioning. In addition, co-stimulatory blockade may decrease recipient conditioning time such that chimerism can be used outside of the living-donor setting.

1.3.2.2. Graft-Versus-Host-Disease

GVHD occurs when immunocompetent cells from the donor bone marrow react against host antigens (149). In bone marrow transplantation for malignancy, GVHD is a common process; occurring in 35-42% of HLA-matched sibling donor-recipient combinations and even more frequently in unrelated HLA-matched, or HLA-mismatched combinations (150-152). The high rates of morbidity and mortality associated with GVHD and the infectious complications

of treating GVHD with steroids (149) have cautioned the use of bone marrow transplants outside the setting of malignancy. Advances in prophylactic treatment, T cell depletion or the co-administration of facilitating-cells may decrease the risk of developing GVHD and assist in expanding the application of bone marrow transplantation in the clinical setting (107,151,153). However, for chimerism-induced tolerance to be justified for islet transplants, the risk of GVHD must be considerably lower than the risks of living with T1DM.

In contrast to the high rates of GVHD seen in human bone marrow transplantation for malignancy, GVHD is rare in murine mixed chimerism models. The rarity of GVHD may be related to the mild conditioning regimens used for murine bone marrow transplantation that leaves more of the host immune system intact. Additionally, the establishment of mixed chimerism may be protective against GVHD. Last, mouse bone marrow may contain fewer T cells of the phenotypic type that most readily causes GVHD. The decreased incidence of GVHD in murine models of mixed chimerism highlights a shortcoming in this model system. It is important to recognize this shortcoming for future clinical translation of mixed chimerism protocols.

1.4. OVERVIEW OF MY THESIS

The establishment of hematopoietic chimerism has the potential to induce a robust form of tolerance to alloantigens and re-establish tolerance to auto-antigens. In relation to clinical islet transplantation for T1DM, chimerism is a potential therapeutic option that may allow the avoidance of immunosuppressive medications while also circumventing alloimmunity and recurrent autoimmunity. However, the clinical translation of chimerism approaches for tolerance induction has been hindered by toxic induction protocols and fears of GVHD. Additionally, in the presence of autoimmunity, chimerism may be more difficult to establish and maintain. Furthermore, in some cases, the presence of chimerism does not guarantee tolerance towards allografts; a phenomenon known as split tolerance. Therefore, the two themes of my thesis are: 1. Understanding the challenges in establishing chimerism in the setting of autoimmunity and creating a protocol to overcome these difficulties; and 2. Defining the mechanisms behind the development of split tolerance. Appreciating the difficulties in chimerism induction in the setting of autoimmunity could potentially impact upon the design of protocols that can induce tolerance to islet transplants given to patients with T1DM. While understanding the mechanisms of allograft tolerance or rejection in the setting of mixed chimerism can provide insight into the development of strategies to overcome split tolerance.

The aim of Chapter two is to introduce the mechanisms of tolerance that are operating after the establishment of chimerism and discuss the occurrence of split tolerance in both animal models and the clinical context. Specific focus is

placed on the difficulties of overcoming recipient alloimmunity for the establishment of chimerism and the development of split tolerance in the non-obese diabetic (NOD) mouse model of human T1DM.

After reviewing the difficulties in developing a clinically relevant conditioning protocol for chimerism induction in NOD mice, in chapter three we identify the previously unrecognized barrier that natural killer (NK) cells impose upon chimerism induction in this mouse strain. In the presence or absence of adaptive immunity, antibody depletion of NK cells *in vivo*, or transplantation of F1 hybrid donor cells to eliminate the "missing-self" trigger of NK cells, was performed to test the NK-mediated rejection of donor bone marrow cells. We also studied the capacity of rapamycin to block the NK cell response against allogeneic cells *in vivo*. We show that depleting NK cells or rendering them inactive greatly improved the level of chimerism obtained in NOD mice. Rapamycin significantly reduced the allogeneic chimerism resistance mediated by NOD NK cells, however, it was a much less effective than NK cell depletion by antibodies. These data indicate that in addition to the known obstacle of NOD adaptive alloimmunity to allogeneic chimerism, we found that NOD NK cells are also a substantial barrier to allogeneic chimerism.

In Chapter four, we investigate the cells responsible for the generation of split tolerance in chimeric NOD mice generated by a non-myeloablative, co-stimulation based conditioning protocol. Using reciprocal bone marrow chimeras between a non-autoimmune mouse strain that does not develop split tolerance after fully allogeneic chimerism induction (C57Bl/6) and a strain that does

(NOD), we show that both NOD radioresistant and radiosensitive cells contribute to the development of split tolerance. We then show that if the NOD adaptive immune system matures in the presence of chimerism, split tolerance does not develop. This finding led us to investigate the effects that each lymphocyte population had on both the induction of chimerism and the occurrence of split tolerance. Depletion of either B or NK cells did not affect the development of split tolerance; however, through a modification of our non-myeloablative chimerism induction protocol to include T cell depletion we demonstrate the effect T cells have on both chimerism induction and split tolerance. Specifically, T cell depletion allows for a higher level of sustainable chimerism and tolerance to donor islet transplants, although these chimeric NOD mice still rejected donor skin grafts, demonstrating residual split tolerance. Taken together, these data demonstrate that pre-existing T cells are a significant contributor to the development of split tolerance in chimeric NOD mice. Also, despite T cell depletion, split tolerance can still develop towards skin grafts; this reflects the contribution of NOD radioresistant cells to the development of split tolerance or the inherent difficulties in establishing tolerance to polymorphic tissue specific antigens located in the skin.

In the last study (chapter five), we explored one of the potential mechanisms of split tolerance: the differential susceptibility of non-vascularized allografts to indirect rejection by CD8 T cells. Using a T cell receptor transgenic system in which only monoclonal CD8 cells were present, we could manipulate the donor-recipient combination to isolate the indirect rejection pathway. We

found that transplanted islet and neonatal heart grafts were accepted, whereas skin grafts were sensitive to indirect rejection. This study also demonstrates that transplant location or tissue-specific antigens do not account for the difference in allograft susceptibility to rejection. Furthermore, through the use of reciprocal bone marrow chimeras, we show CD8 mediated indirect rejection of skin grafts depends on MHC class I expression on recipient radiosensitive cells, and not recipient radioresistant cells (in opposition to a commonly held belief). This study identifies that the differential susceptibility of an allograft to CD8 T cell indirect immunity is a possible explanation for the development of split tolerance in mixed chimeras.

Collectively, these studies identify some of the factors responsible for the challenges of establishing fully allogeneic chimerism and the development of split tolerance in NOD mice. Through the identification of these factors, we have developed a successful minimalistic non-myeloablative induction protocol that abrogates split tolerance towards islets. Finally, split tolerance may reflect an intrinsic property of allogeneic cells or allografts; a differential susceptibility to indirect rejection.

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Chapter 2: Mixed chimerism and split tolerance

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2.1. CHIMERISM AND TOLERANCE

Induction of donor-specific tolerance to transplanted organs or tissues is one of only a few approaches with the potential to eliminate the need for long-term immunosuppressive therapy, while also preventing chronic rejection. Establishing hematopoietic chimerism is one such method of inducing donor-specific tolerance. Chimerism was first associated with tolerance in the observations of Owen in which fraternal cattle twins were shown to be natural chimeras and therefore operationally tolerant of one another (1). It is also likely that the demonstration of acquired tolerance induced by Billingham *et al.* through the injection of "testis, kidney and splenic tissue" into fetal mice involved the creation of hematopoietic chimerism (2).

2.2. TOLERANCE MECHANISMS IN MIXED CHIMERAS

Tolerance in mixed chimerism involves both central and peripheral mechanisms. After bone marrow transplantation, donor stem cells migrate to and proliferate in the host bone marrow compartment (3). Donor stem cell haematopoiesis leads to mixed chimerism and populates the thymus with the hematopoietic cells involved in negative selection. In the thymus, donor and recipient antigen presenting cells will then eliminate both donor-reactive and host-reactive T-cells (4-6). After transplant, donor antigens can be presented to anti-donor T cells 'directly' on the major histocompatibility complex (MHC) of donor cells, or 'indirectly' when processed and presented on the MHC of recipient cells. Importantly, both sets of anti-donor T cells, i.e. those with direct anti-donor

specificity and those with indirect anti-donor specificity, can be made tolerant in the thymus (7,8). Thus, chimerism takes advantage of central tolerance, a robust form of tolerance in a manner similar to how the immune system evolved to eliminate most self-reactive responses (9). However, as discussed in more detail further on, it is unlikely all donor antigens reach the thymus to induce central tolerance.

2.2.1. Central Tolerance

Central tolerance is believed to be the dominant mechanism of tolerance in mixed chimerism. However, although many experiments demonstrate central (deletional) tolerance is occurring in chimeras, few experiments have actually tested whether chimerism is required to induce tolerance to alloantigens. Evidence for the occurrence of central tolerance in non-myeloablative mixed chimerism protocols comes from multiple experimental models. Early experiments demonstrating central deletion in chimerism used donor-recipient mouse combinations that differ in MHC class II I-E expression, thereby allowing the tracking of superantigen reactive T cells that express certain Vβ families. In this manner, it has been shown that donor reactive mature T cells are centrally deleted soon after the induction of chimerism (6). These results were confirmed in a CD8 T cell receptor transgenic mouse model, made chimeric with MHC mismatched bone marrow; the transgenic CD8 T cells were deleted in the thymus (5). Since antigen presenting cells are potent mediators of negative selection in the thymus (10,11), indirect evidence for negative selection in mixed chimeras

comes from the association between donor MHC class II positive cells in the thymus and tolerance to skin (5,12) or kidney grafts (13).

Although the above mentioned studies demonstrate central deletion can indeed occur in the setting of chimerism, none provide evidence that central tolerance is required for mixed chimerism to induce tolerance. Similarly, the fact that thymectomy of chimeras prevents a loss of tolerance upon depletion of the chimeric donor cells (14) is not evidence that central tolerance was required. There is a fundamental difference between demonstrating that central tolerance occurs in the setting of chimerism and demonstrating it is required for chimerism to induce tolerance. Whether central tolerance is required may be at the heart of understanding why one cellular transplant (hematopoietic cells) generates tolerance but another cellular transplant (e.g. pancreatic islets) does not. Or, in other words, is there something unique about hematopoietic cells that allow them to generate tolerance, or is it simply the fact that they are able to migrate to the thymus and induce central tolerance? Our group has investigated the ability of donor tissue transplants vs. donor hematopoietic cells to naturally induce tolerance, i.e. without drug treatment. These studies were aimed at defining the 'rules' of self-tolerance generation, using graft alloantigens as a model. Using this model we could ask, to be treated as self (i.e. induce natural tolerance), does an (allo)antigen present in a recipient need to be present systemically or can it be localized in the periphery? To provide a setting where such natural tolerance has optimal conditions to take hold, we transplanted donor tissue into recipients before the recipient's immune system had begun to develop. In one example, we

grafted male skin onto female immunodeficient (athymic) mice followed several months later by a female thymus graft to allow T cell generation to begin in the recipients (15). These experiments showed passenger lymphocyte chimerism was required for natural tolerance and that it can induce central tolerance. In addition, these experiments also demonstrated chimerism was unable to generate natural peripheral tolerance in adoptively transferred mature splenic T cells (mature T cells were instead immunized), suggesting central deletion was the dominant mechanism. However, although supportive, this study also could not prove that the central tolerance was necessary for the chimerism to induce tolerance. For example, these studies were done in highly lymphopenic immunodeficient mice, and the homeostatic activation that occurs in this setting may have blocked peripheral tolerance induction (16). However, more recently we have shown that chimerism generating passenger lymphocytes from an islet transplant increase the rate of rejection in wild type immunocompetent recipients, supporting the contention that chimerism is immunogenic in the periphery (17); these studies also showed that donor dendritic cells are not the only immunogenic passenger cells in a transplant.

Despite the above studies, there remains an additional issue that requires resolution before making the conclusion that central tolerance is necessary for chimerism to induce natural tolerance. It might be that only newly generated T cells have the appropriate programming to become naturally tolerant upon encountering donor chimerism in the periphery. In the above studies, the immunization of mature T cells may have been due to their functionally mature

programming as opposed to more immature recent thymic emigrants (18,19), which might be more susceptible to tolerance. Perhaps newly generated T cells may be tolerized by encounter with donor chimerism in the periphery, with no requirement for central tolerance. Moreover, the passenger lymphocyte chimerism in the above studies was mainly T cells, which lack MHC class II expression; expression of MHC class II might be a prerequisite for donor cells to induce peripheral tolerance. Only recently was the requirement for central tolerance in–chimerism specifically examined (20). In this study, we designed experiments to directly test whether endogenous, newly produced, T cells exiting the thymus and encountering the chimerism only in the periphery become tolerized or immunized by the donor chimerism. Again, we placed donor cells into immunodeficient recipients prior to immune system development of the recipients. We found that chimerism (donor derived peritoneal leukocytes) would be accepted only if the donor leukocytes were able to establish systemic chimerism, but not when present, in a localized fashion, solely in the periphery. This experiment confirms that newly generated T cells do not become peripherally tolerant of donor leukocytes (for an exception to this rule see ref. 16), and argues that any potential increased susceptibility of newly generated T cells to tolerance induction (21) is insufficient to allow peripheral tolerance to be established against the highly mismatched chimeric donor cells. Additionally, the chimerism in these experiments was composed mostly of B cells that express both MHC I and II. Therefore, peripheral mechanisms alone appear insufficient to generate tolerance to MHC mismatched chimeric cells, as tolerance did not

develop, even in newly generated T cells, unless the chimerism was present systemically. However, these conclusions are also based on studies in a lymphopenic model, and it remains possible that in a non-lymphopenic setting, newly generated T cells may demonstrate an ability to be tolerized in the periphery by chimerism without any need for central tolerance to the donor.

Taken together, our studies indicated that, with the exception of very weakly mismatched transplants (20), natural tolerance to donor tissues transplanted before recipient immune system development cannot be established without donor cells reaching the thymus. This finding suggests a parsimonious solution to the long-standing paradox that transplants given to recipients before immune system development (e.g. grafts given to a fetus) trigger rejection in some cases and tolerance in others. Of historical interest, the key requirement for central mechanisms in tolerance of such 'pre-immunocompetence transplants', and tolerance in general, is predicted by Lederberg's theory (22). This 1959 theory, often misattributed to Burnet, who even years later had not appreciated the advance made by Lederberg's postulate that it is the maturity of the lymphocyte and not the maturity of the animal that is the key solution to tolerance.

Another important example of the critical role of chimerism in tolerance of foreign cells that appear early in life has been elucidated in studies of tolerance to non-inherited maternal antigens (NIMA). This tolerance, caused by maternal cell microchimerism in the fetus, involves a fetal regulatory T cell (Treg) cell response (23-25). The studies discussed above suggest that tolerance to NIMA may require central tolerance of the antigens of the cells that set up the microchimerism (stem

cells). Furthermore, the regulatory mechanisms observed may be in response to the progeny of the maternal microchimerism that differentiates to express a distinct set of antigens (26). Such a scenario predicts that the depletion of Tregs would reveal immune reactivity towards the differentiated progeny of maternal microchimerism (e.g. maternal DCs) without a response to the maternal stem cells that initially established the microchimerism. Whether peripheral tolerance alone is sufficient for this state of natural tolerance to allogeneic cells has not yet been determined. Future investigations will be important to determine if tolerance to NIMA requires central tolerance or is instead due solely to peripheral mechanisms. These peripheral mechanisms may include immune escape as a result of the relatively tiny number of cells introduced, low MHC expression, and/or residence in an 'immune privileged' niche, acting together with the aforementioned Treg responses.

2.2.2. Peripheral Tolerance

Many of the experiments suggesting the key role of central mechanisms in chimerism induced tolerance have used T cell depleting induction protocols (5,6); therefore, peripheral mechanisms of tolerance were thought to play only a minor role. Although T cell depleting protocols are non-myeloablative, the drive towards developing clinically relevant mixed chimerism induction protocols began shifting away from T cell depletion and towards co-stimulatory blockade. This shift brought with it the notion that central deletion cannot account for the tolerization of pre-existing donor-reactive T cells, and peripheral mechanisms must also be present. Subsequently, both therapy-induced deletional and non-

deletional peripheral tolerance mechanisms involved in mixed chimerism were identified. Tracking T cells with Vβ reactive to donor superantigen, in a co-stimulatory based chimerism induction protocol, it was found that directly donor reactive T cells were rapidly deleted in the periphery (27). To ensure total donor reactive T cell numbers were not diluted due to contributions from the recently centrally tolerized T cell repertoire, thymectomized chimeras were shown also to have deleted a subset of donor reactive peripheral T cells. These results were supported by another study that showed peripheral deletion is due to the co-stimulatory blockade and not signalling through the co-stimulatory blocking antibody (28). This study also demonstrated the presence of therapy-induced non-deletional peripheral tolerance mechanisms because tolerance develops in this mixed chimerism model before the elimination of donor-reactive cells. T cell anergy is rapidly seen upon induction of chimerism with co-stimulatory blockade (29,30).

In contrast to anergy, the identification of regulatory mechanisms of tolerance involved in co-stimulatory blockade induced chimerism has been challenging and has sometimes provided conflicting conclusions. For example, some studies have found recipient CD4 cells are required for the induction of chimerism (12,30,31). These results, combined with *in vitro* (31) and *in vivo* (32-34) suppression experiments may point towards a role for regulatory T cells in establishing the chimerism induced tolerance. Additionally, in canine chimerism models of lung or vascularized composite tissue allografts, the frequency of T regs is increased in blood and draining lymph nodes, respectively (35,36).

However, in our own and others' experiments (34,37,38) depletion of CD4 T cells at the time of bone marrow transplantation has enhanced sustainable chimerism. Further arguments against regulatory T cell involvement, at least in maintenance of tolerance, comes in part from experiments where the depletion of CD4 cells months after bone marrow transplant does not affect tolerance (12). Additionally, adoptively transferring splenocytes from chimeric mice into immunodeficient hosts could not induce tolerance to donor skin grafts and injection of naive recipient splenocytes into stable chimeric mice breaks tolerance (29). In another experiment, after BMT some mixed chimeras did not develop T cells; they only developed peripheral granulocytes, B and NK cells. In contrast to chimeric mice that did generate T cells, the chimeras without T cells were not tolerant of donor skin grafts. Even when sorted CD4 CD25 T cells from tolerant animals were transferred into the chimeras that did not generate their own T cells, tolerance towards skin grafts could not be induced (38). However, in some patients who received non-myeloablative conditioning and a renal allograft (39), the recovery of CD4 T cell populations was associated with higher frequencies of $CD25^{high}$ $CD127^{-}$ $FoxP3^{+}$ (40). This high frequency was associated with suppression of anti-donor reactivity in 2/4 patients at 6-12 months post-transplant, but suppressive function was lost after one year.

Although deletion, anergy and regulation may play roles in peripheral tolerance in chimeras, the exact contributions of each may differ between model systems (41). Alternatively, peripheral tolerance may have an important function

in the immediate post bone marrow transplant period, but play a progressively smaller role as central tolerance becomes established.

2.3. SPLIT TOLERANCE

Even in the long-lasting presence of mixed chimerism, some allogeneic tissues from the same donor can be rejected; a phenomenon known as split tolerance (37,42-45). Experimentally, split tolerance occurred when the first mouse mixed chimeras were created using lethal irradiation and bone marrow reconstitution with mixed syngeneic and allogeneic cells (42). After being identified in lethally irradiated mixed chimeric mice, split tolerance was subsequently seen in non-myeloablative mixed chimeras (37,44,45). Beyond mice, split tolerance has also been seen in large animal models (46-48) and human clinical trials (39,49,50) of mixed chimerism. By organ, split tolerance has been seen in large animals towards skin (47,48), kidney (46), heart (51), and hematopoietic cells (39,52,53). With the current goal of creating mixed chimeras using clinically feasible amounts of donor bone marrow and with minimal conditioning, split tolerance may become more prevalent and its mechanisms need to be defined.

A number of factors may be associated with an increased likelihood of split tolerance developing in mixed chimeras (Figure 2.1). Predisposing factors may include immunogenicity of the graft, certain donor-recipient combinations, prior sensitization, location and type of graft (cellular or solid) and minimal conditioning chimerism induction protocols. Additionally, through chimerism

and non-chimerism experiments, a number of explanations have been proposed to explain the differential susceptibility to rejection of various types of tissues. These explanations include the presence of polymorphic tissue specific antigens and variable effectiveness of indirect effector mechanisms to reject certain allografts.

2.3.1. Increasing the Probability of Split Tolerance

2.3.1.1. Immunogenicity of Graft

The inherent immunogenicity of the tissue transplanted to mixed chimeras may predispose the graft split tolerance. Once chimerism is established, it is possible that a subsequent, highly immunogenic, donor tissue graft may be rejected (especially if allelic tissue specific antigens are present). Peripheral tolerance mechanisms operating in mixed chimeras may become overwhelmed in the presence of considerable activating signals and antigen presentation, leading to split tolerance. For example, skin possesses properties of potent antigen presentation by Langerhans cells or dermal DCs and high concentrations of extracellular glycoproteins that may facilitate T cell activation. However, this latter hypothesis does not explain why minor antigen matching prevents rejection of skin grafts in chimeras (37,41,42). Indeed, the difficulty in skin graft acceptance in mixed chimerism is similar to that seen in non-chimerism experiments, where a mis-match for a single antigen is sufficient to cause skin rejection (54). However, being mis-matched for a single antigen is not sufficient for heart rejection, and yet there can be split tolerance to heart (55). Perhaps, non-

vascularized allografts (e.g. skin) may be more susceptible to immune destruction than vascularized grafts (e.g. heart). Skin grafts are subject to ischemic insults, and continuous exposure to microbial flora, that may lead to inflammation and necrosis. This non-specific inflammation may make such non-vascularized grafts more susceptible to subsequent immunological destruction (56). Again, peripheral tolerance mechanisms may become overwhelmed in the presence of significant inflammatory signals leading to split tolerance to the non-vascularized allograft. However, despite the theoretical difference in immunity based on vascularity, skin grafts were rejected much more rapidly than non-vascularized heart grafts in a MHC-mismatched donor-recipient combination (57). Additionally, vascularized skin grafts are rejected in an identical fashion to full-thickness skin grafts (58). Although it is debatable as to the exact property of skin that increases its immunogenicity, conceivably, non-specific inflammation and potent antigen presenting properties may predispose it to rejection. However, since split tolerance can occur to other tissues that, unlike skin, do not posses high immunogenicity the aforementioned factors are not universal and cannot account for split tolerance towards all allografts.

2.3.1.2. Minimal Conditioning

In 1955, the first success at experimentally creating chimerism was done in mice with lethal dose irradiation in order to create an immunological ‘clean slate’ (59). The total destruction of the host haematological system allows the complete reconstitution of this system by donor bone marrow cells (full

chimerism), and the subsequent acceptance of donor skin transplants. However, there are some drawbacks with inducing full chimerism. First, although often successful at inducing chimerism and tolerance to donor tissues, it had already been established that transplantation of tissues that contain significant numbers of immunologically active cells carries the risk of graft-versus-host disease (GVHD) (60,61). GVHD occurs when donor immunocompetent cells react against host antigens and is a major concern in transplant protocols that create full chimerism, especially when involving MHC mismatches. Of note, a subset of chimeras created with myeloablative conditioning and bone marrow transplantation display a desirable graft-versus-lymphoma (GVL) response that is useful in the treatment of some haematological malignancies (62). However, even when the beneficial effects of GVL are taking place the undesirable effects of GVHD can still occur, underscoring the importance of understanding and avoiding GVHD. Second, full chimeras may be relatively immunodeficient, potentially as a consequence of donor T cells maturing in a MHC mis-matched host thymus (63,64). Although this immunodeficiency does not occur in humans, where at least partial MHC matching is performed. Last, the myeloablative conditioning involved in the creation of full chimeras is toxic and is associated with high morbidity, prohibiting its use outside of treatments for malignancies. For chimerism to be widely applicable in transplantation protocols, the risks of GVHD must be minimized and milder conditioning regimens implemented.

Mixed chimeras, organisms with a variable balance of donor and recipient hematopoietic cells, are more clinically favourable than full chimeras. Similar to

full chimerism induction protocols, mixed chimeras were created using lethal irradiation; the difference being that the immune system was reconstituted with a mixture of syngeneic and allogeneic bone marrow (65). These mixed chimeras were tolerant to donor skin grafts and rejected third party skin grafts. However, the donor recipient combination employed in this study was matched for all minor antigens, a situation that artificially avoids split tolerance and yet is not a realistic approach for the clinic. Importantly, the mice were immunocompetent as suggested by their longer survival when compared with full chimeras (42). Since the host hematopoietic system remains intact in mixed chimeras, antigen presenting cells with host MHC are able to interact with positively select T cells, potentially contributing to a more competent immune system than full chimeras (66).

Although efficient at inducing mixed chimerism, the toxicities and undesirable side effects of myeloablative protocols led to the development of non-myeloablative chimerism induction protocols. For example, one successful non-myeloablative protocols to establish mixed chimerism used T cell depleting monoclonal antibodies, low dose total body irradiation (TBI) and additional radiation to the thymus (67). In addition to low dose TBI and T cell depletion, further efforts to minimize host conditioning in order to translate mixed chimerism from experimental to clinical settings have involved the implementation of donor specific transfusion, co-stimulatory blockade, and short-term use of immunosuppressive medications.

Split tolerance may be more likely to occur in minimal conditioning (non-myeloablative) mixed chimerism induction protocols that leave more of the recipient T cell compartment intact. In such circumstances, successful induction of tolerance becomes more dependent on peripheral tolerance, for both direct and indirect donor-reactive T cells. However, with many different chimerism induction protocols, and the relative rarity of experimental split tolerance, it is difficult to isolate the effects each conditioning agent has on the development of split tolerance.

2.3.2. Mechanisms of Split Tolerance

2.3.2.1. Tissue-Specific Alloantigens

In many studies, mixed chimeras maintained donor hematopoietic cells but rejected skin transplants (37,42-45); the cause of which was likely immunity towards polymorphic tissue-specific alloantigens expressed by donor skin but absent from donor bone marrow cells (37,42,68-71). Therefore, although chimerism can induce tolerance towards hematopoietic cells, because bone marrow cells do not express all tissue-specific antigens (TSA) meant tolerance towards skin could not be achieved in many cases. Indeed, skin-specific alloantigens have been identified as targets for rejection (72,73). Split tolerance towards heart allografts has also been observed in both murine and canine models of mixed chimerism (51,55). These data suggests split tolerance is not the unique result of alloreactive T cells recognizing skin-specific antigens, and suggests other polymorphic TSAs may generate split tolerance. We recently proposed a model

for split tolerance requirements, outlining the necessity of TSA polymorphisms (37). Interestingly, previous non-myeloablative mixed chimeras were tolerant to islets (74), seemingly suggestive of a lack of islet-specific alloantigen polymorphisms in the mouse combinations studied. However, the extent of split tolerance due to TSAs is likely to be underestimated based on existing data. Many of the model systems used to evaluate the potential for mixed chimerism to induce tolerance employ donor and recipient combinations that are matched for minor antigens, eliminating the potential for TSA polymorphism to trigger rejection of donor tissues in chimeras. Unfortunately, as mentioned before, minor antigen matching is not something that is feasible in the clinic. Even when minor antigens are not intentionally matched, it is not clear that the limited number of inbred mouse strains used in chimerism studies will reflect the extent of TSA polymorphism in the human population. It seems unlikely that TSAs in humans would lack polymorphisms. The relative rarity of split tolerance in murine studies is quite likely to be misleading. A recent study has shown that all of the various laboratory mouse strains are derived from a limited heritage and consequently have a very limited genetic diversity (75). Therefore, it can be anticipated that inbred mouse strains greatly under-represent the true quantity of polymorphisms in TSAs that exist in wild populations. This raises the question, how many polymorphic TSAs need to be expressed in a donor tissue to generate an immune response that is sufficiently strong enough to cause rejection? Unlike the tolerance to donor bone marrow antigens (which will have the capacity to take advantage of central tolerance), tolerance to allelic variants of TSA will be fully

dependent on peripheral mechanisms. Therefore, with highly immunogenic tissues such as lung and intestine and non-vascularized grafts, such as skin, a very small number of allelic tissue antigens may be sufficient. In contrast, less immunogenic organs such as kidney, heart, and liver may require the donor to express many mismatched TSAs. This scenario would make split tolerance to the most commonly transplanted organs (e.g. kidney) a much more rare event. This is indeed the case in cynomolgus monkeys where kidney allografts survive long-term when given months after bone marrow transplantation (76). We would suggest that the most plausible interpretation of this outcome is that the few allelic TSAs present in the donor kidney do transiently induce an immune response, however, the response is weak and eventually switches to a tolerogenic response as the graft heals in and APC activating danger signals subside. Since central tolerance is not available for TSAs, peripheral tolerance must be induced, and would have to occur even after an initial state of priming to the TSAs. In this scenario, it may be that in order to avoid split tolerance towards highly immunogenic allografts (e.g. skin) the tolerizing agents (e.g. co-stimulatory blocking agents) should not only be administered during infusion of donor bone marrow, but also when the donor tissue/organ is transplanted (37). It will therefore be important to not simply design the duration of our tolerance induction protocols on the length of time required to induce tolerance to the bone marrow cells, but also the length of time required to induce tolerance to TSA of the organ transplanted. Given the wide distribution within the recipient of donor antigens on bone marrow cells, and the relatively restricted location of TSA, it can be

anticipated that it will take longer to induce tolerance to TSA than to bone marrow antigens. Given the existence of polymorphisms in TSA and the potential for split tolerance, it would be advantageous to develop chimerism approaches that generate at least some degree of donor specific regulation (often referred to as 'dominant tolerance'). In this way, regulatory T cells recognizing donor antigens common to donor hematopoietic cells and tissues can inhibit the response to TSA. This approach may involve an alteration of the class of anti-donor response in the few anti-donor T cells that manage to escape elimination, rather than a true tolerance that mimics self-tolerance (77,78).

The concept that TSAs trigger rejection is not the only possible explanation for split tolerance under all conditions. Tissue specific alloantigens as the explanation of split tolerance for skin grafts has been questioned in the past (79,80). Furthermore, tissue-specific alloantigens could not explain the differential susceptibility to rejection of heart, islet or skin transplants in a CD8 T cell transgenic mouse model (56), or islet, skin and hematopoietic cell transplants in a CD4 transgenic model (17).

2.3.2.2. Direct and Indirect Allograft Rejection

Allograft recognition by recipient T cells through either the direct (81) or indirect (82,83) pathway is independently capable of transplant rejection. With high frequencies of allospecific T cells, direct recognition is thought to be the dominant mechanism of acute rejection (84,85). However, indirect allorecognition is also relevant and is the driving force behind chronic rejection

(86,87). With indirect recognition, allogeneic MHC (85) or non-MHC (88,89) antigens can be presented in the context of host MHC to host effector cells. Furthermore, certain transplantation locations (90) and certain antigens (91) may preferentially present via the indirect pathway.

Although indirect alloresponses by CD8 T cells are possible (92,93), CD4 T cells may preferentially respond via the indirect pathway (94-96). Indirectly activated CD4 T cells can provide help to alloreactive cytotoxic T cells (97,98) and stimulate the production of alloantibodies by B cells (99). In addition, CD4 T cells can act as effectors. Without CD8 T cells or B cells, CD4 T cells can mediate transplant rejection when activated solely via the indirect pathway (17,83,88,100).

The central deletion of directly alloreactive T cells in bone marrow chimeras is likely mediated by donor bone marrow derived antigen-presenting cells (5,6,101) and T cells (15) that migrate to the thymus. However, mixed chimeras may not immediately become tolerant to donor antigens via the indirect pathway. Therefore, it is possible to visualize a situation where indirect immunity to transplanted tissues could potentially explain split tolerance. In an experiment to examine the role of T cells that see donor antigen in host MHC alleles (indirect immunity) in split tolerance, we created chimeras in which donor cells had both donor and host MHC alleles. In this manner, we could test if split tolerance in fully MHC mismatched chimeras might be due to the inability to directly present to and tolerize host T cells that have indirect anti-donor specificity. Even under conditions where the donor cells could directly present donor antigens to ‘indirect

pathway' T cells, the indirectly reactive T cells were tolerant of donor hematopoietic cells, but were able to reject donor skin (37). This demonstrated that in mixed chimeras there is indirect tolerance towards donor hematopoietic cell, but not skin TSAs.

An additional potential mechanism of split tolerance is a differential susceptibility of allogeneic tissue types to indirect T cell rejection. Although there is a known hierarchy in the susceptibility of different allografts to rejection (56,80,102,103), it is unknown why the differences exist, and there are many possible explanations. Using a monoclonal population of CD4 T cells recognizing a defined antigen-MHC complex, indirect immunity alone is sufficient to reject skin transplants (17,88) but not heart (104) or thymus (105) transplants. Furthermore, in a wildtype mouse using an MHC mismatched donor-recipient combination, orthotopic corneal allotransplants are rejected exclusively by indirectly activated CD4 T cells (90). In a chimerism model, transgenic CD4 T cells could mount an effective indirect immune response against donor B cells, islets and skin grafts given early post inoculation with donor hematopoietic cells (17). However, within the same animal where donor B cells and skin transplants were being rejected, donor T cells were not eliminated. This demonstration of split tolerance was due to a relative resistance of T cells to undergo indirect rejection.

Direct immunity may play a role in the rejection of some tissues, however, most of the direct alloresponse should be abrogated in mixed chimerism due to central deletion. Although, with increasingly mild mixed chimerism conditioning

protocols (non-myeloablative, co-stimulation blockade based) that leave more of the recipient T cell compartment intact, direct recognition may indeed play a larger role in allogeneic tissue rejection. Taken together, split tolerance may be generated by properties of the host (direct and indirect immunity) or the donor tissue (susceptibility to indirect recognition, and expression of TSA).

2.3.2.3. Dynamic Donor Phenotype

The concept that donor hematopoietic stem cells may have a phenotype that confers resistance to host immunity, either through direct contact inhibition or low expression of MHC molecules (106-108), is receiving increased attention but remains controversial. After bone marrow transplantation for chimerism induction, donor stem cells migrate to and proliferate in the host bone marrow compartment (3,109). These stem cells will then differentiate into myeloid and lymphoid progenitors and be exported into the periphery. Split tolerance may then be possible through a disparity of susceptibility of the differentiating stem cell progeny to rejection by the host immune system. Perhaps this is one of the contributing mechanisms of split tolerance seen in a non-obese diabetic (NOD) mouse model of chimerism (45). In this experimental system, NOD chimeras demonstrated persistent T cell chimerism but rejected other donor hematopoietic cells, including B cells. This result mirrors the outcome in the CD4 transgenic T cell model previously discussed (17). These data demonstrate that different lineages of terminally differentiated hematopoietic cells are not equally susceptible to rejection by the immune system.

Similar to the aforementioned studies on tolerance to NIMA, cellular differentiation as a potential mechanism of split tolerance has also been seen in maternal chimerism with fetal cells (110). During gestation, fetal cells may cross the placenta and establish fetal microchimerism in the mother (111,112). However, in some instances, fetus-derived stem cells may be present at high levels in the bone marrow of sensitized mothers, but not in any peripheral organs (110). This finding may be a result of the maternal immune system's inability to recognize the 'immune privileged' fetal stem cells. As the fetal stem cells differentiate, the maternal immune system may then recognize the foreign cells as they upregulate MHC and reduce immunoregulatory cytokine production (113). These results, in both experimentally induced and natural chimerism, suggest that as the differentiating progeny of donor stem cells change their phenotype they can become susceptible to immune recognition and/or immune effector mechanisms. This immune recognition may then account for split tolerance towards differentiated hematopoietic cell lineages. In contrast, for more terminally differentiated tissues, a changing cellular phenotype is likely to make only a small contribution to the development of split tolerance (111,112).

2.3.3. NOD mouse model and Multiple Mechanisms of Split Tolerance

The induction of chimerism for T1DM holds promise to induce tolerance towards an islet allograft while avoiding recurrent autoimmunity. However, before the chimerism can be applied to islet transplantation, the barriers listed above must be overcome. Fortunately, model systems such as the non-obese

diabetic (NOD) mouse can provide insight into the unique challenges of tolerance induction through mixed chimerism in the presence of autoimmunity. The NOD mouse was developed in 1980 and has been used extensively as a model system for the study of human T1DM (114). Clinical features of diabetes in NOD mice are similar to human T1DM and include abrupt onset of diabetes between 90 and 120 days (equivalent to early adolescence period in humans), hyperglycemia, glycosuria, hypercholesterolemia, ketonuria, polydipsia, polyuria, and polyphagia (114-116). Immunohistological similarities between the human and NOD mouse diseases include T cell mediated β cell destruction leading to extensive pancreatic islet inflammatory cell infiltration (117). Even at a genetic level, NOD mice show similarity to the human disease, as both have a unique major histocompatibility complex (MHC) beta chain mutation that alters the repertoire of peptides that can be presented by this allele (118,119). The fact that diabetes develops spontaneously in NOD mice (not chemically or experimentally induced) allows insight into gene and environmental interactions that gives rise to a complex disease. Furthermore, the availability of genetically manipulated (transgenic or gene knock-out) NOD mice allow dissection of immunological pathways, cells and signaling that drive immune responses (120). Unlike non-autoimmune strains, the multiple immunological abnormalities in NOD mice render their immune system difficult to tolerize towards allo- and auto-antigens. This difficulty in inducing tolerance in NOD mice also extends to chimerism induction (34,45,121). As such, overcoming the barriers of allo- and autoimmunity in the NOD mouse can provide a robust test of chimerism induction strategies. Using

relevant chimerism induction protocols in a relevant model system offers promise for translation of these approaches clinically.

The potential mechanisms behind the development of split tolerance in mixed chimerism are not necessarily mutually exclusive. In NOD mouse models of mixed chimerism, a resistance to tolerance induction (122-125) or a defect in self-tolerance (114,116,126,127) may heighten the potential for split tolerance via additional mechanisms besides those involved in split tolerance to TSAs. NOD mice require more intense conditioning to establish chimerism than other mice strains, and there is a lower incidence of lasting mixed chimerism (34,45,128,129). This NOD mouse tolerance resistance may decrease the efficiency of the chimerism conditioning regimen, thereby preventing complete tolerance to donor antigens (45). In this manner, chimerism may become more dependent on peripheral tolerance, of which there are notable defects in NOD mice (126,127).

Although we hypothesize the NOD mouse strain may be more prone to split tolerance, many chimerism studies in NOD mice do not support this hypothesis. As only a few of the many chimerism protocols involved fully MHC-mismatched combinations (130-133), partial donor-recipient MHC matching may explain the lack of split tolerance (134,135). The difficulty in establishing mixed chimerism in NOD mice may also limit the detection of split tolerance. For example, chimerism induction can lead to initial mixed chimerism that can eventually become full chimerism, thus diminishing the probability of split tolerance (128,132). Furthermore, split tolerance towards hematopoietic cells

may be missed in cases where chimerism levels were not monitored long-term (130,133).

We developed a NOD mouse chimerism conditioning regimen with a radiation-free approach that was successful across full MHC mismatches (45). While induction of autoimmunity in transplantation is an important potential source of split tolerance (136), islet-specific autoimmunity could not explain the rejection of donor islets by these NOD chimeras, as syngeneic islets were not rejected. Additionally, in contrast to chimeric C57BL/6 mice, chimeric NOD mice rejected donor skin grafts even when these grafts were given on the day of bone marrow transplantation (during the tolerance promoting co-stimulatory blockade treatment). The survival of donor T cells may be related to a resistance of donor T cells to indirect rejection (17), despite this being an unusually potent rejection pathway in the NOD mouse (94-96).

These studies identify a number of underlying factors for the development of split tolerance in NOD mixed chimeras. For example, resistance to chimerism induction, donor-recipient combinations, minimal conditioning induction protocols, tissue specific antigens and the ability of indirect effector mechanisms to reject certain allografts could all be contributing. Our NOD mouse chimerism induction protocol across fully allogeneic barriers consistently generates split tolerance; thereby allowing us an opportunity to study the cells and mechanisms involved in the split tolerance in this model. Split tolerance is likely to be a more important obstacle to the success of chimerism approaches than previously

considered, which may impact upon tolerance induction in islet transplantation and other types of donor tissue transplantation.

2.4. CLINICAL CHIMERISM, TRANSPLANTATION AND SPLIT TOLERANCE

The success of hematopoietic chimerism leading to donor-specific tolerance in animal models (137-140) has translated into clinical trials. The augmentation of chimerism in the clinical setting is an attempt at decreasing the amount of immunosuppressive medications transplant patients receive, increasing graft survival and, ideally, mimicking the induced tolerance seen in mice. Although the donor organ can act as a small source of pluripotent hematopoietic cells (141), bone marrow transplantation (BMT) is needed to provide sufficient amounts of donor hematopoietic cells to generate macro-chimerism.

Initially, piloted in only a few patients, bone marrow grafts ($11x10^9$ bone marrow cells (BMC)) were given 21-25 days after kidney transplant with an immunosuppressive regime that consisted of azathioprine, anti-lymphocyte globulin and prednisone (142,143). The early results were encouraging, with decreased levels of kidney graft rejection and decreasing levels of donor responsiveness. Subsequently, a larger study of post-kidney transplant bone marrow administration ($2\text{-}3x10^8$ BMCs/kg) was done with cyclosporine, prednisone, azathioprine and anti-lymphocyte globulin immunosuppression (144). Although not randomized, in this study, there was better kidney graft survival at both 12 and 18 months in the group that received the bone marrow transplant.

However, there were no differences in renal graft function or rejection episodes between the two groups (145). Increased long-term kidney graft survival in patients receiving BMT was also seen in a more recent series that included OKT3 induction, tacrolimus, methylprednisolone and mycophenolate mofetil (146). Also in this study, fewer kidney rejection episodes were seen in the recipients of the BMT ($7X10^8$ BMCs/kg) which was given in two doses on post-operative days four and between days 10 and 14. The success with bone marrow infusion was also seen with liver allografts. In 1997, the first randomized trial of liver allografts with peri-operative BMT showed significant results in favour of multiple donor bone marrow infusions (147). Specifically, both patient and liver graft survivals were greater in patients who received multiple bone marrow infusions over controls who did not receive bone marrow or who received a bone marrow graft on the same day as the liver. In addition, this study showed that cytoablative conditioning was not necessary to improve allograft survival when the recipient was given multiple bone marrow infusions. Recently, the bone marrow after organ transplantation technique has been used as part of the immunosuppressive regime for face allografts (148). In another recent study, kidney transplantation under the cover of total lymphoid irradiation, antithymocyte globulin, cyclosporine, prednisone and mycophenolate mofetil followed by administration of $1x10^6$ $CD3^+$ T cells and $8x10^6$ $CD34^+$ enriched donor hematopoietic cells/kg was attempted (149). Impressively, this regimen established mixed chimerism and tolerance towards the allograft such that all immunosuppressive medications were discontinued six months after transplant. Subsequently, this protocol has led

to stable kidney graft function and withdrawal of immunosuppressives in 8/12 patients (150). Although bone marrow infusions may have shown a benefit for kidney, liver and facial tissue allografts, donor BMT's were not able to increase pancreas or kidney graft survival in simultaneous kidney/pancreas transplants even though there was maintenance of peripheral blood chimerism (151,152). The lack of effect on graft survival with simultaneous kidney/pancreas transplants is in contrast to kidney grafts alone despite similar immunosuppressive therapy (OKT3, tacrolimus, azathioprine and methylprednisolone) and bone marrow dose ($5x10^8$ BMCs/kg). In the studies that monitored peripheral blood chimerism, it is evident that BMT increases the levels of chimerism over controls that do not receive BMT (151,153). However, the split tolerance seen with the persistence of peripheral blood chimerism in the face of solid organ allograft rejection (even in the presence of immunosuppression) demonstrates complexities in the relationship between tolerance and chimerism.

In contrast to the augmentation of chimerism by BMT, donor microchimerism commonly refers to chimerism that is detectable after a solid organ graft only. In these cases, microchimerism is a consequence of the passenger leukocytes migrating out of the transplanted tissue (154) As its name suggests, microchimerism is detectable at very low levels, usually only by molecular mechanisms. Although microchimerism is detectable after liver, intestinal or kidney transplant (144,155-157), there is debate regarding its immunological consequences. The observed immunological consequences of microchimerism range from long-term donor graft acceptance and donor-specific

unresponsiveness (154,158) to having no predictive value for clinical course (159-163) to being associated with graft rejection and high responsiveness to donor antigen (164-166). As such, in humans with long term graft acceptance and immunosuppressive withdrawal, it is not clear as to whether microchimerism is the cause or effect of operational tolerance towards donor antigens. As with macrochimerism, the split tolerance seen with microchimerism outlines the complex relationship between the presence of chimerism and tolerance. Further studies of microchimerism are required to delineate its potential use as an indicator for immunosuppressive withdrawal (167).

Despite the disparate clinical observations seen with microchimerism after solid organ transplant, the identification of microchimerism in long-surviving kidney and liver recipients at the University of Pittsburgh (158) prompted a program to augment chimerism in solid organ recipients by including a peri-operative bone marrow transplant. The unique Pittsburgh method of chimerism augmentation has no patient pre-conditioning and 1-6x10^8 BMCs are given on the same day as the organ allograft, not weeks afterwards (168). Using this method of bone marrow administration, heart (169), lung (170), kidney (171), pancreas (172), liver and kidney/pancreas (173-175) transplants have been performed. As with other BMT along with solid organ grafts, in these studies, chimerism levels in patients that received a BMT were higher than the controls. These studies also demonstrate there is variation in outcomes by enhancing chimerism depending on the organ transplanted. For example, some studies have shown decreases in acute rejection episodes for heart (169,173), and an ability to decrease steroid dose in

kidney, lung or pancreas recipients (170,173,175). However, with respect to kidney transplants, delayed graft function and the incidence of acute and chronic rejection have remained similar between BMT and control patients (168,174). Although there may be a benefit to enhancing chimerism by administering a BMT (with no pre-treatment) along with a solid organ graft, there are few attempts at immunosuppression withdrawal. Therefore, despite chimerism augmentation, true tolerance appears to be rarely achieved without a conditioning regimen.

The induction of mixed hematopoietic chimerism through non-myeloablative pre-conditioning and BMT can induce allograft tolerance in murine (67), large animal (176), and non-human primate models (76,177). However, in contrast to the mouse models, the establishment of chimerism does not guarantee solid-organ tolerance in large animal models. In addition, split tolerance may be observed much more frequently in large animals and humans than in mice; for example, in a canine model, despite high levels of chimerism, donor-specific heart transplants were rejected (51). In contrast to the loss of a solid organ, when mixed chimerism tolerizing strategies are employed in non-human primates, some kidney grafts survived long-term, despite the decline and eventual loss of peripheral chimerism (76,177,178). The first clinical trial of non-myeloablative conditioning and BMT to induce mixed chimerism and tolerance towards kidney transplant were done in patients with multiple myeloma (49,52). Long-term outcomes of these seven patients outline three important points (50). First, non-myeloablative BMT can induce operational tolerance towards kidney allografts as discontinuation of immunosuppression was achieved in 4/7 patients. Second,

graft vs. host disesase (GVHD) occurred in 4/7 patients, raising the importance of this complication after BMT. Third, an unusual form of split tolerance was observed, as most patients lost peripheral chimerism and yet remained tolerant of the kidney allograft. However, in one patient both renal graft tolerance and mixed chimerism was maintained. Subsequently, patient pre-conditioning and BMT for the purpose of inducing tolerance towards kidney allografts was trialed in non-malignant settings (39,149). In this series, 4/5 patients demonstrated tolerance towards the kidney allograft after the withdrawal of immunosuppressive medications. However, mixed chimerism was short-lived with all patients losing peripheral chimerism by day 21 post-BMT, suggesting a state of split tolerance may have developed.

In contrast to experiments showing that chimerism is required for maintenance of tolerance in mice (179), the demonstrations of split tolerance in humans indicate that sustained peripheral macro-chimerism may not be necessary for long-term allograft tolerance. Perhaps, as seen with passenger leukocyte experiments (180) and pre-natal chimerism models (181), induction of a certain level of hematopoietic chimerism is critical to establish rather than maintain allograft tolerance. Or perhaps, since it was not monitored (39), only microchimerism is required for ongoing allograft tolerance.

2.5. CONCLUSIONS

While the strengths of chimerism-induced tolerance are well appreciated (1), there are impediments to this approach. First, less-toxic conditioning

regimens have allowed the translation of chimerism induction into the clinical setting, and importantly, into the non-malignant clinical setting. However, for the routine use of chimerism as an adjuvant for solid organ transplantation, even milder or more targeted regimens must be tried. In addition, shorter protocols must be also be tried as current conditioning regimens that involve many days of pre-treatment make chimerism a possibility only in the living donor situation. Second, the potential for development of GVHD is also a common concern with bone marrow transplantation and chimerism. Last, as seen in both animal studies and human clinical trials, the relationship between chimerism and tolerance is not straightforward. Split tolerance is a serious potential pitfall of chimerism-induced tolerance because it can affect allografts as well as donor hematopoietic cells (2,37,42-44,69,70). Since split tolerance affecting hematopoietic cells can lead to a loss of chimerism with unclear consequences on tolerance, further research into its mechanisms are warranted.

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2.7. FIGURES

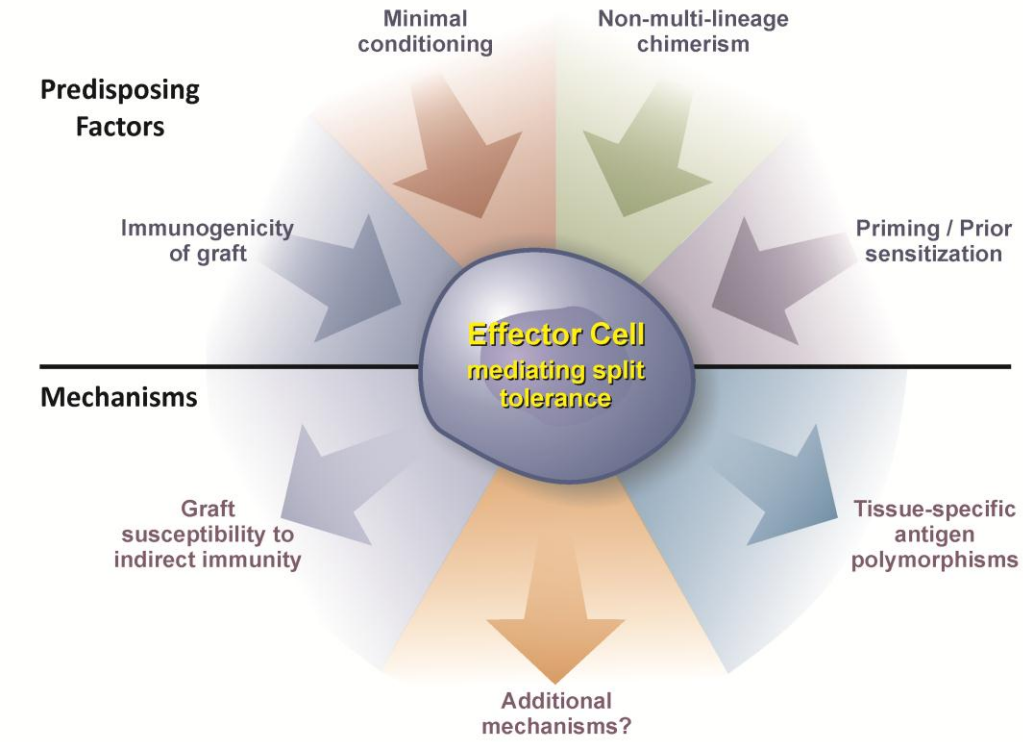

Figure 2.1. Predisposing factors and potential mechanisms for the generation of split tolerance

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Chapter 3: The barrier of natural killer cells in allogeneic chimerism induction in the non-obese diabetic mouse

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3.1 INTRODUCTION

Naturally (1) or experimentally (2,3) acquired systemic, allogeneic hematopoietic chimerism can induce immunological tolerance to the same allogeneic donor. Host alloimmunity, however, is an important barrier to chimerism. Rejection of allogeneic cells occurs by both adaptive and innate mechanisms, the latter by natural killer (NK) cells. NK cells generally eliminate cancerous, virally infected, or stressed cells (4,5). The current paradigm on NK cell function is that they kill target cells that lack expression of MHC class I molecules that the NK cells themselves express ("missing-self") (6,7). Thus, an allogeneic hematopoietic cell expressing only foreign MHC is a susceptible target. Indeed, NK cells vigorously reject allogeneic cells after transplantation (8). When adaptive immunity is absent, NK cells alone can be sufficient to confer rejection (2,9,10).

Nonobese diabetic (NOD) mice are prone to autoimmune diabetes (11). Establishment of allogeneic chimerism can restore self-tolerance to islet autoantigens and may induce allotolerance (12-14). Surprisingly, whether NOD NK cells prevent allogeneic chimerism has never been studied, perhaps because NOD NK cells have various functional defects. NOD NK cells show impairment in both cytotoxic killing of tumour cell lines, and in cytokine production (15-22). Existing data suggest a broadly impaired killing machinery intrinsic to NOD NK cells, with multiple defects in activating signaling pathways (15). Additionally, inadequate expression of the IL-15 gene may contribute to their deficiencies (23). However, the NOD genome contains a greater number of activating Ly-49

receptor genes than the genomes of other mouse strains (24). Moreover, anti-viral immunity by NOD NK cells may be better than previously considered (25). Successful induction of allogeneic chimerism in NOD mice is also particularly challenging due to their general tolerance resistance (26-28). Robust allotolerance is further compromised by increased split tolerance (29). Thus, NK cells may be an overlooked barrier to chimerism in NOD mice.

Here, we provide evidence that NOD NK cells alone, in the absence of adaptive immunity, are sufficient to reject allogeneic hematopoietic cells. Decreasing NOD NK cell frequency by globally depleting antibodies or transplantation of F1 donor cells prevented rejection. Importantly, we demonstrate that blocking NOD NK-mediated rejection of donor bone marrow is relevant to chimerism protocol development, as it significantly increases the level of fully allogeneic chimerism generated by a non-myeloablative approach. We also found that rapamycin, which has been shown to suppress NK cell function *in vitro* (30-32), was able to reduce the alloimmunity of NK cells *in vivo*, suggesting its potential use in overcoming the NK cell barrier. Last, we attempted to induce chimerism by specifically targeting the subset of NK cells mediating rejection of allogeneic cells.

3.2. MATERIALS AND METHODS

3.2.1. Animals. Adult C3H (H-2^k), C57BL/6 (B6; H-2^b), Wt NOD and NOD mice on the recombinase activating gene 1 and IL-2 receptor gamma (RAG/IL-2Rγ)

double KO background (T-, B-, and NK-deficient; H-2^{g7}) mice were purchased from a combination of National Cancer Institute at Frederick (Frederick, MD, USA), Jackson Laboratory (Bar Harbor, ME, USA), and Charles River Canada (Saint-Constant, QC, Canada). Immunodeficient B6 and NOD mice on the recombinase activating gene 1 (RAG1) KO background, originally from Jackson Laboratory, were bred in-house. (NOD×C3H) F1 mice were generated in-house by mating NOD females with C3H males. C3H and NOD day 14 gestation fetuses were generated by timed pregnancies onsite. WT prediabetic NOD mice used for chimerism induction were females between 8-10 weeks of age. This study was approved by the institutional review board at the University of Alberta. All care and handling of animals were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.2. Induction of allogeneic chimerism by transplantation of bone marrow cells (BMCs), fetal liver cells (FLCs), or mature hematopoietic cells. Transplantation of 20 million allogeneic BMCs by a non-myeloablative protocol into WT NOD or B6 mice was previously described (29). Briefly, WT NOD or B6 mice were pre-conditioned with a donor-specific transfusion of 20 million unmodified C3H or (NOD×C3H) F1 spleen cells administered as an intraperitoneal (i.p.) injection on day -7, as well as anti-CD40 ligand (anti-CD40L; MR-1) and recombinant human Cytotoxic T-Lymphocyte-Associated Protein 4 immunoglobulin (CTLA-4-Ig) on days -7, -5 and -3 (each at 0.25 mg i.p.; Bio X Cell, West Lebanon, NH). On day -1, busulfan (BUS;

GalaxoSmithKline Canada), a DNA alkylating agent (33), was given i.p. at 20 mg/kg body weight. On day 0, recipient mice were transplanted intravenously (i.v.) with 20 million unmodified C3H or (NOD×C3H) F1 BMCs, as well as given i.p. injections of anti-CD40L (34), CTLA-4-Ig, and rapamycin (Rapamune®, Wyeth Canada, Montreal, QC) at 3 mg/kg diluted in phosphate-buffer saline (PBS); we previously determined this high dose of rapamycin was required for chimerism in NOD mice (data not shown). After transplantation, anti-CD40L and CTLA-4-Ig were administered on days 2, 4, and 6, while rapamycin was injected daily for 28 days. To transplant allogeneic FLCs, either NOD.RAG1-KO or NOD.RAG/IL-2Rγ-KO mice with prior BUS conditioning were used as recipients. A 1:1 mixture of allogeneic (C3H) and syngeneic (NOD) FLCs, at 15 million cells each, were injected i.v. To generate chimerism with mature hematopoietic cells, we transplanted five million allogeneic B cells i.v. into recipients. B cells, at an average of 96% purity, were enriched from donor spleens by magnetic cell sorting using anti-CD19 or anti-B220 microbeads (MACS® separation column; Miltenyi Biotec, Auburn, CA). Recipients from two of the groups were either given 3 mg/kg rapamycin i.p. on days 0-2 or left untreated. With all 3 methods of chimerism induction, non-specific NK cell depletion was performed in some recipient mice (as indicated) by administering anti-asialo GM1 i.p. (Wako Chemicals USA, Richmond, VA; 35 μL diluted in PBS).

In a separate cohort, NOD.RAG1-KO mice with prior BUS conditioning were used as recipients. Anti-Ly49-W (CWY-3) or anti-Ly49-M (CK-1; both

antibodies were gifts from Kevin Kane, University of Alberta, Edmonton, AB) were administered (each at 0.25mg or 0.5mg i.p.) on days -3, 0 and 3 relative to the day 0 i.v. injection of a 1:1 mixture of allogeneic (C3H) and syngeneic (NOD) FLCs, at 15 million cells each.

3.2.3. Islet isolation and transplantation. Islet isolation was carried out as previously described (35). Three days before transplantation, recipient mice were made diabetic by a single i.p. injection of streptozotocin (Sigma-Aldrich Canada, Oakville, ON) at 200-210 mg/kg body weight. Diabetes was confirmed by a blood glucose measurement of >20.0 mmol/L. Five hundred islets were transplanted into the renal subcapsular space to reverse hyperglycemia.

3.2.4. Flow cytometry. NK cells were detected as $CD49b^{+}CD122^{+}TCR\beta^{-}$ cells. At various time points, chimerism levels were assessed in WT NOD and B6 recipients of C3H BMCs. Chimerism was assessed in immunodeficient NOD mice at least 5 weeks after FLC transplantation. Donor C3H cells were identified by anti-H-$2K^{k}$ antibodies. Donor (NOD×C3H) F1 cells were identified by double positive staining for H-$2K^{k}$ and H-$2K^{d}$. Recipient cells were identified by anti-H-$2K^{d}$ for the NOD model, or anti-H-$2D^{b}$ for the B6 model. When chimerism was induced by allogeneic B cells, C3H donor cells were detected in recipient spleen 3 days after transfer by anti-CD45.2 antibodies. Recipient cells were identified by anti-CD45.1 antibodies. Antibodies were purchased from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA). Data were acquired using either a

FACSCalibur® or LSR II (Becton Dickson, Sunnyvale, CA) flow cytometer and analyzed with CellQuest™ or FCS Express™ (De Novo Software, Los Angeles, CA).

3.2.5. Cell counting and calculation of donor cell and recipient NK cell numbers. After homogenizing mouse spleens into single cell suspensions, manual cell counts were done by a hemocytometer and phase contrast microscopy. The number of donor B cells recovered from spleen was calculated as the product of the total cell count and the percentage of donor B cells within the lymphoid gate. The number of recipient NK cells in spleen was calculated as the product of the total cell count and the percentage of NK cells within the lymphoid gate.

3.2.6. Statistical analysis. A two-tailed Student's *t*-test was used for comparison of means between two groups. One-way analysis of variance and Tukey's multiple comparison tests were used to compare three or more means. All statistical analyses were done using Prism 5 (GraphPad Software, San Diego, CA) with statistical significance defined as $p<0.05$.

3.3 RESULTS

3.3.1. NOD NK cells reject allogeneic passenger hematopoietic cells from an islet transplant. Previously (29), we generated a protocol that induced long lasting mixed chimerism in NOD mice using a fully allogeneic donor/recipient

strain combination (C3H to NOD). Since the donor cells lacked recipient MHC class I molecules, and NOD NK cells have been shown to express the activating receptor Ly-49W specific for H-2D^k (36), NK cell alloimmunity against C3H would be plausible but has not been examined. We therefore chose this combination to begin to investigate allogeneic resistance by NOD NK cells. Taking advantage of the capacity of an islet transplant to generate systemic chimerism via its few passenger cells (2), we transplanted C3H islets into immunodeficient NOD.RAG1-KO mice that have NK cells but not T or B cells, and monitored recipients for chimerism. For comparison, we gave C3H islets to B6.RAG1-KO mice, as these recipients are also fully allogeneic to the donor and yet are not known to express any activating Ly-49 receptor specific for H-2^k (37-40). More than 40 days post-transplant, C3H chimerism, generated by the passenger cells, was detected systemically in the peripheral blood or tissues (spleen and peritoneum) of B6.RAG1-KO mice (3/4 mice) but not NOD.RAG1-KO mice (0/5 mice; Figure 3.1), despite long-term acceptance of the islet transplant in both groups (maintenance of normoglycemia for greater than 100 days). These data suggested that NOD but not B6 NK cells were capable of rejecting allogeneic C3H cells that could otherwise generate systemic chimerism.

3.3.2. NOD NK cells reject allogeneic hematopoietic stem cells and NK cell depletion prevents rejection.

Next, we examined the capacity of NOD NK cells to reject directly injected allogeneic cells rather than passenger cells in a transplant. To accomplish this, we

first attempted to induce allogeneic chimerism in NOD.RAG1-KO mice by giving them C3H FLCs as a source of hematopoietic stem cells that are devoid of any contaminating T cells. Interestingly, NOD.RAG1-KO recipients of 20 (n=7) or 40 (n=3) million C3H FLCs did not become chimeric when assessed at 4-14 weeks after transplantation (data not shown). Hypothesizing that allogeneic cells were rejected by NOD NK cells, and due to our interest in studying mixed chimerism, we repeated our experiments with the following modifications. NOD.RAG-KO mice were given a mixture of C3H and NOD FLCs as a source of allogeneic and syngeneic hematopoietic stem cells, respectively, to induce mixed allogeneic chimerism. To test the capacity of NK cells to resist chimerism, we treated a separate cohort of NOD.RAG1-KO mice with anti-asialo GM1 to deplete their NK cells, before FLC transplantation. In another approach to test the ability of NK cells to resist allogeneic chimerism, we gave NOD.RAG/IL-2Rγ-KO mice (41) a mixture of C3H and NOD FLCs. Flow cytometric analysis of the peripheral blood before transplantation showed that NOD.RAG1-KO mice given anti-asialo GM1 had a decreased proportion of NK cells ($CD49b^+CD122^+$) compared to the untreated cohort (0.1±0.2% versus 26.2±2.0%; Figure 3.2A). Approximately 5 weeks after reconstitution, we analyzed the peripheral blood of recipients for systemic chimerism. Mice that were untreated and contained NK cells did not become chimeric (Figure 3.2B and C). In contrast, all mice that had been depleted of NK cells showed mixed allogeneic chimerism (25.7±8.1% of the cells within the lymphoid gate were C3H; Figure 3B and C). Similarly, all NOD.RAG/IL-2Rγ-KO mice became chimeric (37.1±3.0% of the cells were

C3H). Thus, NOD NK cells were responsible for the rejection of allogeneic hematopoietic stem cells; removal of NK cells prevented rejection, and allowed the generation of systemic chimerism.

3.3.3. NOD NK cells are a barrier to chimerism induction using a fully allogeneic donor and a non-myeloablative induction protocol.

Since chimerism induction in WT NOD mice is difficult to achieve ((29,42) and our unpublished observations), it is important to assess the contribution of NOD NK cells in this overall tolerance resistance. Using a previously successful non-myeloablative protocol (29), we examined the contribution of NOD NK cells to chimerism resistance in WT mice. Pre-diabetic NOD mice, or B6 mice for comparison, were conditioned and transplanted with fully allogeneic C3H BMCs. A second cohort of NOD mice was treated with the same regimen and received the NK cell-depleting antibody anti-asialo GM1. Since NK cells can become activated when self MHC proteins are not encountered (missing-self hypothesis), we used another approach to render NOD NK cells inactive against the donor BMCs. We gave this third cohort of NOD mice BMCs from (NOD×C3H) F1 donor mice.

Under the current conditions, the standard chimerism inducing protocol was not as effective at generating chimerism as it was in our earlier studies (29). However, consistent with our previous results (29), both the incidence and the level of chimerism achieved in NK-sufficient NOD mice were significantly less than those achieved in B6 mice (data not shown). In contrast, when NK cells

were depleted, NOD mice became chimeric both at a higher incidence and to a higher level than when the host NK cell compartment was untouched (Figure 3.3). Similarly, when NK cells could not become activated against the donor BMCs (F1 donors), chimerism was achieved at a significantly increased level compared to when parental C3H BMCs were transplanted. Although inhibition of donor NK cells has the potential to augment chimerism (43), the increased chimerism with F1 donor cells suggests that specifically reducing the recipient's NK response to the donor augments chimerism. Moreover, the mixed chimerism achieved was initially multi-lineage (data not shown) and the frequency of donor cells was quite stable across the first 13 weeks post-transplant (Figure 3.3B). Together, these results indicate that NOD NK cells in WT mice are a barrier to chimerism induction using fully allogeneic donors.

3.3.4. Rapamycin reduces NK cell alloimmunity but is less effective than NK cell depletion.

Besides depleting NK cells, we tested whether NK cell alloimmunity in our model could be overcome by conventional immunosuppression. Rapamycin, a nonspecific immunosuppressant that can promote allogeneic chimerism (44-46), has been shown by some in *vitro* studies to suppress NK cell activity (30-32). However, the capacity of rapamycin to block NK function *in vivo* has not been examined. Our finding that NK cell depletion (Fig. 3.3) augmented chimerism in a protocol that included rapamycin, suggested that rapamycin might be ineffective at blocking NK cells *in vivo*.

To directly test the *in vivo* effects of rapamycin on NK cells, we challenged NOD-RAG1-KO mice with purified fully allogeneic C3H B cells (Figure 3.4A) with or without injecting the recipients with rapamycin, and assessed rejection 3 days later. A third group received anti-asialo GM1 to deplete their NK cells before transfer of donor cells (Figure 3.4B-D). We studied the NOD NK cell response to allogeneic B cells rather than bulk spleen cells because, in this way, we could monitor the rejection of a homogenous cell population, avoiding any possible differential NK cell killing of the various types of hematopoietic target cells present in bulk spleen cells. Moreover, since our recipients were immunodeficient, target killing could be offset by their rapid homeostatic proliferation, particularly if T cell targets were used (47). In contrast, B cells would be expected to undergo little homeostatic proliferation within the time that we studied rejection (48). We quantified donor cells recovered from the recipient spleen, the site at which the majority of NK killing of allogeneic cells occurs (49). Compared to untreated mice, we recovered a significantly greater number of donor cells in the spleens of rapamycin-treated mice (Figure 3.4C). However, mice depleted of NK cells harbored a significantly greater number of donor cells in their spleens than mice given rapamycin (Figure 2.4C). When we compared the number of recipient NK cells between untreated mice and mice given rapamycin, there was no significant difference (Figure 2.4D). Thus, rapamycin reduced the killing of allogeneic cells by NOD NK cells, but its suppressive effect was not as potent as NK cell depletion by antibodies and did not occur by decreasing NK cell numbers. Similar observations were made when

we challenged NOD-RAG1-KO mice with fully allogeneic cells from a different donor (FVB; Figure 3.5), and when we performed similar experiments in B6-RAG1-KO mice (Figure 3.6).

3.3.5. Depleting NOD NK cells that express Ly-49W does not prevent rejection of donor hematopoietic stem cells. Our experiments thus far involved the global depletion of recipient NK cells with anti-asialo GM1 or the non-specific immunosuppressive, rapamycin. Therefore, we next attempted to isolate the specific NK cell subtype that is mediating the rejection of allogeneic cells and tailor our induction therapy accordingly. NOD NK cells express the activating receptor Ly49-W which recognizes H-2D^k as well as the activating receptor Ly49-M which has an unknown specificity (although protein sequence homology predicts interaction with H-2K). Antibodies to both Ly49-W and Ly49-M were generated previously, and we found them to deplete their targets when administered at 0.25mg i.p. (data not shown). To determine if specifically depleting NK cells expressing Ly49-W or Ly49-M would allow for the acceptance of allogeneic FLCs, we administered antibodies specific for these receptors to NOD.RAG1-KO mice that were given a mixture of C3H and NOD FLCs as a source of allogeneic and syngeneic hematopoietic stem cells, respectively, to induce mixed allogeneic chimerism. Approximately 5 weeks after reconstitution, we analyzed the peripheral blood of recipients for systemic chimerism. Similar to the results in figure 3.2, in this cohort, mice that were untreated did not become chimeric whereas mice that had been depleted of NK

cells with anti-asialo GM1 showed mixed allogeneic chimerism. NOD.RAG-KO mice treated with anti-Ly49W or anti-Ly49M did not become chimeric (Fig 3.7), but did develop T and B cells of NOD origin (data not shown). Despite doubling the dose of antibodies we were still unable to generate chimerism (data included in Figure 3.7). Thus, the depletion of NOD NK cells specifically expressing the activating Ly49-W or Ly49-M receptors was unsuccessful at inhibiting the rejection of allogeneic C3H hematopoietic stem cells.

3.4. DISCUSSION

Mixed hematopoietic chimerism is difficult to induce and maintain in the NOD model of human type-1 diabetes (29,42). NK cells have long been known to be a barrier to hematopoietic stem cell engraftment (50,51); however, their role in chimerism induction in an autoimmune-prone host has not been investigated. Perhaps, NK cells have been overlooked in NOD mice due to their impaired cytotoxic killing of multiple NK-sensitive tumour cell lines and their impaired cytokine production. Although poor cytotoxic killing could be partially reversed by stimulation with IL-12 and IL-18 or interferon-α/β (15), and other defects could be partially reversed by incorporation of the NK1.1 gene segment from B6 mice into the NOD genome (19), the overall mechanism that accounts for the broadly defective function of NOD NK cells has remained unclear. Although, dampened signaling through the NKG2D activation pathway (16) or inadequate IL-15 gene expression (23) are possibilities. Recently, Orr et al. reported that NOD NK cells congenic for NK1.1 expression were poor producers of granzyme

B despite normal degranulation, thus pointing to a defect that might explain why multiple signaling pathways appear defective in NOD NK cells (25). However, this hypothesis does not appear consistent with our observation that NOD NK cells appear better than B6 NK cells at killing C3H passenger cells (Figure 3.1).

Since NK killing of aberrant cells is mediated by a concert of stimulating and inhibitory signals, and NOD mice have a large repertoire of activating Ly49 receptors, it seems plausible that when stimulated with an appropriate ligand, robust NK activation can ensue. Indeed, studies of NOD NK cells functioning in the presence of their activating receptors implicate these cells in the development of diabetes in NOD mice (52). Our overarching conclusion from this study is that despite the functional and synthetic deficiencies of NOD NK cells when stimulated with tumour cell lines, we have demonstrated that NOD NK cells resist allogeneic chimerism. The ability of NOD NK cells to be potent killers of C3H hematopoietic cells indicates that any functional deficiency in NOD NK cells is activation and/or inhibition pathway-specific, and not the result of a deficiency in any of the downstream intracellular signals common to all activation pathways.

Our observation that B6 NK cells were unable to reject H-2^k cells was consistent with previous findings (53,54). Mechanistically, poor rejection by B6 NK cells of C3H cells expressing H-2^k is potentially explained by the presence of inhibitory Ly-49 receptors capable of binding H-$2D^k$ and/or H-$2K^k$ (Ly-49A, Ly49-C, and Ly-49I) in addition to a lack of activating receptors for H-2^k (36-40). In contrast, the presence of Ly-49W, an activating receptor that can bind H-$2D^k$ and is expressed by NOD NK cells, is a plausible explanation for the robust

rejection of C3H cells by NOD.RAG1-KO mice (36). Importantly, Ly-49W has been shown to be a functional activating receptor that triggers killing of susceptible target cells (36). However, NOD NK cells also express the activating receptor Ly-49D, but Ly-49D-mediated killing of susceptible target cells by NOD NK cells is impaired (15). Since both these receptors presumably use a similar signaling pathway of activation (36,55), it is not immediately clear how their functional outcomes can be different. Moreover, the involvement of activating Ly-49 receptors in the rejection of FVB cells by NOD NK cells (Figure 3.5) is less clear, since it is not known whether any activating receptor that can bind MHC class I molecules of the H-2^q haplotype exists (39,40).

To provide further evidence for allogeneic resistance by NOD NK cells, we challenged NOD.RAG1-KO mice with a mixture of syngeneic and allogeneic FLCs and observed no chimerism. Importantly, when NK cells were depleted by anti-asialo GM1, this led to mixed allogeneic chimerism, confirming that the absence of chimerism was due to rejection by NOD NK cells. This result also suggests that the establishment of allogeneic chimerism in B6.RAG1-KO but not NOD.RAG1-KO islet transplant recipients was not due to differential survival of C3H passenger cells in the two different MHC-mismatched environments, irrespective of recipient NK cell activity. Additionally, in WT NOD mice, allogeneic chimerism incidence and level could be significantly enhanced with the depletion of recipient NK cells or the restoration of inhibitory ligands. This indicates a prominent role of NOD NK cells in resisting allogeneic chimerism in this model. In a broader context, these data suggest that in the absence of

adaptive immunity, or when adaptive immunity is suppressed, host NK cells pose a residual but sufficiently potent barrier to chimerism not only in non-autoimmune recipients (49,56), but also in diabetes-prone recipients with 'altered' NK function. Thus, removal of host NK cells would be greatly beneficial to chimerism induction (56-58) for islet transplantation, particularly when they express a relevant activating receptor for donor MHC ligands. It is interesting to note that removal of host NK cells is even beneficial when applying a chimerism induction protocol that uses anti-CD40L (Figure 3.3). In contrast, NK cell depletion strongly reduces the tolerance promoting ability of anti-CD40L in islet transplantation (59). Since this study suggests that anti-CD40L works in an NK cell dependent fashion, clarification of the mechanism of action of anti-CD40L in chimerism versus tissue transplantation is needed.

Besides depleting NK cells (50,58,60,61), overcoming the NK cell barrier can also be achieved by infusing a sufficiently large dose of donor cells to overwhelm the cytotoxic capacity of NK cells (56,62,63), or blocking NK cell function either specifically (61,64) or nonspecifically (30-32,56,65,66). In this study, we attempted to block the function of NOD NK cells by rapamycin. Rapamycin is a potent anti-fungal antibiotic; however, it is also a nonspecific anti-proliferative compound that is known to act on T cells, B cells, mast cells, lymphoid and non-lymphoid tumor cells, smooth muscle cells, hepatocytes, and fibroblasts (67-69). In BMC transplantation, rapamycin inhibits anti-donor immunity induced by recipient T cells thereby facilitating chimerism induction (44). Rapamycin also acts synergistically with other immunomodulatory agents

to promote allogeneic chimerism (45,46). The beneficial effect of rapamycin in chimerism induction is conventionally attributed to its inhibition of T cell alloimmunity; whether it also affects NK cells in the setting of BMC transplantation had not previously been investigated. Indeed, we observed that NOD.RAG1-KO mice given rapamycin (and even B6.RAG1-KO mice) rejected allogeneic cells less effectively than untreated mice (Figures 3.4-3.6). This suppressive effect was rather limited, since NK cell depletion led to significantly better survival of donor cells. Interestingly, we could not generate allogeneic chimerism by reconstituting NOD.RAG1-KO mice with allogeneic FLCs, when the recipients were only given rapamycin (our unpublished observations). The mechanism(s) by which rapamycin suppresses mouse NK cell function *in vivo* will require further studies. However, *in vitro* studies of rat and human NK cells have shown the inhibitory effects of rapamycin to include decreased upregulation of CD69, decreased proliferation upon stimulation, decreased cytotoxicity and cytokine production, altered expression of activating and inhibitory receptors, and altered distribution of NK cell subsets (30-32). Loss of NK cells *in vivo* (32) has been reported after rapamycin treatment, but this was based on the proportion of NK cells present in blood rather than absolute quantification. In contrast, we did not find any change in NK cell numbers in mice treated with rapamycin compared to untreated mice (Figures 3.4 and 3.6).

In order to narrow the spectrum of conditioning during chimerism induction, we attempted to identify the NOD NK subtype responsible for rejection of allogeneic cells. Likely NK subtypes are those expressing the activating

receptor Ly-49W, which can bind H-2D^k (36). However, specifically depleting NOD NK cells expressing Ly-49W did not allow for the induction of chimerism. Our attention then focused on the activating receptor Ly-49M that has an unknown ligand, but high sequence homology to Ly-49W in its carboxy-terminal carbohydrate recognition domain (39). However, chimerism could not be established upon depleting NK cells expressing Ly-49M. Since globally depleting NK cells with anti-asialo GM1 could generate chimerism, there may be other NK cell subtypes that are involved in the rejection of allogeneic cells. If there is a redundant role for the Ly-49W and Ly-49M activating receptors in NOD mice, perhaps depletion of NK cells carrying either receptor is required to inhibit killing of their target cells. Alternatively, the number and type of activating receptors expressed by each NK cell can vary (70). Antibodies may preferentially deplete those NK cells with many targets expressed on their cell surface. The spared NK cells that express only a few activating receptors are then capable of killing their target. Similarly, the low levels of receptor on the surface of these NK cells would also make them difficult to detect with flow cytometry, thus giving the appearance of their efficient depletion.

In conclusion, our data indicate that despite the previously observed hyporesponsiveness of NOD NK cells in other settings, they can confer a substantial resistance to the generation of allogeneic chimerism, and do so in the presence or absence of adaptive immunity. We also found that rapamycin reduced the killing of allogeneic cells by NK cells *in vivo*, but as a stand-alone agent the suppression was fairly limited.

3.5. ACKNOWLEDGEMENTS

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3.6. AUTHOR'S CONTRIBUTIONS

DPA participated in research design, performance of research, data analysis and writing the paper. WFNC participated in research design, performance of research, data analysis and writing the paper. CCA participated in research design, data analysis and writing the paper

3.7. FIGURES

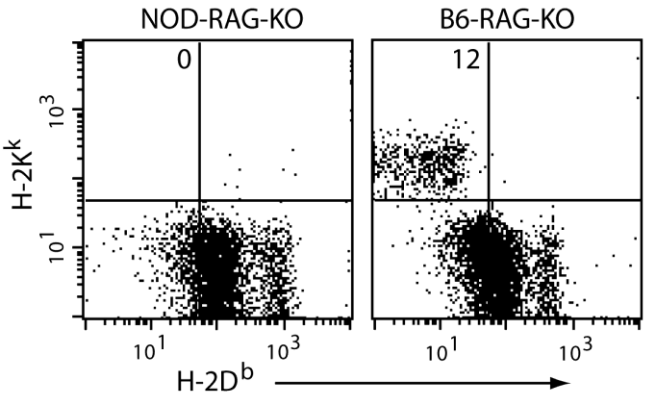

Figure 3.1. NOD.RAG1-KO NK cells rejected allogeneic C3H passenger cells that could otherwise generate systemic chimerism. NOD.RAG1-KO (n=5) and B6.RAG1-KO (n=4) mice were made diabetic and given C3H islet transplants. More than 40 days later, the presence of C3H (H-2K^{k+}) passenger cells was examined in the peripheral blood, or in the spleen and peritoneum. Recipient cells were distinguished by H-2D^{b} expression. Representative plots are shown from peripheral blood analysis, with the relevant quadrant percentage indicated (gated on lymphoid cells). None of the NOD.RAG1-KO and all but one of the B6.RAG-KO mice became chimeric.

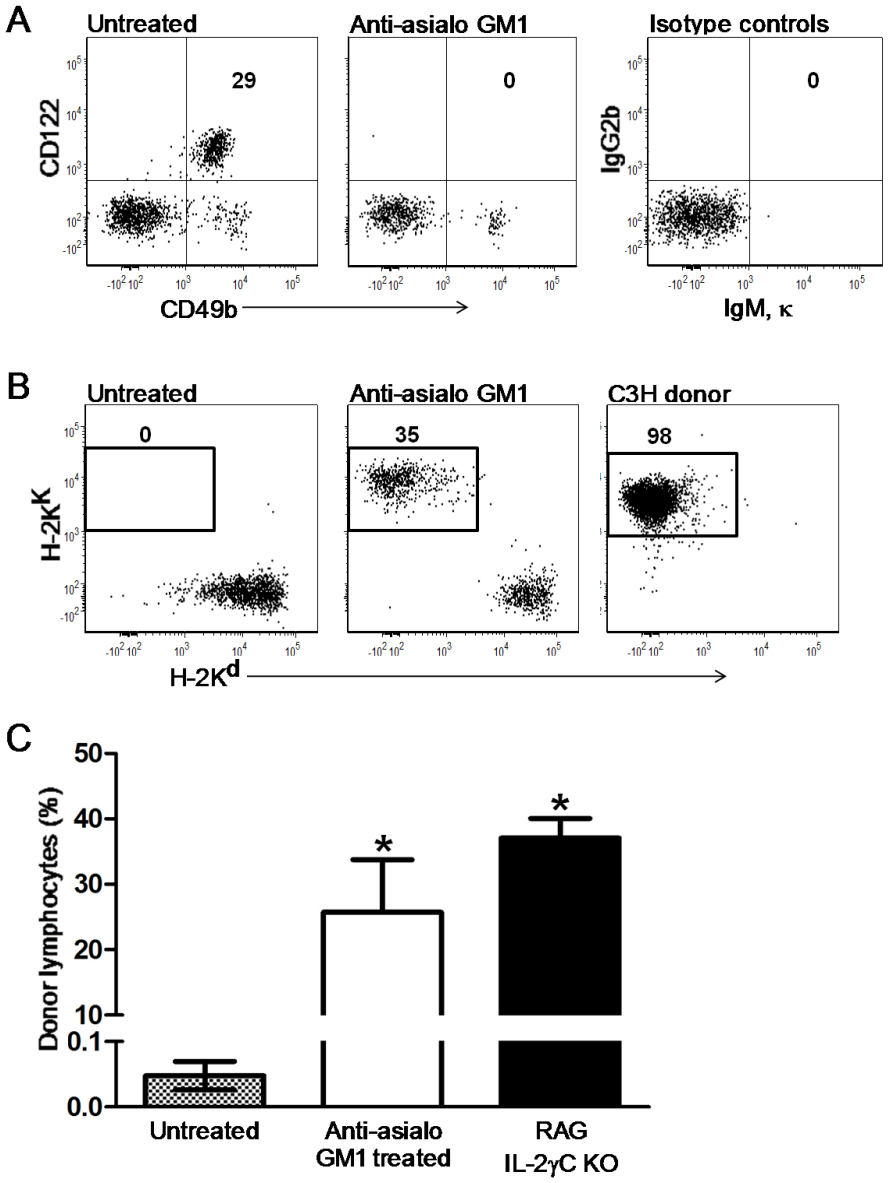

Figure 3.2. NOD.RAG1-KO NK cells rejected allogeneic hematopoietic stem cells and NK cell depletion prevents rejection. NOD.RAG1-KO mice were either untreated (n=5), or injected with anti-asialo-GM1 on day -3 (n=8). Two days later, these two groups, in addition to a third group of NOD.RAG/IL-2Rγ-KO mice (n=4), were conditioned with BUS. (A) Analysis of peripheral blood NK cells ($CD49b^+CD122^+$) in mice 3 days after injection of anti-asialo GM1

compared to untreated mice. A representative plot from each group, along with isotype controls, is shown. The percentage of NK cells within the lymphoid gate is displayed. (B) One day after BUS conditioning, all three groups received a mixture of 15 million C3H and 15 million NOD FLCs i.v. Approximately 5 weeks later, peripheral blood of recipients was analyzed for the presence of donor (H-2K^{k+}) versus recipient (H-2K^{b+}) cells. Representative plots from untreated mice and mice treated with anti-asialo GM1 are shown along with their percentages of donor cells within the lymphoid gate. Peripheral blood of a C3H mouse is shown as a positive control. (C) Mean and standard error of donor lymphocyte chimerism in each of the three groups. Asterisk indicates statistical significance compared to untreated mice.

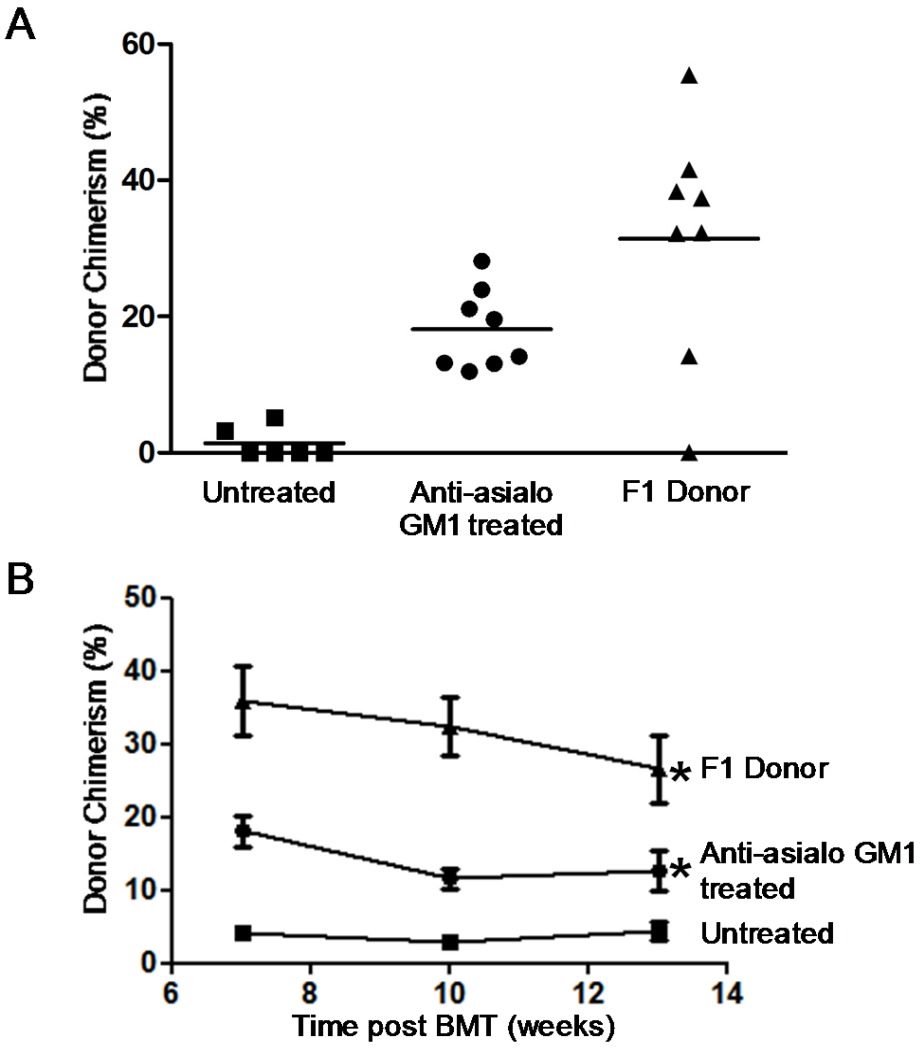

Figure 3.3. NOD NK cells in WT mice are a barrier to chimerism induction using fully allogeneic donors. WT NOD mice receiving fully allogeneic C3H BMCs were conditioned with a non-myeloablative chimerism protocol with or without the administration of anti-asialo GM1 on days -3 and 0 and weekly thereafter for 4 weeks, for a total of 6 injections. Another group of WT NOD mice receiving (NOD×C3H) F1 BMCs were conditioned with the non-myeloablative chimerism protocol alone. (A) When NK cells were depleted, NOD mice could be made chimeric at a higher incidence (8/8 mice) than the non-

depleted group (2/6 mice). Similarly, when NOD NK cells could not become activated against the donor BMCs, the incidence of chimerism was increased (7/8 mice). (B) Initially and when followed long-term, both the NK-depleted and F1 donor groups demonstrated a significantly increased level of chimerism compared to the NK-replete group. Asterisks indicate statistical significance compared to untreated mice.

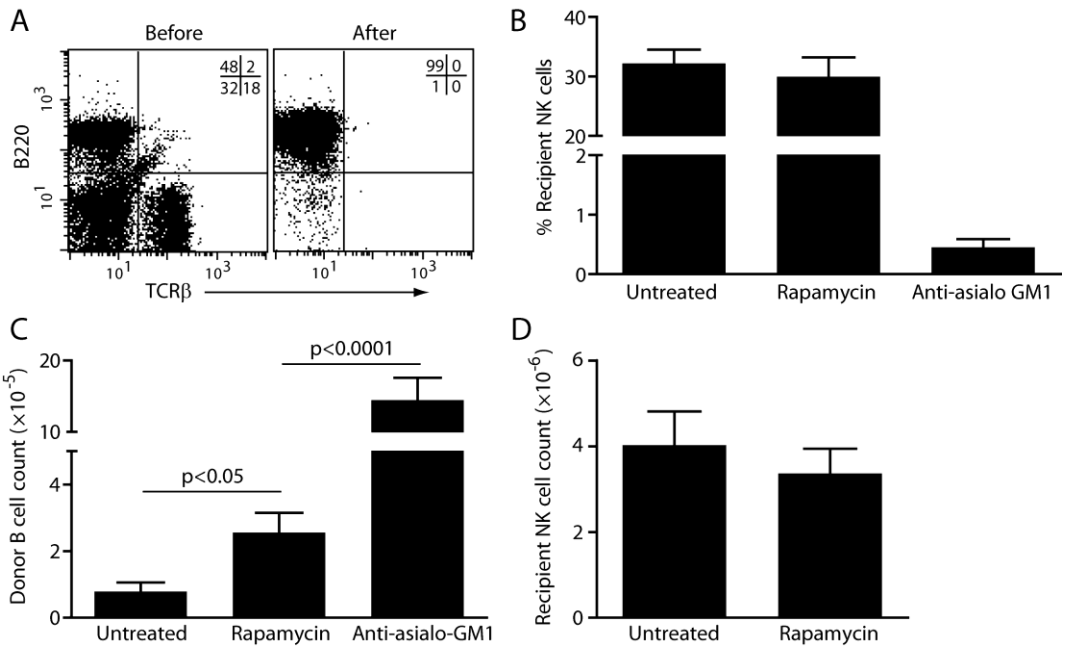

Figure 3.4. Rapamycin reduced the rejection of allogeneic cells by NOD NK cells but its suppressive effect was limited and not mediated by depletion. NOD.RAG1-KO mice were either left untreated (n=7), injected with rapamycin, 3mg/Kg, (n=10), or injected with anti-asialo GM1 on day -3 (n=3). C3H B cells were transplanted i.v. on day 0. (A) An example of purity assessment of donor B cells before and after magnetic sorting. (B) On day 0, before transplantation of allogeneic B cells, some of the recipients (n=3 for all groups) were analyzed for the proportion of peripheral blood NK cells by flow cytometry. Both untreated mice and mice receiving rapamycin had a significantly higher proportion of NK cells than mice receiving anti-asialo GM1 ($p<0.001$). (C) Three days after transfer, donor B cells were quantified in the spleen for all mice in all groups. C3H B cells were distinguished from recipient cells based on the expression of CD45.2 versus CD45.1, respectively. Levels of statistical significance between relevant groups are indicated. (D) Recipient NK cells in the spleen were

quantified on day 3 in all untreated mice and all mice given rapamycin. No statistical significance was found. In all bar graphs, mean and standard error are depicted.

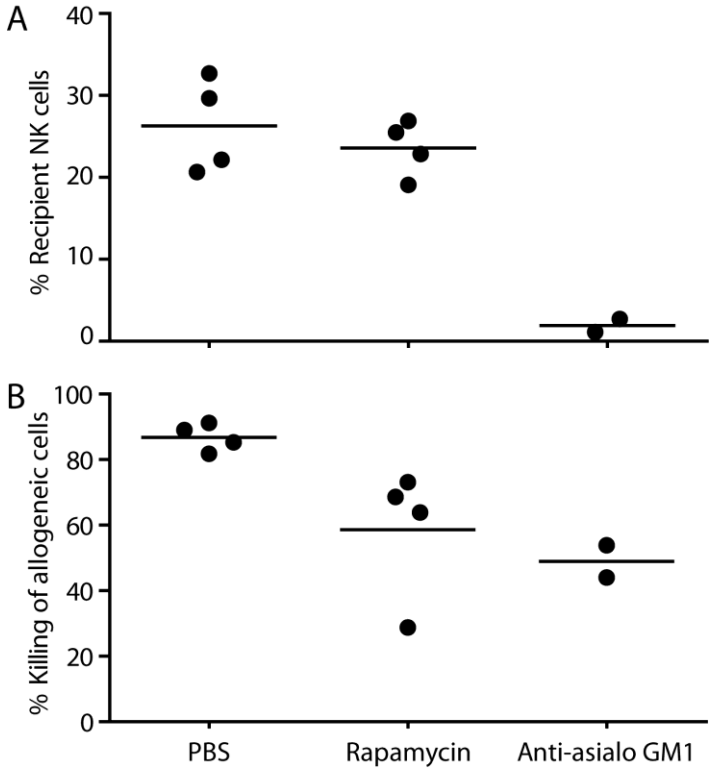

Figure 3.5 Rapamycin reduces the rejection of fully allogeneic FVB cells by NOD NK cells. NOD.RAG1-KO mice (n=2-4) were either injected with PBS, rapamycin at 3 mg/kg, or the NK cell-depleting antibody anti-asialo GM1 i.p. 3 days before receiving a 1:1 mixture of allogeneic FVB and syngeneic NOD B cells i.v. (4-5 million cells of each donor). The syngeneic B cells co-injected with allogeneic cells served as a control for the specificity of host NK cell immunity and allowed us to determine the percent allogeneic killing. (A) Flow cytometric analysis of peripheral blood on the day of B cell transfer, before B cells were injected. The proportion of recipient NK cells ($CD49b^{+}CD122^{+}TCR\beta^{-}$) was determined and shown for each mouse. (B) Three days after B cell transfer, spleens were harvested to assess target killing. The number of allogeneic or syngeneic B cells recovered from the spleen was calculated as the product of the

total cell count and the percentage of allogeneic or syngeneic B cells within the lymphoid gate (determined by flow cytometry; CD19 staining for B cells, D^q/L^q for FVB, and D^b for NOD). To calculate the percent killing of allogeneic cells, we used the following formula: [(Number of syngeneic B cells – Number of allogeneic B cells) ÷ Number of syngeneic B cells] × 100. Rapamycin-treated mice showed significantly less allogeneic killing than untreated mice ($p<0.05$, unpaired two-tailed Student's *t*-test). For both A and B, horizontal bar depicts the mean of each group.

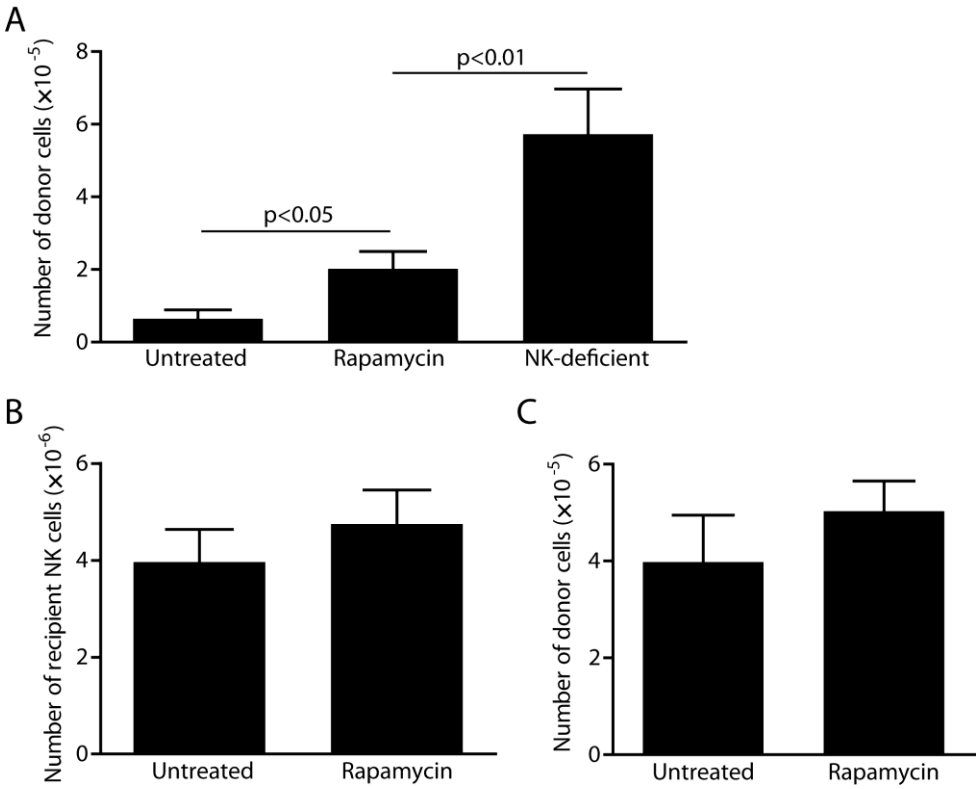

Figure 3.6 Rapamycin specifically reduces the rejection of allogeneic cells by B6 NK cells without affecting NK cell numbers. (A) B6.RAG1-KO mice were either left untreated (n=9) or injected with rapamycin, i.p. on days 0-2 (n=11). BALB/c B cells were transplanted i.v. on day 0 into these two groups, in addition to a third cohort of untreated C57BL/10-RAG1/IL-2Rγ-KO mice genetically deficient for T, B and NK cells (NK-deficient; n=9). Three days after transfer, donor B cells were quantified in the spleen. Levels of statistical significance between relevant groups are indicated. Rapamycin-treated mice contained a significantly greater number of donor cells in their spleens than untreated mice. However, donor cell recovery was significantly higher in RAG1/IL-2Rγ-KO mice than rapamycin-treated mice. (B) Recipient NK cells in the spleen were quantified on day 3. No statistical significance was found between untreated and

rapamycin-treated mice. (C) Untreated (n=9) or rapamycin-treated (n=8) B6-RAG1-KO mice expressing CD45.2 were transplanted i.v. with CD45.1 congenic B6 B cells. Three days later, donor B cells were quantified in the spleen. No statistical significance was found, indicating that rapamycin did not confer a survival advantage to the target cells independent of its effect on NK cells. In all bar graphs, mean and standard error are depicted.

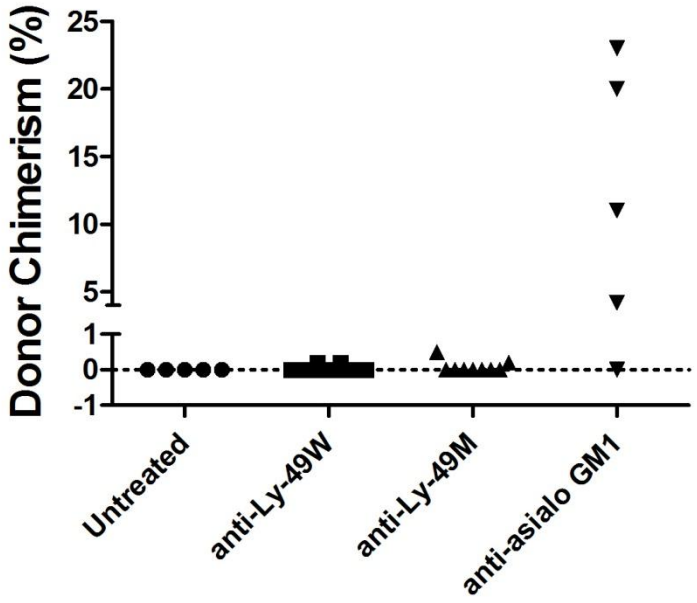

Figure 3.7. Depleting NK cells expressing Ly49W or Ly49M is unsuccessful at inducing chimerism. NOD.RAG1-KO mice were either untreated (n=5), or injected with either anti-asialo-GM1 (n=5), anti-Ly-49W (n=12), anti-Ly-49M (n=9) on days -3, 0, and 3, relative to FLC injection. One day prior to the injection of a mixture of 15 million C3H and 15 million NOD FLCs i.v. recipient mice were conditioned with BUS. Approximately 5 weeks later, peripheral blood of recipients was analyzed for the presence of donor (H-2K^{k+}) cells.

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Chapter 4: Identification and targeting of cells causing split tolerance allows fully allogeneic islet survival with minimal conditioning in NOD mixed chimeras

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4.1. INTRODUCTION

Clinical islet transplantation is a minimally invasive approach to restore functional β cell mass and obtain the benefits of normoglycemia (1). Regardless of insulin independence, islet transplantation can stabilize some secondary complications of diabetes when compared to optimal medical therapy (2,3). However, islet transplantation subjects recipients to a lifetime of immunosuppression, thereby increasing risk of infection and malignancy (4). Furthermore, there is an added challenge to the restoration of islet mass in the setting of autoimmunity; despite immunosuppressive medications, the recurrence of autoimmunity after pancreas transplant is at least 5-6% in the early posttransplant period, and may contribute to the inexorable loss of endocrine function years after transplantation (5).

Donor-specific tolerance induction, through the generation of mixed allogeneic chimerism, is one potential approach that may eliminate need for long-term immunosuppressive therapy, while also preventing chronic rejection. Owen first associated chimerism with tolerance after demonstrating fraternal cattle twins were natural chimeras and, therefore, operationally tolerant of one another (6). In mixed chimeras, tolerance involves both central (7-10) and peripheral mechanisms (11-13), however, even in the presence of chimerism, it is possible for certain donor tissues or cells to be rejected, a phenomenon known as split tolerance (14-17). In addition to its occurrence in mice, in both large animal models (18-20) and human trials (21,22) chimerism induction and solid organ grafting can be associated with split tolerance. In these cases, split tolerance

usually develops such that peripheral blood chimerism is lost, with the preservation of the solid organ graft; however, the reverse can also occur (23). Although the relevance of the maintenance of chimerism in the presence of solid organ tolerance is controversial (21,23-25), rejection of an organ allograft is clearly not desirable. Therefore, a thorough understanding of the mechanisms of tolerance operating in mixed chimerism and the subtleties of why tolerance develops towards some tissues and not others would be valuable. This knowledge would facilitate design of chimerism induction therapies that ensure the recipient will indeed be tolerant of all donor tissue.

Non-obese diabetic (NOD) mice are an autoimmune mouse strain (26) that are resistant to tolerance induction (27,28) and represent the most challenging inbred mouse model to induce chimerism using non-myeloablative protocols (14,29,30). A clinically feasible non-myeloablative protocol that generates lasting multilineage and fully allogeneic chimerism in NOD mice has yet to be achieved. We have previously developed a non-myeloablative protocol that can generate mixed allogeneic chimerism across full MHC plus minor mismatches (14,30), however, the long-lasting chimerism was not multilineage. Furthermore, in contrast to chimeric C57BL/6 mice, chimeric NOD mice demonstrated multiple levels of split tolerance, such that islets, skin, and some hematopoietic cell lineages were rejected. Understanding this autoimmune model may help identify barriers to chimerism induction that will aid in tailoring efficient, clinically relevant, non-myeloablative induction protocols capable of generating fully tolerant chimeras. In the current study, we isolated the contributions of both the

radiosensitive and radioresistant cellular compartments to the development of split tolerance. Furthermore, we found split tolerance was caused primarily by lymphocytes, and specifically T cells. After the implication of T cells in NOD resistance to tolerance, we developed a refined chimerism induction protocol based on recipient NOD T cell depletion. This potentially clinically relevant, non-myeloabalative protocol was successful at generating chimeric NOD mice that are diabetes free and accept donor islet allografts while remaining immunocompetent.

4.2. MATERIALS AND METHODS

4.2.1. Animals. Adult female B6.NOD-(*D17Mit21-D17Mit10*) mice, abbreviated here as B6.g7 (H-2^{g7}), were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Wildtype NOD (NOD.wt; H-2^{g7}), NOD mice on the recombinase activating gene (RAG) 1-knockout (KO) background, B cell deficient NOD.129S2(B6)-*Igh-6*^{tm1Cgn} (NOD.µMT) and C3H (H-2^{k}), originally from Jackson Laboratory, were bred in-house. NOD mice expressing green fluorescent protein (GFP; NOD-Tg (UBC-GFP) 30Scha), originally a gift from Dr R. Gill (Barbra Davis Center for Diabetes; University of Colorado), were bred in-house. NOD.GFP, NOD.wt and C3H day 15 gestation fetuses were generated by timed pregnancies onsite. All mice used for chimerism induction were females between 8-10 weeks of age. All care and handling of animals were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

4.2.2. Creation of lethal irradiation full bone marrow chimeras. Bone marrow cells (BMC) were isolated from the femurs and tibias of donor NOD.GFP and B6.g7 mice. Recipient NOD.GFP and B6.g7 mice were lethally irradiated with 1200 RAD in split doses over two days, and then immediately transplanted intravenously (i.v.) with $20x10^6$ unmodified BMCs from the opposite strain. Alternatively, NOD.GFP FLC→B6.7 full chimeras were generated by the i.v. injection of lethally irradiated B6.g7 mice with $20x10^6$ unmodified NOD.GFP FLC. Irradiated mice were administered TMX/SMP antibiotics in their drinking water for the duration of the experiment.

4.2.3. Induction of allogeneic non-myeloablative chimerism. Transplantation of $20x10^6$ allogeneic BMCs by a non-myeloablative protocol into NOD.wt, NOD.μMT, NOD.GFP→B6.g7 and B6.g7→NOD.GFP chimeric mice was described in depth previously (14). Briefly, recipient mice were pre-conditioned with an i.v. donor-specific transfusion of $20x10^6$ unmodified C3H spleen cells on day -7, anti-CD40 ligand (anti-CD40L; MR-1) and recombinant human Cytotoxic T-Lymphocyte-Associated Protein 4 immunoglobulin (CTLA-4-Ig) on days -7, -5 and -3 (each at 0.25 mg i.p.; Bio X Cell, West Lebanon, NH). On day -1, busulfan (BUS; GalaxoSmithKline Canada) was given i.p. at 20 mg/kg body weight. On day 0, recipient mice were transplanted i.v. with $20x10^6$ unmodified C3H BMCs, as well as given i.p. injections of, anti-CD40L, CTLA-4-Ig, and rapamycin (Rapamune®, Wyeth Canada, Montreal, QC) at 3 mg/kg diluted in phosphate-buffer saline (PBS). After transplantation, anti-CD40L and CTLA-4-

Ig were administered on days 2, 4, and 6, while rapamycin was injected daily for 28 days. Where indicated, NK cell depletion was performed with anti-asialo GM1 (Wako Chemicals USA, Richmond, VA; 35 uL diluted in PBS) administered on days -3, 0 and 3 related to the day of BMT.

Chimerism induction based on T cell depletion was performed by administering anti-CD4 (GK1.5) and anti-CD8 (53.6.7) on days -5 and -1 (each at 0.25 mg i.p.; Bio X Cell, West Lebanon, NH). BUS was administered one day before the day 0 transplantation of $20x10^6$ allogeneic C3H BMCs i.v. along with a single i.p. injection of anti-CD40L. After BMT, rapamycin was injected daily for 28 days.

To generate chimeras in immunodeficient hosts, recipient NOD.RAG1-KO mice were conditioned with BUS (day -1) and anti-asialo GM1 (days -3, 0 and 3). Mixed chimeras were generated by an i.v. injection of a 1:1 mixture of allogeneic (C3H) and syngeneic (NOD.wt) FLCs, at $15x10^6$ cells each. To generate full chimeras, recipient NOD.RAG1-KO mice were transplanted i.v. with 100 million unmodified C3H BMCs.

4.2.4. Flow cytometry. Antibodies against TCR, CD4, CD8, CD11b, CD11c, CD19, CD49b, CD122, H-2 K^k and H-2 D^b were purchased from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA). Donor C3H cells were identified by anti-H-$2K^k$ antibodies and recipient cells were identified by anti-H-$2K^d$ or GFP. Data were acquired using a LSR II (Becton Dickson, Sunnyvale,

CA) flow cytometer and analyzed with FCS Express™ (De Novo Software, Los Angeles, CA).

4.2.5. Skin transplantation. Full thickness trunk skin was transplanted onto the dorsum of recipient mice. Briefly, approximately 1cm^2 of donor skin was secured with sutures to the recipient graft bed. The skin grafts were then bandaged and left intact for seven days. The grafts were inspected daily and considered rejected at the time when >90% surface area was necrotic.

4.2.6. Islet isolation and transplantation. Islet isolation was carried out as previously described (31). Briefly, three days prior to transplantation, recipients were made diabetic by an i.p. injection of streptozotocin (Sigma-Aldrich Canada, Oakville, ON) at 185 mg/kg body weight. Diabetes was confirmed by a blood glucose measurement of >20.0 mmol/l. 500 donor islets were transplanted into the renal subcapsular space. Islet graft survival was monitored by measuring blood glucose. The graft was considered rejected when blood glucose level exceeded 15 mmol/L on two consecutive readings over two days.

4.2.7. Statistical analysis. An unpaired two-tailed Student's *t*-test was used for comparison of means between two groups and a log-rank test was used to compare survival curves. All statistical analyses were done using Prism 5 (GraphPad Software, San Diego, CA) with statistical significance defined as $p<0.05$.

4.3. RESULTS

4.3.1. NOD bone marrow has the capacity to generate split tolerance towards donor hematopoietic lineages but not donor islets. In order to isolate the effects NOD radiosensitive and radioresistant tissues have on split tolerance, reciprocal radiation bone marrow chimeras were generated between female tolerance resistant NOD.GFP and MHC matched tolerance competent B6.g7 mice. NOD.GFP mice develop diabetes with a similar incidence to NOD.wt mice (Fig. 4.1A) and demonstrate split tolerance when made chimeric with fully allogeneic C3H bone marrow transplant by our non-myeloablitive regimen (data not shown). To ensure transplanted bone marrow cells (BMC) could mature into a fully functional adaptive immune repertoire, and to decrease the chances of graft-versus-host disease, we chose to match the MHC when generating full chimeras. To this end, we used B6.g7 congenic mice, which carry the NOD MHC H-2^{g7} (I-A^{g7} I-E^{null} D^{b} K^{d}), to generate full chimeras with NOD.GFP mice. Matching MHC in full chimeras should not interfere with our assessment of split tolerance after allogeneic C3H chimerism induction as we have previously shown that the NOD split tolerance phenotype is not located within the MHC region (11). After five weeks, NOD.GFP→B6.g7 and B6.g7→NOD.GFP full chimeras were assessed for hematopoietic cell reconstitution (Fig. 4.1B and C). Although less than two percent of NOD.GFP BMC's were composed of T cells (data not shown), we were concerned that these few, pre-existing NOD.GFP derived T cells may be sufficient to cause split tolerance when transferred into B6.g7 mice.

Therefore, to ensure all NOD.GFP cells developed in the B6.g7 host, we also generated a cohort of NOD.GFP→B6.g7 chimeras using NOD.GFP day 15 gestation fetal liver cells (FLC), which have no mature T cells. The hematopoietic cells in these reconstituted B6.g7 mice were 78.6±1.4% NOD.GFP in origin. In agreement with prior studies (32), none of the NOD.GFP→B6.g7 or B6.g7→NOD.GFP full chimeras developed diabetes, however insulitis was present in the NOD.GFP→B6.g7 group (data not shown).

Using our irradiation free, co-stimulation based, non-myeloablative protocol (14), we induced allogeneic (C3H donor) hematopoietic chimerism in NOD.GFP→B6.g7, NOD.GFP FLC→B6.g7, B6.g7→NOD.GFP and in control B6.g7→B6.g7 and NOD.GFP→NOD.GFP radiation chimeras (Fig. 4.2A). In all groups, initial C3H chimerism was multi-lineage, consisting phenotypically of T ($CD4^+$ and $CD8^+$), B ($CD19^+$) and NK ($CD49b^+CD122^+$) cells, macrophages ($CD11b^+$) and dendritic cells ($CD11c^+$). When monitored long-term, the multi-lineage nature of the C3H chimerism was stable in B6.g7→NOD.GFP and B6.g7→B6.g7 groups, suggesting that these mice were tolerant of all hematopoietic lineages (data shown for CD4 T and B cells; Fig. 4.2B). This tolerance is in contrast to the split tolerance seen in NOD.GFP→NOD.GFP mice where it is especially noticeable that donor B cell frequency decreases and is lost, while CD4 T cell frequency increases (Fig. 4.2B). The NOD.GFP→B6.g7 chimeras could be divided into two sub-groups, those with high or low initial chimerism levels. Interestingly, NOD.GFP→B6.g7 mice with high C3H chimerism maintained all hematopoietic lineages; however, mice with low C3H

chimerism, demonstrated split tolerance towards donor B cells, similar to NOD.GFP→NOD.GFP mice. We then re-analyzed the B6.g7→NOD.GFP and B6.g7→B6.g7 mice with low chimerism levels and confirmed that split tolerance towards hematopoietic cells did not develop (Fig. 4.2B). Thus, split tolerance was not simply a reflection of low chimerism levels but instead additionally required hematopoietic cells to be of the NOD genetic background.

Next, we wanted to determine if the tolerance or split tolerance towards C3H hematopoitic cell progeny in NOD.GFP→B6.g7 and B6.g7→NOD.GFP chimeras extended to islet transplants. Since the C3H chimerism levels had a wide range (Fig 4.2A), we transplanted C3H islets into mice of various levels of chimerism in order to rule out any confounding effect the level of chimerism may have. As seen in Figure 4.3A, all NOD.GFP→B6.g7, B6.g7→NOD.GFP and B6.g7→B6.g7 chimeras accepted donor C3H islets long-term. In contrast, those mice that did not receive C3H bone marrow and a NOD.GFP→NOD.GFP recipient of C3H bone marrow rejected donor islets, despite the maintenance of chimerism. The quick rejection of allogeneic islets by most of the NOD.GFP→B6.g7 mice that received conditioning, but not C3H bone marrow, demonstrates that these mice are immunocompetent. Additionally, immunocompetence was demonstrated in mice that accepted C3H islets by the rejection of third party B6.RAG1-KO islets (Fig. 4.3A).

We further tested the tolerance of NOD.GFP→B6.g7, NOD.GFP FLC→B6.g7 and B6.g7→NOD.GFP radiation chimeras towards skin grafts. During the nonmyeloablative allogeneic chimerism induction protocol, we

transplanted skin from donor (C3H), syngeneic and third party mice. As expected, both the NOD.GFP→B6.g7 and B6.g7→NOD.GFP chimeras quickly rejected third party skin transplants. NOD.GFP→B6.g7 chimeras rejected donor skin transplants, indicating that the capacity for split tolerance to skin can be conferred with NOD bone marrow alone (Fig. 4.3B). This split tolerance was not due to contaminating T cells in NOD bone marrow, as NOD FLC also had the capacity to generate split tolerance to skin. The B6.g7→NOD.GFP chimeras also demonstrated split tolerance towards C3H skin grafts; however, the rate of rejection was significantly slower than the NOD.GFP→B6.g7 mice ($p=0.018$). The slower rate of rejection may be related to the higher levels of chimerism seen in the B6.g7→NOD.GFP mice (47±11) than the NOD.GFP→B6.g7 (34±4) mice. However, irrespective of the rate of rejection or chimerism level, all NOD.GFP→B6.g7 and B6.g7→NOD.GFP chimeras demonstrated split tolerance towards skin. Together these results show that both the radiosensitive and resistant components of the NOD genetic background are essential for islet rejection in mixed chimeras. In contrast, either of the NOD components is sufficient for donor skin rejection, although the radiosensitive hematopoietic cells make a greater contribution to split tolerance.

4.3.2. NOD lymphocytes developing in the presence of chimerism do not generate split tolerance. Although we have shown evidence that the split tolerance phenotype in NOD chimeras resides in both radioresistant and hematopoietic cells, we next wanted to identify the cells involved in its

generation. To isolate the effects on the generation of split tolerance of NOD lymphocytes that have developed in the presence of chimerism, immunodeficient NOD.RAG1-KO mice had their immune system reconstituted with a mixture of allogeneic C3H and syngeneic NOD.wt FLCs. Therefore, all NOD T and B cells developed in the presence of C3H chimerism. These mice remained stable mixed chimeras for greater than 20 weeks (Fig. 4.4A). Furthermore, the chimerism was multi-lineage, with no significant loss of any cell lineage (data shown for CD4 T and B cells; Fig 4.4A). The stability of the chimerism in these mice is in contrast to the split tolerance seen in NOD.wt mice where overall chimerism levels decline and CD4 T cells become the predominant cell lineage (See previous sections). Although unlikely, the maintenance of chimerism in this experiment could be due to a competitive advantage of C3H FLCs over NOD FLCs. Therefore, in an additional experiment, NOD.RAG1-KO mice were made full C3H chimeras by transplanting $40x10^6$ BMCs. These C3H→NOD.RAG1-KO full chimeras were then challenged solely with 20 million NOD.wt FLCs. Similar to the results obtained with the mixture of C3H and NOD FLCs transplanted into NOD.RAG1-KO mice, the transfer of NOD FLCs alone into C3H→NOD.RAG1-KO full chimeras generated a state of mixed chimerism with no signs of split tolerance towards hematopoietic cells (Fig. 4.4B). These data suggest that the split tolerance that NOD mice develop under non-myeloablative conditioning is generated from pre-existing, non-tolerized lymphocytes and not from cells that have developed in the presence of chimerism.

4.3.3. NOD CD4 and CD8 T cells mediate split tolerance. Since pre-existing lymphocytes appeared to be the cause of split tolerance towards C3H hematopoietic cells, we next wanted to determine the specific NOD lymphocyte lineage involved. Through modifications to the chimerism induction protocol used in the above experiments, we isolated the effects that eliminating NK, B and T cells have on split tolerance development. Beginning with NK cells, we tested whether anti-asialo GM1 treatment on days -3, 0 and +3 relative to C3H BMT converts split tolerance into full tolerance. In agreement with our previous data (30), we were able to induce chimerism at a greater frequency and level than without NK cell depletion (Fig. 4.5A). However, these chimeric mice still showed split tolerance towards both certain hematopoitic cell lineages and islet transplants (Fig. 4.5B and 4.6A). Focusing on donor hematopoietic cells, CD4 T cells had the highest frequency while B cells appeared rejected. In addition, C3H islets were rejected as quickly as in control mice. Therefore, it appears that NK cells are not central to the development of split tolerance. To isolate the effects of B cells on chimerism induction and split tolerance, we used as the recipient of our conditioning protocol and BMT, the NOD.μMT mouse that does not develop endogenous B cells (33,34). These mice became chimeric with similar frequency and levels as the NOD.wt mice. Similar to the results with NK depletion, these mice also demonstrated split tolerance towards donor hematopoietic cells (Fig. 4.5). Through a significant modification of our induction protocol, the last lymphocyte subgroup that we tested was T cells. Since T cells were being depleted, the DST and most of the costimulation blockade were omitted from the

protocol because these treatments act primarily through T cells. Similar to the results of others, we could not induce chimerism in NOD mice with CD4 or CD8 depletion alone (29). Therefore, we used a protocol that involved both CD4 and CD8 depletion on days -5 and -1 relative to BMT as well as a single dose of anti-CD40L antibody on the day of transplant. This protocol was successful at inducing C3H chimerism at a high incidence and a significantly higher level than protocols that did not include T cell depletion. None of the chimeric NOD mice developed diabetes (data not shown). The chimerism levels and donor hematopoietic cell subsets remained constant long-term (Fig 4.5B); with no trends to T cell dominance and B cell elimination. These mice were also tolerant of donor islets transplants, while retaining the ability to reject third party islets (Fig 4.6A). However, the T cell depletion protocol was insufficient to render chimeric mice tolerant towards donor skin grafts transplanted during the conditioning period (Fig 4.6B). Taken together, the elimination of NOD T cells along with a single dose of anti-CD40L antibody and a short course of rapamycin can induce chimerism with high incidence, and also render these chimeras operationally tolerant to all donor hematopoietic cells lineages and islets; however, split tolerance is still seen towards skin grafts.

4.4. DISCUSSION

Generating mixed allogeneic chimerism has long been recognized as a method of inducing operational tolerance towards allografts (35). Mixed chimerism is also clinically applicable, with trials attempting to take advantage of

the robust tolerance associated with chimerism (21,36). Additionally, in the case of transplantation for an autoimmune disease, chimerism may be able to "re-educate" the immune system to avoid recurrent autoimmunity (37). Although chimerism is usually associated with tolerance, even in the setting of mixed chimerism, rejection of allografts or components of chimerism can occur. Previously we showed that the propensity for this 'split tolerance' is greater in autoimmune-prone NOD recipients. We have chosen to investigate the mechanisms of tolerance in mixed chimerism with the aim of identifying the reasons why split tolerance occurs. Many mechanisms behind the occurrence of split tolerance have been proposed, which are reviewed in Ref (38). We believe that split tolerance may become more prevalent as chimerism induction protocols become less severe and require less recipient conditioning. Reduced conditioning leaves more of the recipient T cell repertoire intact and these cels must be made tolerant via peripheral mechanisms alone. Therefore, in the current study, our goal was to identify the cells/tissues that promote split tolerance in NOD chimeras and develop a protocol to generate fully-tolerant chimeras.

Through the use of radiation chimeras between tolerance resistant NOD.GFP and tolerance susceptible B6.g7 mice, we have shown the potential to develop split tolerance after fully allogeneic BMT has components in both the NOD radioresistant and radiosensitive cellular compartments. The radiosensitive compartment implicates NOD hematopoietic cells in the development of split tolerance, while high on the list of candidate cells in the radioresistant compartment are dermal APCs (discussed below) or thymic stromal cells. NOD

mice display defective central tolerance (39-41) (although this has been recently contested (42)) and NOD thymic epithelium is sufficient to generate autoreactive T cells (43). Therefore, despite chimerism, a defect in central tolerance may allow the export of mature T cells capable of mediating split tolerance. There is a greater contribution to the generation of split tolerance from the radiosensitive compartment of NOD mice because despite chimerism, some NOD.GFP→B6.g7 mice were capable of rejecting donor B cells and skin, whereas B6.g7→NOD.GFP chimeras only rejected donor skin (the most immunogenic tissue tested). Different from both of these groups were NOD.wt mice (14) and control NOD.GFP→NOD.GFP mice, which demonstrated split tolerance towards B cells, skin and islets. The most likely explanation for these findings is that radioresistant and radiosensitive cellular compartments have differential effects on the generation of split tolerance, and both compartments are required together for the rejection of less immunogenic tissues. Subsequent experiments implicated pre-existing lymphocytes as the cause of split tolerance, as NOD lymphocytes developing in the presence of chimerism were tolerant of donor HSCs. These data indicate that if all recipient T cells have a chance at central tolerance to the donor, then there is no split tolerance, and furthermore suggest that central tolerance is sufficiently intact in chimeric NOD mice. Consistent with a key role for central tolerance in preventing split tolerance, elimination of T, but not B or NK cells could decrease the occurrence of split tolerance after allogeneic BMT. Only if T cell depletion was part of the chimerism induction protocol were we able to generate tolerance to islets in NOD chimeras. These results indicate NOD split

tolerance is partially a result of pre-existing T cells; however, other mechanisms of split tolerance must be present as T cell depletion did not allow for tolerance towards donor skin grafts. The continual display of split tolerance towards skin demonstrates that despite the central tolerance offered by mixed chimerism, peripheral tolerance may still be overwhelmed by the presence of polymorphic tissue-specific antigens (TSA). Tolerance to TSA requires peripheral tolerance, of which there are defects in the NOD mouse (44). Although polymorphic TSAs may be present in other tissues, the high susceptibility of skin to indirect rejection (15) makes it difficult to induce tolerance towards. Therefore, we hypothesize that unless some degree of donor-recipient antigen matching is performed, there will be split tolerance towards skin grafts in mice strains with defects in peripheral tolerance.

NOD T cells are known to resist tolerance induction (27,28,45) and may therefore resist both central (7-9) and peripheral (11,12,46) tolerance mechanisms acting in mixed chimeras. These non-tolerized T cells can then mediate split tolerance through the recognition of polymorphic TSAs (47) or against targets that have a susceptibility to indirect rejection (15). T cells are indeed capable of generating split tolerance as previous work in our lab has shown that monoclonal CD4 T cells alone are able to eliminate certain allogeneic targets such as B cells, but not others (15), a split tolerance remarkably similar to that seen in NOD mice. Although we have identified T cells as a major contributor to the development of split tolerance in NOD mice, T cells must be activated by antigen presented on APCs. NOD APCs display a defect in the ability to induce peripheral tolerance

(related to a functional deficiency in both T regs and dendritic cells) (44) which may be important in the development of full versus split tolerance after co-stimulation-based chimerism induction protocols. Although chimerism takes advantage of central tolerance (7-9), with co-stimulatory-based induction protocols, more of the recipient cellular compartment is left intact and must be subject to peripheral tolerance mechanisms (11,12,46). Dermal APCs are resistant to radiation (48); therefore, NOD APCs may indeed be the radioresistant cell responsible for the development of split tolerance towards skin in B6.g7→NOD GFP chimeras. If this is the case, split tolerance may be solely due to NOD hematopoietic cells (T cells and APCs), as a limitation of our radiation chimera approach is an inability to remove all hematopoietic derived cells.

In our study, we found that the depletion of CD4 and CD8 cells along with a single dose of anti-CD40L were necessary to induce chimerism and long-term acceptance of all hematopoietic cell lineages and islets. Experiments preformed with only CD8 depletion or CD4 depletion were unsuccessful at inducing chimerism. Similarly, studies where we omitted anti-CD40L were unsuccessful at inducing chimerism (data not shown). Perhaps anti-CD40L is required in this protocol to tolerize any non-deleted T cells as our data suggest pre-existing T cells are primarily responsible for the development of split tolerance in NOD mice. Previously, others have found 3Gy TBI, anti-CD8 and anti-CD40L without CD4 depletion could induce chimerism in 50% of mice and make them tolerant towards skin grafts (29). In this case, they concluded the effect of anti-CD40L is to tolerize pre-existing CD4 T cells. The mice in this experiment appeared fully

tolerant; however, the donor-recipient strain combinations were partially MHC matched, a situation that may reduce the occurrence of split tolerance (47). In contrast, we have used the fully mismatched C3H strain as a donor, and both NOD T and NK cells act as potent barriers to successful BMT in this combination (30). The requirement of anti-CD40L in our experiments argues against the previous interpretation that NOD mice resist costimulation-based tolerance inducing therapy (27,49). Upon review of these previous studies, NOD resistance to tolerance induction may be instead due to a resistance of the DST aspect of these protocols; as there was no difference in allogeneic skin graft survival given to NOD and B6 mice when DST was omitted and only anti-CD40L administered (49). Although chimerism induction protocols that involve T cell depletion are currently more clinically relevant (21) than the use of anti-CD40L (50,51), the potential to avoid split tolerance and powerful tolerizing effect of interrupting the CD40-CD40L pathway cannot be ignored. As such, novel co-stimulation blockade therapies using anti-CD40 are being tried with success at inducing chimerism (52,53). It will be of interest to see if the eventual loss of chimerism seen in this protocol could be avoided if some amount of T cell depletion is performed.

Aside from being successful at generating chimerism across full MHC barriers in NOD mice, which are both autoimmune and have a resistance to tolerance induction, the chimerism induction protocol we used, based on T cell depletion, has significant advantages over other approaches. First, the protocol is irradiation-free, minimalistic and clinically feasible. T cell depletion and

rapamycin are currently used for solid organ transplant (54,55), and busulfan has been used in human patients (56,57) and in non-human primate bone marrow transplant recipients (53). While anti-CD40L is not available clinically due to risk of thromboembolic complications, alternative promising approaches with co-stimulatory blockade are being developed (52,53). Second, our protocol generates mixed chimerism across fully mismatched barriers and autoimmune barriers. Many other protocols for generating chimerism were only tested in the setting of some degree of MHC matching between donor and recipient (29,58,59); a combination that is less clinically feasible. Although there has been previous successes at generating chimerism in NOD mice using fully mismatched donor-recipient strains, there was a tendancy for chimerism to become full (60), or eventually lost if not induced with mega-doses of bone marrow and the infusion of donor CD8 T cells (61). Third, the chimerism obtained with our protocol is multi-lineage and stable long-term. Although the clinical relation of sustained chimerism to solid organ tolerance is controversial, and often associated with split tolerance (21,22), we have shown long-term acceptance in NOD mice of both allogenic hematopoietic cells and islet grafts.

We have identified a potentially clinically relevant induction protocol based on T cell depletion that generates NOD mixed chimeras and have also implicated pre-existing T cells as a major cause of split tolerance in the NOD mice. The need to overcome additional factors that enhance the likelihood of split tolerance, such as transplantation into highly sensitized recipients, can be anticipated.

4.5. AKNOWLEDGEMENTS

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4.6. AUTHOR'S CONTRIBUTIONS

DPA participated in research design, performance of research, data analysis and writing the paper. RP and AMJS participated in performance of research. CCA participated in research design, data analysis and writing the paper.

4.7. FIGURES

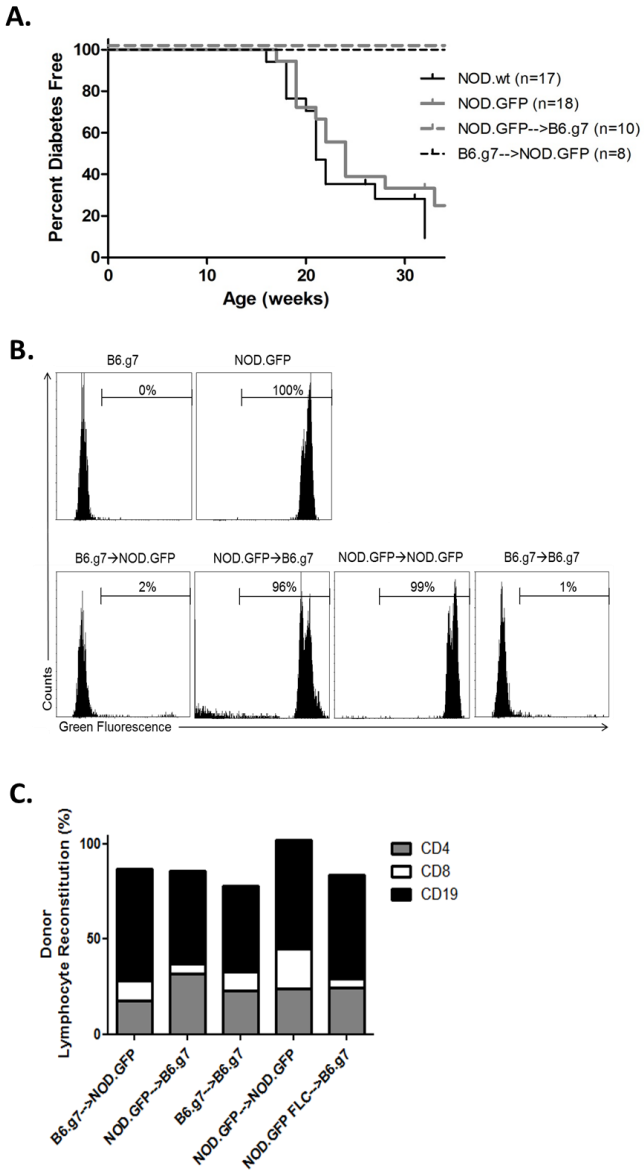

Figure 4.1. Characterization of NOD.GFP→B6.g7 and B6.g7→NOD.GFP radiation chimeras. **A.** Diabetes incidence of NOD.wt, NOD.GFP, and radiation

chimeras. **B.** After five weeks, to allow for hematopoietic cell reconstitution, peripheral blood of full chimeras was assessed by flow cytometry. Full chimerism was assessed in NOD.GFP→B6.g7 and B6.g7→NOD.GFP mice by gating on peripheral blood live cells and determining the expression of GFP. Lymphocytes of reconstituted B6.g7 mice were 94.0±1.0% NOD.GFP in origin, and reconstituted NOD.GFP mice were 96.3±0.6% B6.g7 in origin. **C.** Donor lymphocyte gated CD4 T, CD8 T and B cell frequency in peripheral blood at five weeks post-radiation and BMT.

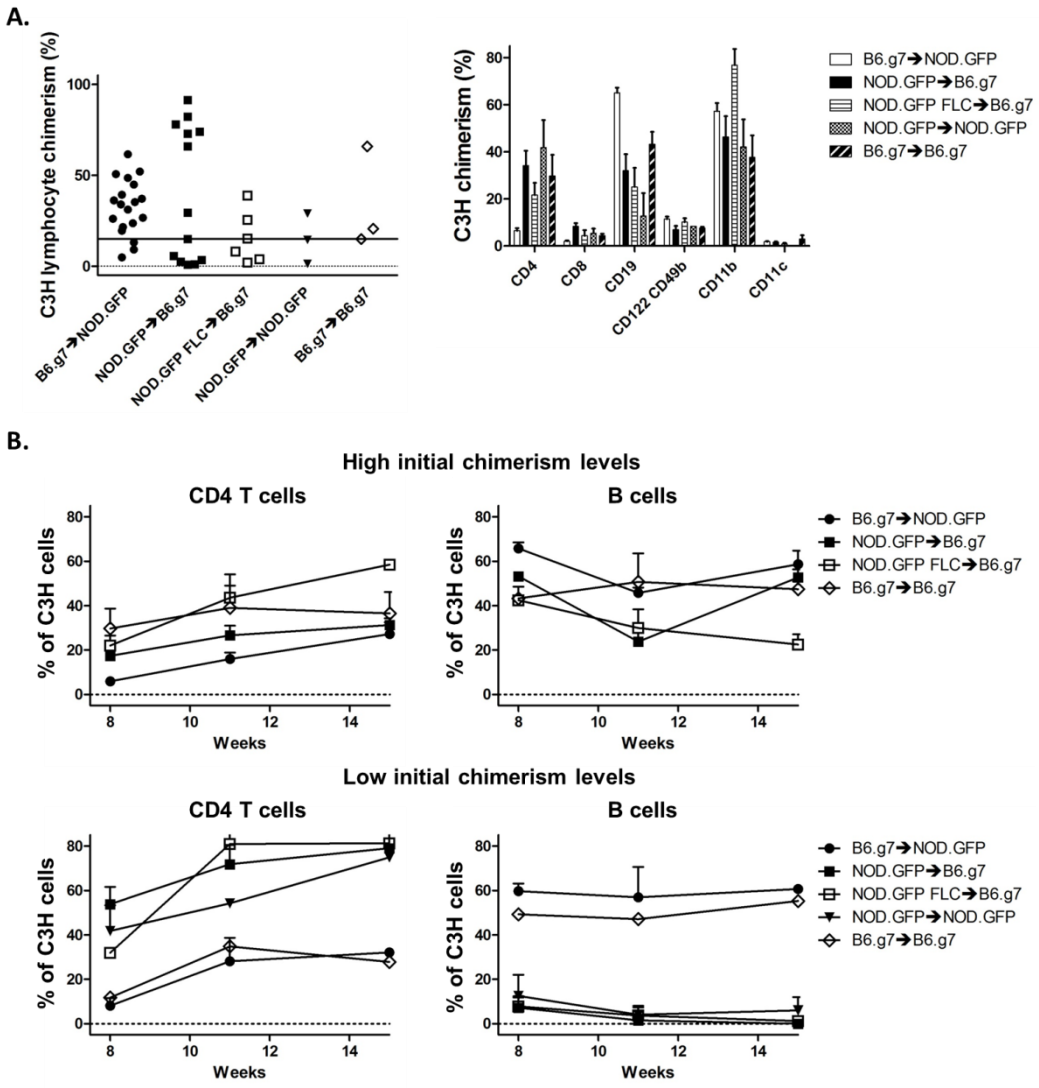

Figure 4.2. Split tolerance develops towards progeny of HSCs only in NOD.GFP→B6.g7 mice with low C3H chimerism levels. The full chimeras (donor and host as indicated) were created by lethal irradiation and BMT. 1-3 weeks after full chimerism was confirmed they were given non-myeloablative conditioning and a C3H BMT. **A.** *Left,* Eight weeks post-BMT, peripheral blood C3H chimerism was assessed. Mixed chimerism was generated in 19/19 B6.g7→NOD.GFP, 13/15 NOD.GFP→B6.g7 (this group generated mice with either high or low chimerism levels; above or below solid line at 15%,

respectively), 6/6 NOD.GFP FLC→B6.g7, 3/3 B6.g7→B6.g7 and 3/5 NOD.GFP→NOD.GFP mice. *Right,* In all chimeric animals, peripheral blood was analyzed by flow cytometry for the presence of multilineage C3H chimerism. Total chimerism levels, CD4, CD8, CD19 and CD49bCD122 cell proportions are taken from the lymphocyte gate, whereas CD11b and CD11c proportions are taken from the live cell gate. **B.** Long-term monitoring of C3H CD4 T and B cell chimerism. *Top panels,* Mice that had high (>15%) initial chimerism levels and *bottom panels,* mice that had low (<15%) initial chimerism levels. 15% was chosen as the cut-off because no mice with greater than this level of chimerism in the NOD.GFP→B6.g7 group showed evidence of split tolerance.

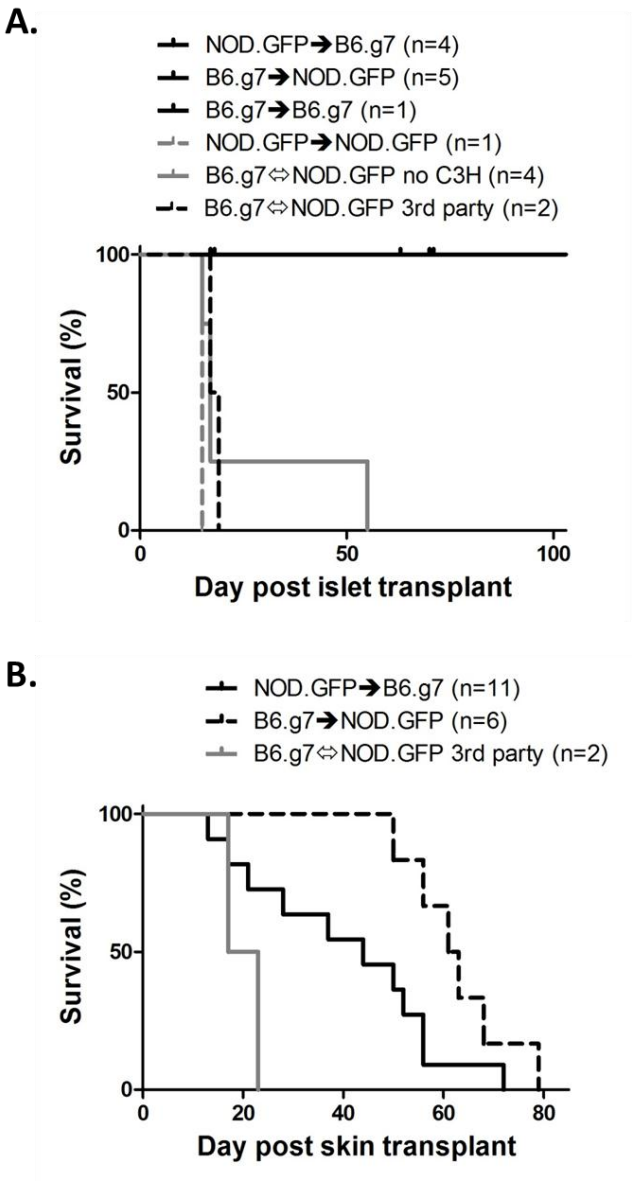

Figure 4.3. NOD.GFP→B6.g7 and B6.g7→NOD.GFP chimeras accept donor C3H islets but are split tolerant towards C3H skin grafts. A. Survival of islet transplants. Between 10 to 12 weeks after conditioning and C3H BMT, NOD.GFP→B6.g7, B6.g7→NOD.GFP, B6.g7→B6.g7 and NOD.GFP→NOD.GFP chimeric mice were made diabetic with streptozotocin

and then given C3H islet transplants. As controls, NOD.GFP→B6.g7 and B6.g7→NOD.GFP mice that received conditioning, but no C3H BMT, were similarly given a C3H islet transplant. The outcome of these groups were similar and combined as "NOD.GFP⇔B6.g7 no C3H". After greater than 100 days, a donor nephrectomy was performed to ensure the recurrence of hyperglycemia and for islet histological analysis (data not shown). After donor nephrectomy, some mice in each group were then transplanted with third party (B6.RAG1-KO) islets. The outcome of these groups were similar and combined as "NOD.GFP⇔B6.g7 3rd party". **B.** Skin was transplanted onto NOD.GFP→B6.g7, NOD.GFP FLC→B6.g7, and B6.g7→NOD.GFP mice during the nonmyeloablative allogeneic chimerism induction protocol. As both the NOD.GFP→B6.g7 (n=7) and NOD.GFP FLC→B6.g7 (n=4) groups rejected donor skin transplants with similar kinetics, these groups were combined (labeled as NOD.GFP→B6.g7). Skin survival was significantly different between NOD.GFP→B6.g7 and B6.g7→NOD.GFP ($p=0.018$). Some NOD.GFP→B6.g7 and B6.g7→NOD.GFP chimeras also received a third party (B6.RAG1-KO) skin transplant. The outcome of these groups were similar and combined as "NOD.GFP⇔B6.g7 3rd party".

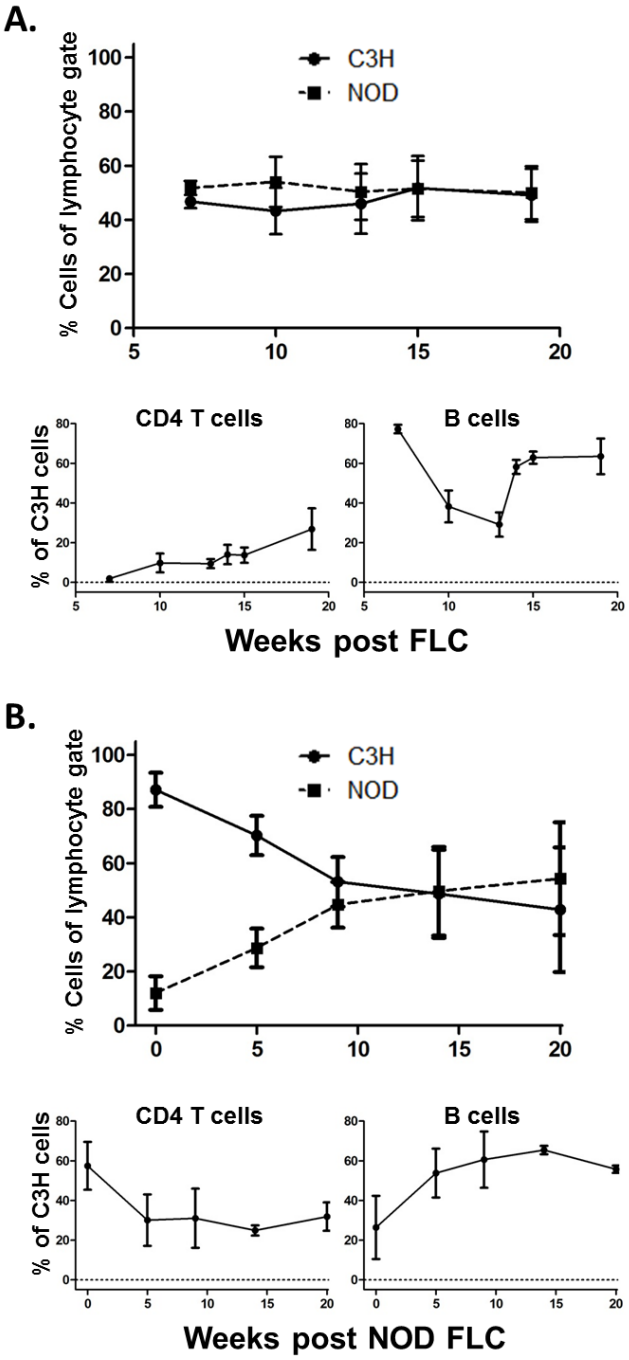

Figure 4.4. Stable mixed chimerism develops in NOD.RAG1-KO mice when recipient lymphocytes develop in the presence of allogeneic cells. **A.** *Top,* Peripheral blood was assessed for the presence of allogeneic and syngeneic

lymphocytes in immunodeficient NOD.RAG1-KO mice that had their immune system reconstituted with a mixture of allogeneic C3H and syngeneic NOD.wt FLCs. *Bottom,* Long term-monitoring of donor lymphocyte gated CD4 T and B cells in these chimeric mice. **B.** *Top*, C3H→NOD.RAG1-KO full chimeras were injected with 20 million NOD.wt FLCs (arrow indicates time of injection) and had their peripheral blood monitored long-term for the presence of allogeneic and syngeneic lymphocytes. *Bottom,* Long term-monitoring of donor lymphocyte gated CD4 T and B cells in these chimeric mice.

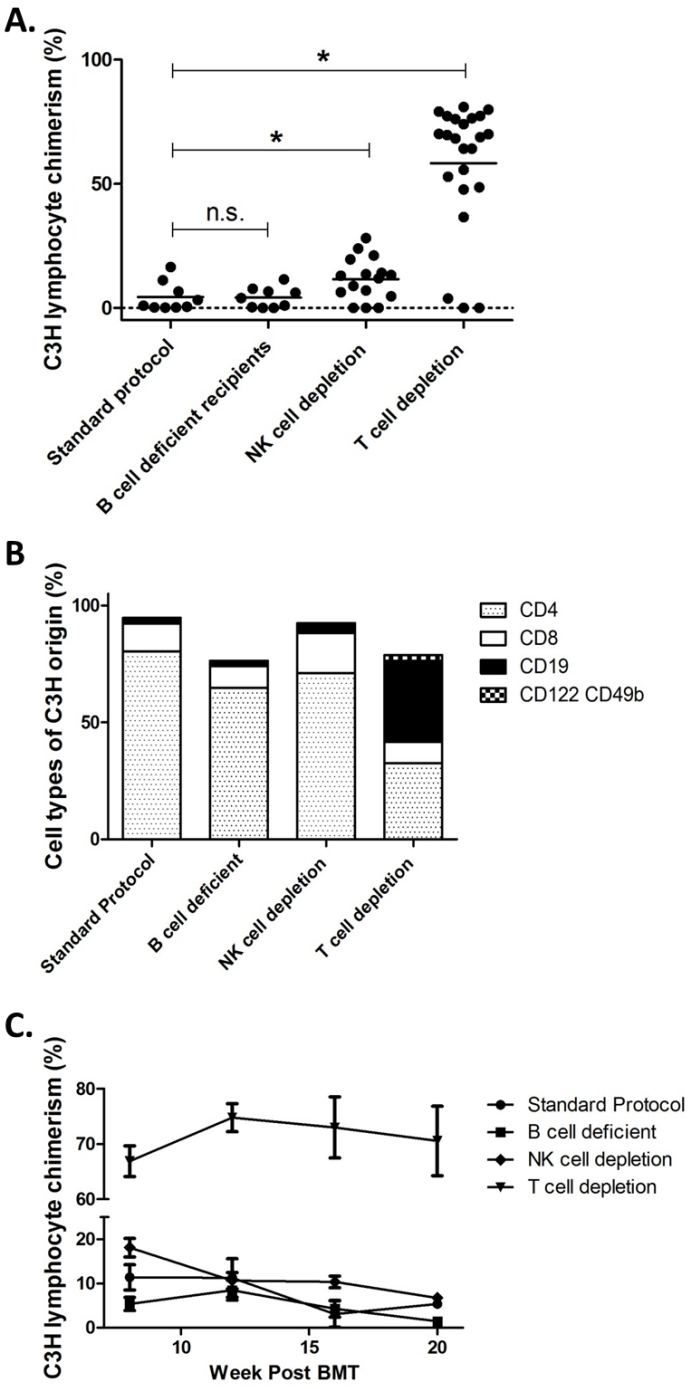

Figure 4.5. NOD T cells resist chimerism induction and cause split tolerance.

A. C3H chimerism level in peripheral blood at eight weeks post-BMT. Wildtype

NOD and B cell deficient NOD mice treated with a non-myeloablative, co-stimulation based chimerism induction protocol prior to the transplantation of 20 million C3H BMCs. Other groups of mice received a modified chimerism induction protocol that included NK or T cell depletion. **B.** At 20 weeks post-BMT, peripheral blood was analyzed for donor lymphocyte composition by flow cytometry. **C.** Peripheral blood was monitored over time for the presence of allogeneic lymphocytes. The mean and standard error was calculated by including only mice that had chimerism levels above zero for each chimerism induction protocol. Asterisks denote statistical significance.

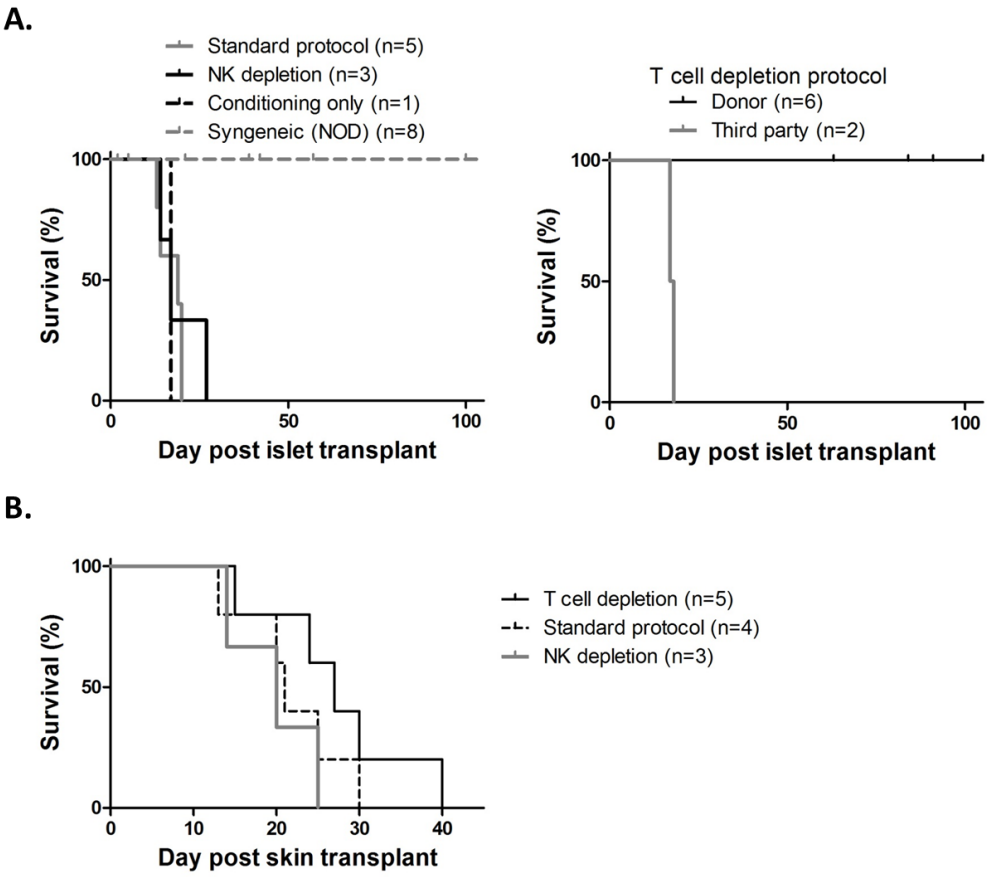

Figure 4.6. Chimerism induction that includes T cell depletion allows tolerance to donor islets. A. At 10-12 weeks post-BMT, chimeric mice were treated with streptozotocin to induce diabetes. ***Left*** Once diabetic, chimeric mice generated by either our co-stimulation blockade-based protocol or a protocol that also included NK depletion were transplanted with donor (C3H) islets. As a control, a single mouse treated with co-stimulation blockade that did not receive a BMT was also transplanted with C3H islets. After the C3H islets were rejected, syngeneic (NOD) islets were transplanted into these chimeric mice. ***Right*** After diabetes induction, mice made chimeric via the T cell depletion protocol received

either donor (C3H) or third party (B6.RAG-KO) islets. **B.** During BMT conditioning, some mice in each protocol group received donor (C3H) skin grafts.

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Chapter 5: Indirect allograft rejection by CD8 T cells requires MHC class I expression on radiosensitive cells

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5.1. INTRODUCTION

Immune recognition of allografts via the indirect pathway is an important contributor to graft rejection (1). Although direct recognition of allografts is thought to be the dominant mechanism in acute allograft rejection (2), it is believed that indirect recognition is a major cause of chronic rejection (3,4). Direct activation of T cells occurs when these cells recognize intact donor major histocompatability complex (MHC) on donor antigen presenting cells. Indirect activation of recipient T cells (cross-priming) occurs when alloantigens are processed and presented in the context of recipient MHC class I or II. Both CD4 and CD8 T cells are capable of being activated and effecting graft rejection via the indirect pathway (5,6). However, alloantigen recognition and rejection via the indirect pathway requires further explanation. This is because of the question of how a CD8 T cell that is indirectly primed by specifically recognizing its cognate foreign antigen in the context of self-MHC generates a cytotoxic response to that foreign antigen on an allograft (where it would be presented in the context of non-self-MHC). This rejection may be due to a degenerate effector response by the indirectly activated CD8 T cells whereby they can recognize foreign antigen even in the context of non-self MHC. However, this would break the rules of MHC restriction when donor and recipient MHC are mismatched, making this possible mechanism unlikely to have a large role. Instead, the mechanism of rejection may be a result of indirectly activated CD8 T cells expressing death inducing ligands and inflammatory cytokines near the allograft site causing tissue destruction. However, this situation would lead to non-specific tissue destruction by cytotoxic

T cells in the presence of the allograft with potentially deleterious effects on surrounding healthy host tissues.

In an attempt to solve the question of how indirectly primed CD8 T cells mediate an effector response, T cell receptor (TCR)-transgenic mice have been used to eliminate direct reactivity. In these experiments, it was shown that female Matahari T cells (TCR-transgenic CD8 T cell population that specifically recognizes the male H-Y antigen in the context of H-2D^{b}) can indirectly reject male allogeneic skin grafts by a mechanism dependent on interferon-γ (6). Furthermore, it was concluded that H-Y antigen must be processed and presented in the context of MHC class I on recipient vascular endothelium in order for indirectly primed CD8 T cells to be effectors in graft rejection. In this way, the graft would be deprived of blood supply, resulting in loss of viability. Therefore, this model predicted that skin allografts will be susceptible to indirect rejection because of the nature of its post-transplant neo-vascular blood supply (recipient derived). It also predicted that heart allografts would not be susceptible to indirect rejection because the post-transplant blood supply of these grafts is predominately donor derived. Indeed, experimental evidence with non-vascularized skin and vascularized heart allografts supported this conclusion. However, no direct *in vivo* experiment was presented in this study to show that MHC class I expression on recipient-derived vascular endothelium was actually required for indirect rejection (6). Therefore, it is possible that other mechanisms are responsible for indirect CD8 rejection *in vivo*. In addition, the proposed model suggested that other allografts with a recipient derived blood supply (such

as islet allografts) would be susceptible to indirect rejection. Although Valujskikh *et al*. have demonstrated a novel pathway of destruction of grafts by effector T cells, it is not clear whether this mechanism occurs *in vivo* or if it is generalizable to other cellular and solid grafts.

Our research interests into the mechanisms of split tolerance have shown that allografts can exhibit a differential susceptibility to indirect rejection, which can be a contributing factor in this phenomenon (7). Specifically, our previous studies show that allogeneic islets, skin and B cells are susceptible to indirect rejection by CD4 T cells; however, allogeneic T cells were not rejected by this mechanism. Recognizing the importance of CD8 T cells in allograft rejection and its potential role in split tolerance, we wanted to investigate the mechanisms of indirect CD8 effector mechanisms. Furthermore, we wanted to determine if the observed differential susceptibility of allografts to indirect rejection by CD4 T cells could be extrapolated to CD8 T cells. In the current study, using the Matahari TCR-transgenic mouse model, we demonstrate that CD8 indirect rejection occurs for skin but not for islet or hearts given as non-vascularized grafts; indirect CD8 mediated rejection was not universally capable of rejection of non-vascularized grafts. We show that prior priming of Matahari T cells or transplant location does not alter the susceptibility of a non-vascularized allograft to indirect rejection. Lastly, in a direct test of the requirement for cross-presentation on host radioresistant cells such as endothelial cells, we show that MHC class I expression is not necessary on recipient radioresistant tissues for CD8 mediated indirect rejection; however, MHC class I is required on recipient

radiosensitive cells in order for indirect rejection to occur. Taken together, our results suggest that the cross-presentation of donor antigen on recipient derived neovascularization of non-vascularized allografts cannot be the primary explanation for indirect rejection. Instead, our data suggests that cross-presentation by host APCs underlies CD8 mediated indirect rejection.

5.2. MATERIALS AND METHODS

5.2.1. Animals. Adult C3H (H-2^k), C57BL/6 (B6; H-2^b) and B10.BR (H-2^k) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and Charles River Canada (Saint-Constant, QC, Canada). Adult CBA mice (H-2^k) were obtained from NCI Frederick (Frederick, MD, USA). B6 mice on the recombinase activating gene (RAG)-1 knockout background (B6.RAG1-KO) and B6 mice expressing green fluorescent protein (B6-Tg (UBC-GFP) 30Scha/J), originally from Jackson Laboratory, were bred in-house. B6.RAG1-KO.$K^{b-/-}$ $D^{b-/-}$ mice (referred to hereafter as B6.RAG1-KO Class I-KO) were generated by crossing B6.RAG1-KO with B6.H-$2K^{btm1}$-H-$2D^{btm1}$N12 (from the NIAID Exchange Program, NIH: 004215; B6.RAG1-KO Class I-KO) (8). B6.RAG1-KO GFP mice were generated by crossing B6.RAG1-KO with B6.GFP mice described above (B6.RAG1-KO GFP). Female Matahari mice (6) on the B6.RAG1-KO background were bred at University of Alberta. This study was approved by the institutional review board at the University of Alberta. All care and handling of

animals were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

5.2.2. Skin Transplantation. Full thickness trunk skin was transplanted onto the dorsum of recipient mice. Briefly, 1cm^2 of donor skin was cleared of all lose areolar subcutaneous tissue and secured with sutures to the recipient graft bed. The skin grafts were then bandaged and left for seven days. Skin grafts were considered rejected at the time when >90% surface area was necrotic. In representative animals, histological analysis was performed on rejected or surviving skin grafts.

5.2.3. Islet isolation and transplantation. Islet isolation was carried out as previously described (9). Briefly, three days prior to transplantation, recipients were made diabetic by a single i.p. injection of streptozotocin (Sigma-Aldrich Canada, Oakville, ON) at 185 mg/kg body weight. Diabetes was confirmed by a blood glucose measurement of >20.0 mmol/L. Islet graft survival was monitored by assessing blood glucose level, with the graft considered rejected when blood glucose level exceeded 15 mmol/L on two consecutive readings over two days. In some experiments, recipient female Matahari mice were primed with a single i.p. injection five million irradiated B6.RAG1-KO splenocytes five days prior to islet transplantation.

5.2.4. Heterotopic Neonatal Heart Transplantation. Hearts from <36 hour old male or female CBA, B6.RAG1-KO or C3H neonates were transplanted either under the skin of the ear pinna or under the renal capsule of female Matahari recipients. Briefly, hearts from donor mice were procured and one ventricle was cut in half such that the donor graft was approximately three quarters of a heart. The operative procedure for recipients of an intra-abdominal heart graft was similar to the islet transplantation, with the neonatal heart being placed in renal the subcapsular space. For ear grafts, recipient mice were anesthetized with isofluorane and the dorsum of the ear was cleaned with 70% ethanol. A 2-mm slit was made on the skin of the ear in such a way that only epidermis and dermis were penetrated but not cartilage. A small pocket of 2- to 3-mm diameter was made with curved forceps. The prepared heart was then inserted into the recipient ear pocket with no suturing or vascular anastomosis. Excess liquid and air was removed from the ear pocket with sterile cotton tips. After 70 or 100 days, survival of heart tissue was assessed by visually inspecting the graft for the presence of muscle contractions. In addition, grafts under both the renal capsule and ear pinna were assessed histologically using hematoxylin and eosin as well as Masson's trichrome staining.

5.2.5. Creation of differential MHC I expressing bone marrow chimeras with a monoclonal CD8 T cell population. To generate mice with MHC class I expression exclusively on either radiosensitive or on radioresistant tissues, reciprocal bone marrow chimeras were generated between female B6.RAG1-KO

Class I-KO mice and female B6.RAG1-KO GFP mice. Bone marrow cells (BMC) were isolated from the femurs and tibias of donor mice by crushing the bone with a sterile mortar and pestle and then passing the suspension through a 70μm nylon cell strainer (BD Biosciences; Bedford, MA). Single cell suspensions were then counted using trypan blue exclusion. Recipient mice were lethally irradiated with 1200 RAD in split doses over two days, and then immediately transplanted intravenously (i.v.) with 20 million unmodified BMCs from the opposite strain. Irradiated mice were administered TMX/SMP (Strathcona Prescription Centre Pharmacy, Edmonton, AB) in their drinking water for the duration of the experiment. Five weeks later, full chimerism was assessed by flow cytometry. At this time, female Matahari mice were primed with a single i.p. injection of five million irradiated B6.RAG1-KO male splenocytes. Five days later, splenocytes were isolated from these primed Matahari mice and were FACS sorted for T cells ($CD8^+TCR^+$). 2.5-7.5 x 10^5 sorted Matahari T cells were then injected i.v. into the B6.RAG1-KO Class I-KO→B6.RAG1-KO GFP or B6.RAG1-KO GFP→ B6.RAG1-KO Class I-KO full bone marrow chimeras. The presence of Matahari T cells was confirmed in the recipients by flow cytometry (data not shown).

5.2.6. Flow cytometry and cell sorting. Matahari T cells were detected and sorted as $CD8^+TCR^+$ cells using an Influx^{TM} cell sorter (Becton Dickson). Post-sort T cell purity was 85-95%. Full chimerism was assessed in immunodeficient B6.RAG1-KO Class I-KO→B6.RAG1-KO GFP or B6.RAG1-KO GFP→

B6.RAG1-KO Class I-KO mice 5 weeks after BMC transplantation by gating on live monocytes expressing $CD11b^+$ and determining the expression of GFP and MHC class I proteins, K^b and D^b. Antibodies against TCR, CD8, CD11b, MHC K^b and MHC D^b were purchased from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA). Data was acquired using an LSR II (Becton Dickson, Sunnyvale, CA) flow cytometer and analyzed with FCS Express™ (De Novo Software, Los Angeles, CA).

5.3 RESULTS

5.3.1. Matahari mice cannot reject islets through an indirect pathway

Female Matahari are a TCR transgenic mouse strain which have a monoclonal population of CD8 T cells specific for the WMHHNMDLI peptide of male antigen (H-Y) in the context of MHC D^b (10). Previously, it has been shown that MataHari mice are capable of rejecting non-vascularized skin grafts via the indirect pathway (6). We hypothesized that a primary factor controlling the potency of the indirect rejection response is the level of indirect presentation of donor antigens by recipient APCs within the graft. To begin to test whether our hypothesis or the currently accepted alternative, that the vascularized vs. non-vascularized nature of donor grafts is the key determinant of indirect rejection, we assessed indirect rejection of islet grafts. Islet grafts, like skin grafts, are non-vascularized (i.e. are revascularized by neoangiogenesis). Consistent with previous studies (6), we found that MataHari female mice can indirectly reject

male C3H skin grafts (Table 1). However, we found the monoclonal CD8 T cells of the MataHari mouse were incapable of rejecting male C3H islet transplants via the indirect pathway (Table 1). This indirect rejection deficiency occurs despite displaying an ability to directly reject donor male B6.RAG1-KO islets in an extraordinarily rapid fashion. In order to confirm the finding that MataHari cannot reject islets solely via the indirect pathway, we transplanted these mice with male CBA, B10.BR and B6.RAG1-KO Class I-KO islets. In each case, the islet grafts survived for greater than 100 days (Table 1) and hyperglycemia was confirmed upon donor nephrectomy (data not shown). We also tested whether the acceptance of male C3H islets was due to lack of indirect priming. In order to rule out a deficiency in T cell priming, we tested whether pre-immunization of the female MataHari mice with male antigen prior to islet transplantation would allow for indirect rejection of islet grafts. Immunization with five million male splenocytes was previously determined to be adequate for Matahari priming (RT unpublished results). However, despite priming, all female MataHari recipients failed to indirectly reject C3H islet grafts. Therefore, even with priming to male antigen, MataHari CD8 T cells are unable to reject MHC mismatched or MHC deficient islets via the indirect pathway.

5.3.2. Altering antigen location does not result in susceptibility to indirect rejection

One potential explanation for the differential ability of Matahari mice to indirectly reject non-vascularized grafts is due to the location of the donor tissue (i.e. skin in

the case of skin grafts vs. renal subcapsular in the case of islets). In order to test if non-vascularized allograft location influences indirect rejection by CD8 cells, we transplanted Matahari mice with non-vascularized neonatal heart grafts either in proximity to the skin, under the ear pinna (11), or under the kidney capsule (12). When transplanted under the ear, 3/4 male C3H and 3/4 male CBA heart grafts showed visual signs of muscle contraction at 70 or 100 days post-transplant. In contrast, 0/5 male B6.RAG1-KO heart grafts could be seen contracting, even when observed early post-transplant (day 35). Half of the heart grafts from female C3H (n=4) or B6.RAG1-KO (n=4) could be seen contracting at 70 days. Histological analysis demonstrated well organized cardiac myocytes with no lymphocytic infiltration in all male CBA and C3H and female C3H and B6.RAG1-KO neonatal heart grafts placed under the ear pinna (Figure 1A). In contrast, male B6.RAG1-KO grafts showed extensive fibrosis, such that no cardiac muscle fibers could be visualized. Results of neonatal heart grafts placed under the kidney capsule were similar; at 100 days, contractions were seen in male CBA (n=4/4), but not male B6.RAG1-KO grafts (n=0/7). Histological analysis of male CBA and female C3H heart grafts showed intact cardiac myocytes (Figure 1B). After retrieval of the kidney bearing the control male B6.RAG1-KO graft, neonatal heart was not seen on gross pathology or histological sections. We assumed that similar to the quick rejection of B6.RAG1-KO islets, these heat grafts were completely destroyed by the time the grafts were removed at 100 days. Therefore, we removed some grafts at 30 days post-transplant; however, the grafts could still not be seen, likely a reflection of

rapid graft destruction. The long-term survival of male C3H neonatal heart grafts suggest that transplant location is unlikely to be the major determining factor in the ability of MataHari mice to reject a non-vascularized graft via the indirect pathway.

5.3.3. MHC Class I is required on radiosensitive cells but not on recipient derived vascular endothelium for indirect rejection by CD8 T cells

To directly test the concept that indirect rejection by CD8s is due to cross-presentation of donor antigen on radioresistant host vascular endothelium we examined the requirement for MHC class I expression by radiosensitive cells (such as APCs) or radioresistant cells (such as vascular endothelium). We generated reciprocal radiation chimeras between B6.RAG1-KO GFP and B6.RAG1-KO Class I-KO mice and then transferred primed and sorted MataHari T cells. After transfer of MataHari T cells into either B6.RAG1-KO Class I-KO→B6.RAG1-KO GFP or B6.RAG1-KO GFP→B6.RAG1-KO Class I-KO chimeras, these mice were challenged with male or female skin grafts from C3H or B6.RAG1-KO donors (figure 2). Chimeras lacking MHC class I on radiosensitive cells accepted male C3H skin grafts for greater than 80 days (n=1) or 100 days (n=2) (Figure 3A). Histological analysis of the accepted C3H grafts demonstrates some dermal fibrosis but preserved hair follicles and sebaceous glands (Figure 3B). These mice also accepted donor female B6.RAG1-KO skin for greater than 100 days; however, male B6.RAG1-KO skin was quickly rejected (rejected on days 16, 17, and 37; n=3), characterized by significant dermal and

subdermal fibrosis. These data suggest that MHC class I is required on radiosensitive cells in order for CD8 mediated indirect rejection. The lack of rejection in this experiment is not due to incomplete priming of MataHari T cells, as our method of priming these T cells prior to transfer has previously been determined to be efficient (RT unpublished observations). In contrast to the results above, B6.RAG1-KO GFP→B6.RAG1-KO Class I-KO chimeras (lacking Class I on redioresistant cells) were capable of rejecting both male C3H and B6.RAG1-KO skin grafts. Although the rejection of male B6.RAG1-KO skin occurred much faster (rejected on day 18 and 22; n=2), male C3H skin was eventually rejected. The rejected male C3H skin graft began showing visual signs of dermatitis, hair loss and contraction on day 80 and was completely inflamed by day 100 (n=1). Histological analysis revealed evidence of rejection with dermal fibrosis, loss of hair follicles and sebaceous glands (Figure 3). When compared to male C3H grafts that were not rejected from the B6.RAG1-KO Class I-KO→B6.RAG1-KO GFP group, male C3H skin in the B6.RAG1-KO GFP→ B6.RAG1-KO Class I-KO group was fibrotic and lacked subdermal architecture (Figure 3B). As a positive control, B6.RAG1-KO GFP→B6.RAG1-KO GFP chimeras that had MHC class I present on all tissues were able to reject both C3H (rejected on day 55; n=1) and B6.RAG1-KO (rejected on day 16 and 17; n=2) male skin grafts. Although a range of Matahari T cells were transferred into the chimeras, in each case immunocompetence of the T cells was demonstrated by the direct rejection of B6-RAG1-KO skin grafts. As negative controls, some B6.RAG1-KO Class I-KO→B6.RAG1-KO GFP or B6.RAG1-KO

GFP→B6.RAG1-KO Class I-KO full chimeras did not receive any Matahari T cells prior to C3H and B6.RAG1-KO skin grafting. These mice accepted male C3H or B6.RAG1-KO skin grafts long-term (n=4). These results demonstrate that despite the lack of MHC class I on radioresistant tissues, indirect skin graft rejection can still occur. Taken together, for CD8 indirect rejection to occur, there is a requirement of MHC class I to be present on radiosensitive cells but not radioresistant cells. Rejection of C3H male skin grafts in chimeras lacking MHC class I on radioresistant cells suggests a key role for host APCs in indirect rejection and a lack of requirement for indirect presentation by host vasculature.

5.4. DISCUSSION

In the present study, we investigated the ability of MataHari mice to reject non-vascularized transplants via the indirect pathway. We found that the H-Y specific monoclonal population of CD8 T cells present in MataHari mice could indirectly reject male skin grafts; however, male islet grafts were resistant to indirect rejection even in pre-primed recipients. The lack of indirect rejection by CD8 T cells towards some non-vascularized grafts in our experiments requires further explanation. Previously, it has been found that primed Matahari T cells could respond in vitro to cultured aortic endothelial cells that were co-incubated with H-Y peptide. Then, *in vivo* experiments showed that skin could only elicit an indirect CD8 response if grafted onto a recipient that expressed MHC H-2^b. Taken together, the authors concluded that for indirect CD8 rejection, MHC class I is required on recipient derived vascular endothelium in order to display

reprocessed male antigen. However, this conclusion was not directly tested *in vivo*. In addition, the proposed effector pathway of cross-primed CD8 T cells predicted that other non-vascularized grafts, such as pancreatic islet or neonatal heart, would be rejected by a similar mechanism. However, the lack of indirect rejection by MataHari CD8 T cells that we observed with both islet and neonatal heart grafts suggests the presence of other mechanisms of indirect CD8 rejection. We therefore sought to determine if MHC class I is actually required on recipient radioresistant cells or recipient radiosensitive cells for indirect CD8 rejection to occur. Through the use of bone marrow chimeras, our preliminary data show that MHC class I was required on recipient radiosensitive cells for the indirect rejection of skin grafts by CD8 T cells. However, for indirect rejection of skin grafts, MHC class I need not be present on recipient radioresistant tissues; tissues such as the neovascular endothelial cells that grow into the non-vascularized transplants. These experiments are currently being replicated to increase animal numbers in order for definitive conclusions to be made. Although the presentation of male antigen by MHC class I on recipient vascular endothelial cells, as proposed by Valujskikh *et al.,* may be occurring, it is not required for indirect rejection of non-vascularized transplants by CD8 T cells and therefore cannot explain the differential sensitivity of vascularized vs. non-vascularized grafts to CD8 indirect rejection. The difference between our current results and previous studies could be due to the number of recipient APC or the amount of donor antigens presented by the recipient APCs within the graft. If there is enough indirect presentation to CD8 T cells occurring at the graft site by either of

these mechanisms, then it could result in non-specific release of inflammatory cytokines. Inflammation and subsequent local destruction of surrounding parenchymal tissues would then occur. In this manner, recipient vascular endothelium may indeed be the target of rejection, only as a result of 'collateral damage'.

Based on the "transplantation hierarchy" our results showing that monoclonal Matahari CD8 T cells can indirectly reject male skin grafts but not male islet grafts is expected. It is expected because the susceptibility of skin to rejection is greater than the susceptibility of islets (13-15) However, the exact reasons behind a tissue's differential susceptibility to indirect rejection are unclear. Potential reasons for the differential susceptibility include size and location of the graft or the presence of tissue specific antigens (16,17). In our case, we used a monoclonal TCR-receptor transgenic T cell population specific for a known antigen. Therefore, although tissue specific antigens are present in skin (18-20), they cannot account for the differential outcome we observed. In order to investigate the effect of antigen location on CD8 T cell indirect rejection, we transplanted Matahari mice with a non-vascularized male CBA, C3H or B6.RAG-KO neonatal heart in two different locations; the ear pinna and under the kidney capsule. CD8 T cells must be activated in secondary lymphoid tissue and then home back to the site of antigen exposure. One possibility was that lymphatic drainage from skin delivers male antigen to regional lymph nodes in a more efficient manner than lymphatic drainage from the kidney, allowing for more efficient indirect priming of T cells. However, we used pre-primed

MataHari T cells, reducing or eliminating any differences in priming between the groups. Alternatively, skin possesses properties of potent antigen presentation by Langerhans cells or dermal DCs and high concentrations of extracellular glycoproteins that may facilitate re-stimulation of T cells (21,22). The high numbers of APCs may migrate out of the transplanted graft and travel to the draining lymph nodes where they can be phagocytosed by recipient cells and their antigens cross-presented. Or, more recipient APCs will populate skin grafts and mediate indirect rejection in the graft itself. Islets contain few passenger lymphocytes and therefore fewer donor antigens will escape the subcapsular space of the kidney (7). However, we found that regardless of transplantation site, MataHari mice could not reject neonatal heart grafts via the indirect pathway. The failure to indirectly reject male CBA or C3H heart grafts is in contrast to the rapid direct rejection of male B6.RAG-KO neonatal hearts in both transplant locations. The ideal experiment for the effects of antigen location would be to transplant skin under the kidney capsule. However, when we preformed skin transplants under the kidney capsule of Matahari mice, there were health complications that required experiment termination (RT unpublished observations). Taken together, our data suggest that antigen location or the presence of tissue specific antigens could not account for the differential susceptibility of non-vascularized transplants to undergo indirect rejection. Instead, the intrinsic properties of the particular type of tissue grafted most likely underlie the differences in susceptibility to indirect rejection, properties which have yet to be identified. It has been previously shown that when both direct and

indirect rejection pathways are available, the intrinsic ability of non-vascularized grafts to stimulate CD8 T cells or the number of responding CD8 T cells required for skin or islet rejection does not account for the rejection hierarchy (16). However, these variables may be operating when rejection is limited to the indirect pathway, or other factors such as size of graft may be behind the cause of why one non-vascularized graft is accepted and another rejected.

We have identified a requirement for MHC class I on radiosensitive cells for CD8 T cell mediated indirect rejection. However, the reasons behind the differential susceptibility of allografts to indirect rejection could not be elucidated in the current study. To explain why some allografts are indirectly rejected and others not, further research is necessary to identify all cytokines and cells involved in CD8 mediated indirect allograft rejection.

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5.6. AUTHOR'S CONTRIBUTIONS

DPA and RT participated in research design, performance of research, data analysis and writing the paper. CE participated in performance of research and data analysis. RP and AMJS participated in performance of research. CCA participated in research design, data analysis and writing the paper

5.7. TABLES AND FIGURES

Table 5.1. MataHari mice are capable of rejecting skin but not islet transplants via the indirect pathway.

Donor	MHC haplotype (rejection pathway)	Skin graft survival (days x n)	Islet graft survival (days x n)
B6.RAG1-KO male	H-2^b (Direct and Indirect)	12x2, 14x5, 19, 20	2, 2, 3x5, 4x3
C3H male	H-2^k (Indirect)	24, 25, 27, 33x2, 34, 37, 38	>100x12 >100x5*
C3H female	H-2^k (None)		>100x2
CBA male	H-2^k (Indirect)		>100x2
B10.BR male	H-2^k (Indirect)		>100x4
B6-RAG1-KO Class I-KO male	Class $I^{-/-}$ (Indirect)		>100x4

Female MataHari mice received a skin or islet graft from the donor mice indicated. Islet transplant data is pooled from two separate experiments. Number of mice and days of survival are listed for each group.

*Recipient Matahari mice were primed five days prior to islet transplantation

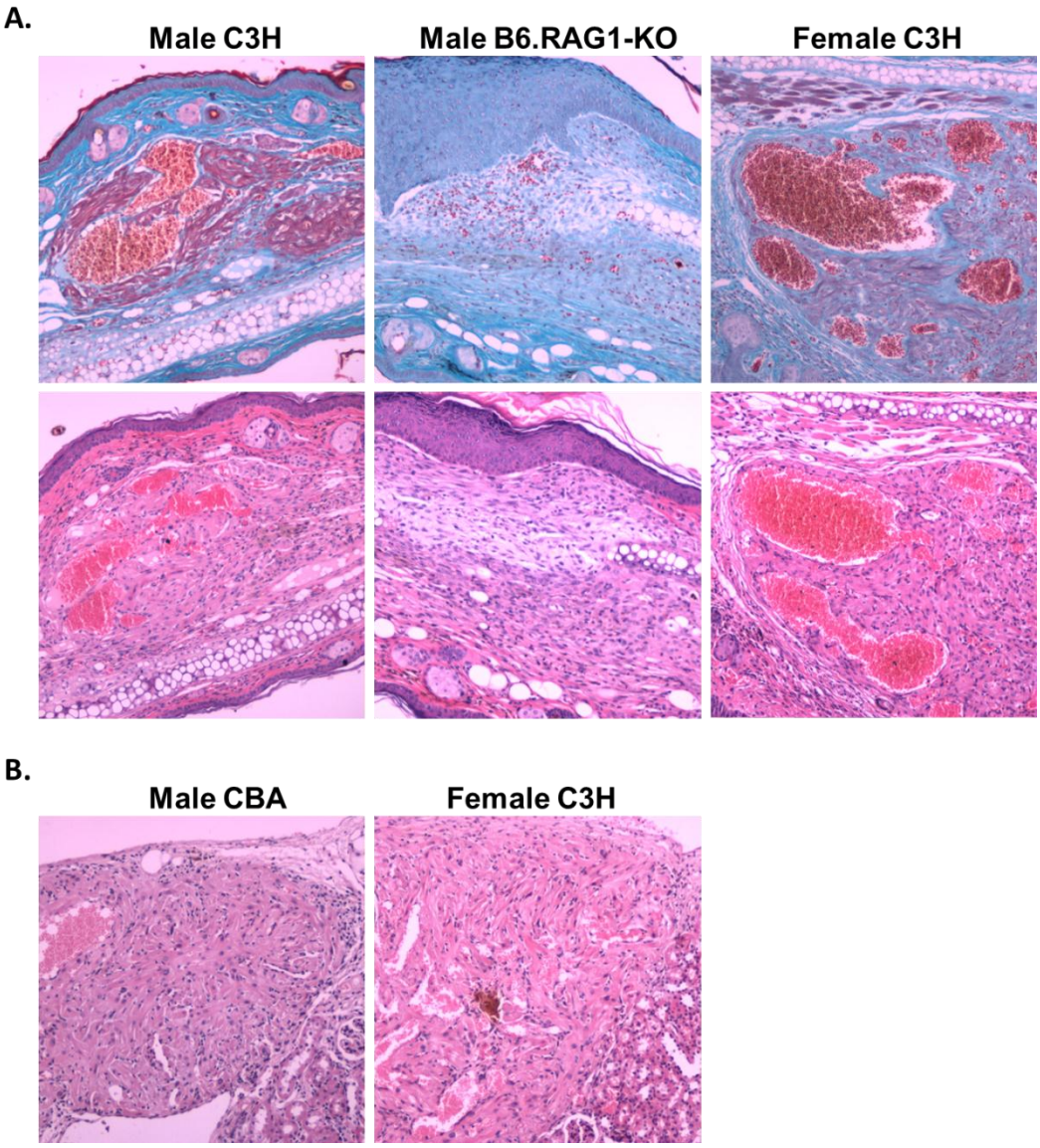

Figure 5.1. Histology of heterotopic neonatal heart allografts transplanted into female MataHari mice. MataHari mice were transplanted with neonatal hearts under the kidney capsule or under the ear pinna. **A.** Heart grafts from donor C3H mice transplanted under the ear were retrieved after 70 days and examined histologically. The histological section of the male B6.RAG1-KO heart transplanted under the ear is from a graft at 35 days post-transplant. *Top panels*

Masson's trichome staining and *bottom panels* hematoxylin and eosin staining of sequential sections. **B.** Heart grafts from male CBA and female C3H under the kidney capsule of MataHari recipients were retrieved 100 days post-transplant and examined histologically with hematoxylin and eosin staining. The photographs shown are representative of the histological findings of all animals in that group.

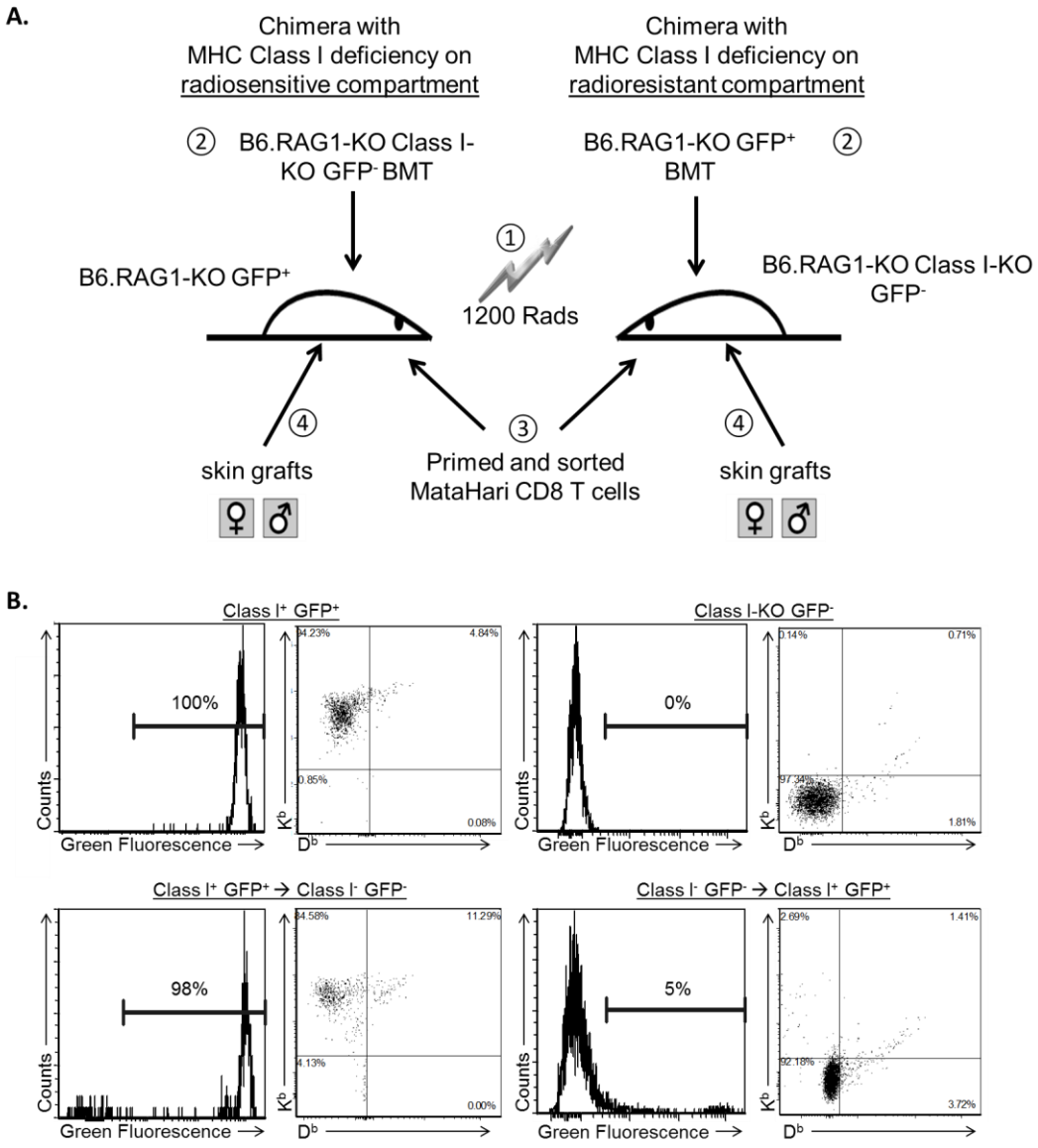

Figure 5.2. Experimental system for isolating MHC class I to either radiosensitive or radioresistant cellular compartments. **A.** 1. Recipient mice were lethally irradiated in split doses. 2. BMT from the reciprocal strain and followed by five weeks for hematopoietic reconstitution 3. Transfer of primed and sorted Matahari T cells into bone marrow chimeras. 4. Skin graft from male or female C3H or male B6.RAG1-KO donors. **B.** After step 2, peripheral blood of

full chimeras was assessed by flow cytometry. Top panels depict profiles of BMT donor mice while bottom panels depict profiles of chimeras post BMT. Based on GFP expression in the live cell gate, reconstituted Class I-KO mice were 98.0±1.0% GFP^+ in origin, and reconstituted GFP^+ mice were 95.3±0.6% Class I-KO in origin. There was consistently low D^b staining in the live cell gate of mice due to all mice being on the RAG1-KO background.

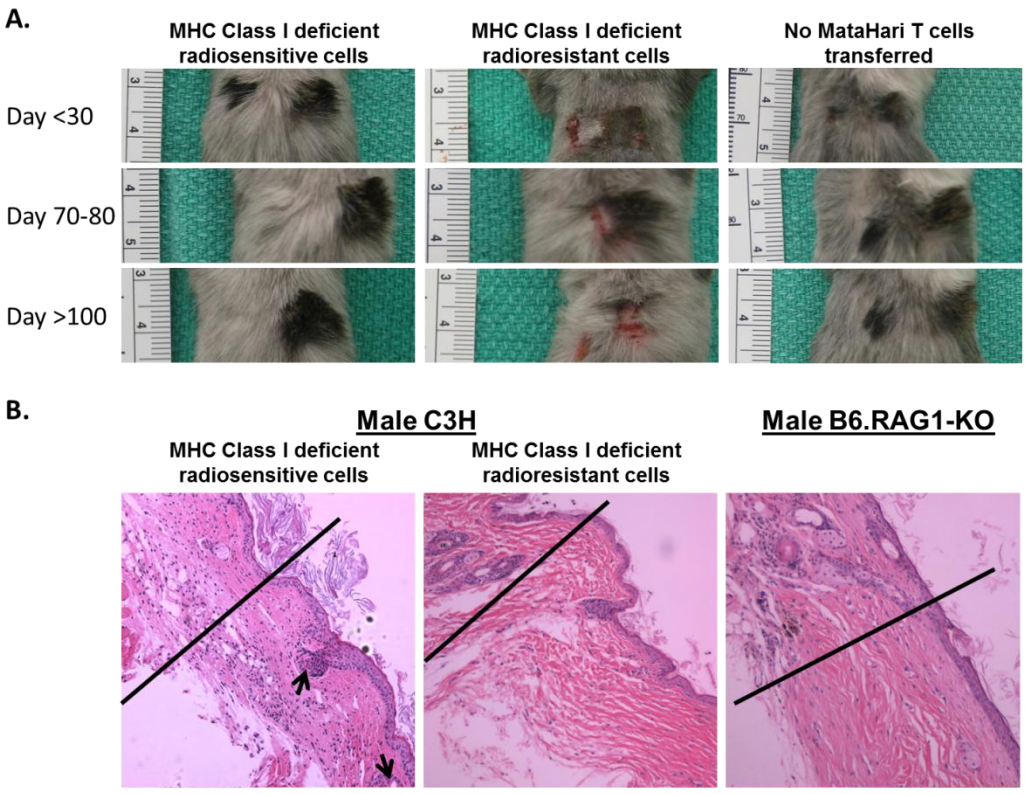

Figure 5.3. Gross morphology and histology of skin grafts from radiation chimeras injected with MataHari T cells. Female B6.RAG1-KO Class I-KO→B6.RAG1-KO GFP and B6.RAG1-KO GFP→B6.RAG1-KO Class I-KO chimeric mice received a skin graft from the donor mice indicated. **A.** Male B6.RAG1-KO skin was transplanted on the left and male C3H skin was transplanted on the right side. Note the early loss of the left sided B6.RAG1-KO graft in mice that had MataHari T cells transferred. **B.** At the time of rejection or after 100 days, skin grafts were retrieved for histological analysis. Hematoxolyn and eosin stained sections are oriented such that recipient skin is located in the top left and donor skin in the bottom right of each picture (black lines approximate the donor-recipient junctions). Arrows indicate hair follicles and sebaceous

glands in the donor skin. The photographs shown are representative of the histological findings of all animals in that group.

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Chapter 6: Future Directions

6.1. FUTURE DIRECTIONS

In this thesis I performed studies that implicated NK and T cells as barriers to overcome in the establishment of chimerism in an autoimmune model of T1DM. I then rationally designed a protocol in an attempt to address these challenges. I have also identified T cells as the main contributors to the development of split tolerance in this model and have identified an allografts susceptibility to indirect rejection as a potential contributing factor behind the development of split tolerance. Although these studies offer insight into the mechanisms of split tolerance and the beginnings of a means to avoid its occurrence, additional experimental refinements are required to enhance our understanding of this phenomenon.

6.1.1. Enhancing Peripheral Tolerance

Split tolerance can occur in mixed chimerism because central deletion cannot generate tolerance towards all allogeneic polymorphic tissue specific antigens (TSAs); therefore, peripheral tolerance mechanisms are required for allograft acceptance. As such, split tolerance can potentially occur towards any organ that expresses TSAs, however intrinsic properties of the organ itself may increase the likelihood of split tolerance developing. For example, due to many skin-specific antigens (1,2), split tolerance commonly occurs towards allogeneic skin grafts. However, although an important factor, TSAs are not the only explanation for the development of split tolerance; especially since other organs, besides skin, express TSAs and split tolerance is less likely to occur to those

organs. Other factors that help explain skin graft rejection are: a high sensitivity to indirect rejection (3) and a poor ability to survive local tissue damage. Since it is not possible to alter the immunogenic properties of most allografts, a potential solution to avoid split tolerance is to enhance the peripheral tolerance mechanisms operating in mixed chimerism.

Augmenting dominant peripheral tolerance through regulatory T cells (T regs) holds potential to abrogate split tolerance towards highly immunogenic allografts. However, the amount and duration of peripheral tolerance provided by T regs in mixed chimerism may differ between protocols (4-8). Therefore, before T reg augmentation is attempted, the extent of T reg function in our NOD mixed chimerism model must be established, especially since there are known defects in NOD T regs (9). A good place to begin is by assessing the frequency of both donor and recipient T regs in mixed chimeras at various time points. To assess function, T regs from NOD chimeras could be isolated and in vitro suppression assays preformed. It would then be useful to attempt chimerism induction with the co-injection of cells with regulatory properties to determine if enhancement of dominant peripheral tolerance can indeed abrogate split tolerance. For example, there has been recent clinical success with chimerism induction through the co-injection of plasmacytoid pre-dendritic "facilitating" cells along with hematopoietic stem cells (HSC) (10).

6.1.2. Defining the pathways of rejection causing split tolerance

Allograft susceptibility to CD4 or CD8 T cell mediated indirect rejection is a potential mechanism behind the development of split tolerance. However, with the exception of islets, the studies that determined an allograft's differential sensitivity to indirect rejection were performed in non-autoimmune mouse strains (3,7,11). Therefore, it would be ideal to determine if these potential mechanisms of split tolerance apply in NOD chimeras. Our current studies indicated that both CD4 and CD8 T cells were involved in the generation of split tolerance in NOD chimeras. Taken together, four potential T cell response pathways may generate split tolerance; donor antigen recognition via CD4 direct or indirect pathways or CD8 direct or indirect pathways. By modifying recipient-donor strain combinations, and transplanting tissues deficient in MHC class I or II, the contribution of each type of rejection to the development of split tolerance can be clarified.

6.1.3. Cells and effector mechanisms involved in split tolerance

During the current studies, we have identified pre-existing T cells as a major contributor to the development of split tolerance in NOD chimeras. Alloreactive memory T cells are a known hindrance to transplantation because they do not require the same stimulation to become active as a naïve T cell (12). Determining the importance of these T cells in mediating split tolerance would be a benefit, especially since the memory T cell component is a subset of T cells that must be made tolerant by peripheral mechanisms alone. The contribution of

memory T cells to the development of split tolerance could be determined by transferring NOD memory T cells into immune deficient NOD.RAG1-KO mice and assessing subsequent chimerism induction. Determining the involvement of memory T cells in split tolerance would demonstrate a role for targeted lymphoablation of these cells during chimerism induction.

Although we have identified T cells as a major contributor to the development of split tolerance in NOD mice, T cells must be activated by antigen presented on APCs. NOD APCs display a peripheral tolerance defect (13) which may be important in the development of full versus split tolerance after co-stimulation-based chimerism induction protocols. Identification of the APCs that activate donor reactive T cells (for example dermal APCs in skin transplantation) may allow for their targeted depletion. Another step in delineating the mechanisms of split tolerance is to determine the cytokine signals involved in activating T cells and the class of immune response elicited. By understanding T cell effector pathways, it may be possible to block these pathways and avoid allograft rejection in NOD mixed chimeras.

B cell tolerance must also be assessed in NOD mixed chimeras. The separate assessment of B cell tolerance is especially important because T cell tolerance does not ensure that B cell tolerance has been achieved. B cell tolerance to transplanted tissues and against beta cell autoantigens can be evaluated by searching for reactive allo- and auto-antibodies. Although B cell tolerance was demonstrated previously in NOD mixed chimeras (14), the importance of avoiding antibody-mediated rejection demands that it should be assessed with any

new chimerism induction protocol. If B cell tolerance is not achieved, refinements to the conditioning protocol that include B cell depletion or cyclophosphamide may be of benefit.

6.1.4. Refinement of chimerism induction strategies

Through identification of the lymphocytes responsible for chimerism resistance and split tolerance in NOD chimeras, we were able to generate a chimerism conditioning protocol that was effective at chimerism induction and avoided split tolerance to all but the most immunogenic tissue tested. However, despite the promising ability of the majority of this protocol to be clinically translatable, establishing chimerism with no graft-versus-host disease and overcoming split tolerance, it remains a protocol that requires anti-CD40L. Since anti-CD40L is not available for clinical use (15), we began testing whether anti-CD40L can be replaced with cyclophosphamide (CYP). CYP is an alkylating agent used clinically for the treatment of leukemia, lymphoma (16), and in some cases as an induction agent for lupus nephritis (17). More recently, it has been used as an agent to prevent GVHD after BMT (10,18). In our preliminary studies, we have found that lymphocyte depletion protocols that include CYP are indeed capable of generating fully allogeneic mixed chimeras in NOD mice that are tolerant of donor hematopoietic cells and islets (see Appendix and Fig A.1). Further studies will include variations in timing and dose to determine the optimal protocol and determine if tolerance towards skin grafts is possible. Furthermore, it will be important to examine whether successful CYP-based chimerism

induction protocols in pre-diabetic NOD mice are effective in generating chimerism and tolerance post onset of spontaneous diabetes.

6.1.5. Experimental limitations

During our experimental approaches to generate operational tolerance through mixed allogeneic chimerism, we have strived to hold clinical translation of our methods in high regard. It is for this reason we have employed use of the NOD mouse. The combination of autoimmunity and a resistance to allogeneic tolerance seen in this mouse strain makes chimerism induction difficult and thus serves as a very stringent model. We have also induced chimerism across fully allogeneic barriers using feasible amounts of bone marrow. Last, our protocols use reagents that are (for the most part) clinically available. However, despite our attempts, the results of our experimental model may not be predictive of successful clinical translation. For example, clean housing and young age may be aspects of murine models that make them reductive. In addition, the decreased incidence of GVHD after BMT in mice compared to humans may be explained by the low frequency of T cells in murine bone marrow and the high level of activation of T cells in human bone marrow (19).

Mice in most of our chimerism experiments were housed in a clean facility and used at a relatively young age. Combined, these two conditions of our experimental animals may influence the number and frequency of memory cells (20,21). Since memory T cells have been shown to contribute to solid organ (22-24) and bone marrow rejection (25) and do not require the same signals for

activation as naïve cells (26), a relatively low number of these cells in our experimental animals may artificially decrease the conditioning required for successful chimerism induction. In contrast, the higher number of memory cells in adult humans may increase the conditioning required in the clinic. In our experiments in NOD mice, the underlying effects of mouse age and the presence of diabetes at the onset of chimerism induction are indeed relevant. Although others have even shown a reversal of diabetes with the establishment of mixed chimerism (27,28), our attempts at chimerism induction in spontaneously diabetic mice using our co-stimulation blockade based protocol was unsuccessful at diabetes reversal (unpublished observations). In addition, when tried on older, non-diabetic mice, our co-stimulation blockade based protocol had limited success. However, we did not attempt our refined induction protocol (based on T cell depletion) in older or spontaneously diabetic animals. In older animals, co-stimulation alone may not be able to tolerize the increased number of memory cells and some degree of depletion is required. Similarly, animals with active autoimmune disease may require deletion of some activated T cells before the remainder can be tolerized. Future aims characterizing the memory T cell population and attempting chimerism induction with T cell depletion in older NOD mice could provide valuable information about the effects of memory T cells on chimerism conditioning protocols.

Despite the aforementioned limitations of murine models, experimental model systems are a valuable resource before clinical translation. Experimental

results obtained in such models are beneficial; however, they should be interpreted while recognizing the limitations of the system itself.

6.1.6. Stem cell approaches for the treatment of organ dysfunction

Functional replacement of whole organs and tissues by stem cell based approaches hold promise for the treatment of end-stage organ failure. While conventional allogeneic organ transplantation would benefit from tolerance induction through chimerism strategies or the use of T regs, autologous stem cell based organ transplantation would be revolutionary; not only would donor organ shortages be mitigated, immunosuppression could be avoided. Two stem cell based technologies that are rapidly gaining plausibility are autologous stem cell growth on decelluarized biomaterial or synthetic matrices and in vitro differentiation of stem cells into cells of interest.

Autologous cell seeding of a scaffold biomatrix holds promise for the generation of dimensionally tailored, non-immunogenic tissues for transplant. In fact, proof-of-concept reports of tissue engineered artificial nanocomposite scaffold reseeded with autologous stromal cells have been successful for tracheal transplantation (29). Furthermore, there is growing experience with different scaffolding materials that are seeded with cells for use in bladder repair or replacement (30). The bioreactor approach of seeding a scaffolding material with autologous cells may be successful in replacing tissues that have minor metabolic functions, such as the trachea or bladder. However, generating tissues with complex metabolic or secretory function, such as liver, is more difficult (31).

Therefore, further research into whole organ generation on three dimensional scaffolds from stem cells is necessary to advance this approach. In contrast to the generation and transplantation of whole organs for end-organ failure, cellular transplants can potentially be used for the replacement of destroyed islets in patients with T1DM. Two major challenges with this approach are the enticing the differentiation of stem cells into beta cells and, once differentiated, achieving glucose-dependent insulin secretion (32). Recent work characterizing the molecular pathways involved in the generation of functional islet-like clusters from HSC derived precursors holds promise to manipulate potential targets for improving beta cell differentiation (33). However, in the case of organ transplantation for autoimmune disease, stem cells may be attacked just as native tissues once were; therefore, some degree of immune modification would still be required.

6.2. CHIMERISM AND SPLIT TOLERANCE IN THE CLINIC

The immune system provides protection to the organism from a diverse array of pathological agents and malignant cellular transformation agents (34,35). However, the extensive repertoire of antigens that the immune system has evolved to recognize and respond against is a hindrance to the contemporary field of transplantation. The innate and adaptive immunological barriers against organ and cellular transplantation are compounded when alloreactivity (36) and memory responses are considered (37). In order to overcome these substantial barriers,

allogenic transplantation requires immunosuppression. Immunosuppressive medications have allowed transplantation to become a reliable treatment option for patients with end-stage organ failure. Despite the shortcomings of immunosuppressive medications (38,39), the success of transplantation for end-stage organ disease has expanded its application to other diseases that have established medical therapies, such as Type 1 Diabetes Mellitus (T1DM) (40,41). However, potent immunosuppression alone may not avoid the chronic activation of the immune system that leads to chronic rejection (42) or to the recurrence of autoimmunity (43). Futuristic goals of organ replacement through the transplantation of matrix-grown syngeneic organs or reversing organ failure by stem cell therapy should be pursued (44). However, with these options currently unavailable, organ transplantation would benefit significantly from the induction of immunological tolerance.

The immune system does not have the capacity to generate self-tolerance by either peripheral or central tolerance mechanisms alone. The requirement for central tolerance is demonstrated in the Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy syndrome (45-48). In this syndrome, a single gene disruption that negates some aspects of negative selection in the thymus causes a breakdown in self-tolerance. The requirement for peripheral tolerance in immune homeostasis is demonstrated by the immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (49,50). The autoimmunity seen in this disease is caused from a single gene defect that causes the loss of the regulatory T cell component of peripheral tolerance. Relating to transplantation,

it would follow that in order to achieve allograft tolerance to the same extent as natural self-tolerance, both central and peripheral mechanisms must be employed. Therefore, attempts at organ transplantation solely with the induction of peripheral tolerance (51,52) could benefit from a concomitant induction of central tolerance.

The establishment of allogeneic hematopoietic chimerism has the potential to induce both central (53-55) and peripheral tolerance (56,57). Through its unique ability to induce central tolerance, chimerism mediates the early deletion of potentially alloreactive T cells. Whereas peripheral tolerance controls pre-existing donor-reactive T cells or donor-reactive T cells that escape central deletion. Through these means, chimerism may reverse autoimmunity through the re-establishment tolerance towards normal self-antigens (58,59). However, split tolerance is an example of how chimerism does not guarantee all donor cells or tissues will avoid generating an immune response. Central tolerance may be incapable of deleting alloreactive T cells that recognize polymorphic tissue specific antigens (TSA), and peripheral tolerance may be incapable of deleting or regulating large numbers of pre-existing alloreactive T cells. In either case, alloantigens can be the target of immune reactivity, despite the presence of chimerism.

Clinical attempts at tolerance induction through the generation of mixed chimerism are often associated with split tolerance (60,61). In these cases, split tolerance usually develops such that chimerism is lost, with the preservation of the solid organ graft. However, large animal and murine models have demonstrated

that the reverse situation can occur; where the organ or tissue allograft is lost despite the maintenance of chimerism (14,62). Therefore, it is essential to completely understand the mechanisms of tolerance operating in mixed chimerism, and furthermore, understand the subtleties of why tolerance develops towards some tissues and not others. This knowledge of tolerance may assist in the design of chimerism induction therapies that do not require extensive conditioning and will ensure the recipient will indeed be tolerant of all donor tissue. In this manner, chimerism approaches may be broadened for use in clinical islet transplantation.

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Appendix: Cyclophosphamide and chimerism induction in non-obese diabetic mice

A.1. INTRODUCTION

Although many aspects of our chimerism induction protocols in NOD mice are clinically translatable, they remain protocols that require anti-CD40L. Since anti-CD40L is not available for clinical use (1), we began testing whether anti-CD40L can be replaced with cyclophosphamide (CYP). CYP is an alkylating agent used clinically for the treatment of leukemia, lymphoma (2), and in some cases as an induction agent for lupus nephritis (3). More recently, it has been used as an agent to prevent GVHD after BMT (4,5). Previously, cyclophosphamide has been used quite extensively in both solid and cellular transplantation experiments in mice (6-9). Focusing specifically on bone marrow transplantation (BMT), CY has been used most commonly in combination with TBI and T cell depleting antibodies. These studies have shown that CY can increase the level of chimerism when combined with T cell depleting antibodies and can decrease the amount of TBI required to establish chimerism in B6 to B10 chimeras (10). The mechanism of CY action is thought to be mainly through the destruction of donor-reactive T cells (11).

In relation to NOD mice, previous studies have shown that the administration of a high dose of CY (200 mg/Kg) leads to the rapid, synchronous development of diabetes (12) via a mechanism thought to be dependent on regulatory T cell function (13). However, to the best of our ability, we have not found any protocols where CY is used in NOD mice as an agent for chimerism induction. Therefore, our aim is to determine if CY can be used as an agent to induce chimerism in NOD mice.

A.2. MATERIALS AND METHODS

A.2.1. Animals. Adult C3H (H-2^k) and NOD mice were purchased originally from Jackson Laboratory (Bar Harbor, ME, USA) and Charles River Canada (Saint-Constant, QC, Canada), were bred on-site. Pre-diabetic NOD mice used for chimerism induction were females between 8-10 weeks of age. All care and handling of animals were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

A.2.2. Induction of allogeneic chimerism by transplantation of bone marrow cells. Transplantation of $20x10^6$ allogeneic (C3H) bone marrow cells (BMCs) by a non-myeloablative protocol into NOD mice was performed by one of three different protocols that included the administration of 150mg/Kg CY (Sigma Canada).

Protocol 1: CY and NK depletion. Recipient NOD mice were pre-conditioned with NK cell depletion by administering anti-asialo GM1 intraperitoneal (i.p.) (Wako Chemicals USA, Richmond, VA; 35 □L diluted in PBS) on day -3. On day -1, busulfan (BUS; GalaxoSmithKline Canada), a DNA alkylating agent (14), was given i.p. at 20 mg/kg body weight. On day 0, recipient mice were transplanted intravenously (i.v.) with $20x10^6$ unmodified C3H BMCs. CY was administered ip on day +2 and rapamycin (Rapamune®, Wyeth Canada, Montreal, QC) at 3 mg/kg diluted in phosphate-buffer saline (PBS) was administered i.p. on daily from day +4 to +28.

Protocol 2: CY, NK depletion and donor specific transfusion (DST). Recipient NOD mice were pre-conditioned with a DST of $20x10^6$ unmodified C3H spleen cells administered as an i.v. injection on day -4. On day -3, NK cell depletion was performed by administering anti-asialo GM1. CY was administered on day -2 and BUS was given on day -1. On day 0, recipient mice were transplanted i.v. with $20x10^6$ unmodified C3H BMCs. Rapamycin was then given daily from day 0 to +28.

Protocol 3: CY, NK and T cell depletion. Recipient NOD mice were pre-conditioned on day -3 with NK cell depletion by administering anti-asialo GM1. Also on day -3, T cell depletion was performed by administering anti-CD4 (GK1.5) and anti-CD8 (53.6.7) (each at 0.25 mg i.p.; Bio X Cell, West Lebanon, NH). BUS was administered one day before the day 0 transplantation of $20x10^6$ allogeneic C3H BMCs i.v. After BMT, CY was administered i.p. on day +2 and Rapamycin was given daily from day +4 to +28.

A.2.3. Islet isolation and transplantation. Islet isolation was carried out as previously described (15). Three days before transplantation, recipient mice were made diabetic by a single i.p. injection of streptozotocin (Sigma-Aldrich Canada, Oakville, ON) at 180 mg/kg body weight. Diabetes was confirmed by a blood glucose measurement of >20.0 mmol/L. Five hundred islets were transplanted into the renal subcapsular space to reverse hyperglycemia.

A.2.4. Flow cytometry. At various time points, chimerism levels were assessed in NOD recipients of C3H BMCs. Antibodies against TCR, CD4, CD8, CD11b, CD11c, CD19, CD49b, CD122, H-2K^{k} and H-2D^{b} were purchased from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA). Donor C3H cells were identified by anti-H-2K^{k} antibodies and recipient NOD cells were identified by anti-H-2K^{d}. Data were acquired using a LSR II (Becton Dickson, Sunnyvale, CA) flow cytometer and analyzed with FCS Express™ (De Novo Software, Los Angeles, CA).

A.3. RESULTS

A.3.1. CY based conditioning can generate chimerism and tolerance to islets. Using protocols 1-3, we attempted chimerism induction using fully allogeneic C3H donor bone marrow in pre-diabetic NOD mice. Protocols 1 and 2 were completely unsuccessful at chimerism induction, however, protocol 3 was successful at inducing chimerism in 3/5 mice (Figure A.1A). Over time, the level of donor chimerism declined in all three of these mice, with one mouse losing chimerism completely (mouse #5; Figure A.1B). At approximately 10 weeks post-chimerism induction, we challenged the three chimeric mice with an C3H islet transplant. Mouse #3 and #5 accepted islets for >100 days, and returned to hyperglycemia after the islet graft-bearing kidney was removed (data not shown). Despite the maintenance of chimerism, mouse #4 rejected donor islets at day 23.

None of the mice in protocols 1-3 developed spontaneous diabetes (data not shown).

A.4. DISCUSSION

In our preliminary studies, we have found that lymphocyte depletion protocols that include CYP are indeed capable of generating fully allogeneic mixed chimeras in NOD mice. Although the chimerism showed decline in all cases and was lost in one case, some of these NOD chimeras accepted donor islets.

Although not tested in the current experiment, the nature of the chimerism should be assessed in future experiments in order to determine if split tolerance develops towards C3H hematopoietic cells. In addition, it should be determined if tolerance towards skin grafts is possible. Immunocompetence needs to be assessed by the transplantation of third-party islet and skin grafts.

Future studies will need to include variations in timing and dose to determine the optimal protocol. Also, the requirement of CY in these protocols needs to be assessed. Only 2 mice in a separate cohort were treated with protocol 3 without CY and these mice did not become chimeric (data not shown); however, these numbers need to be increased in order to ensure CY is actually required. Furthermore, it will be important to examine whether successful CYP-based chimerism induction protocols in pre-diabetic NOD mice are effective in generating chimerism and tolerance after the onset of spontaneous diabetes.

A.5. FIGURE

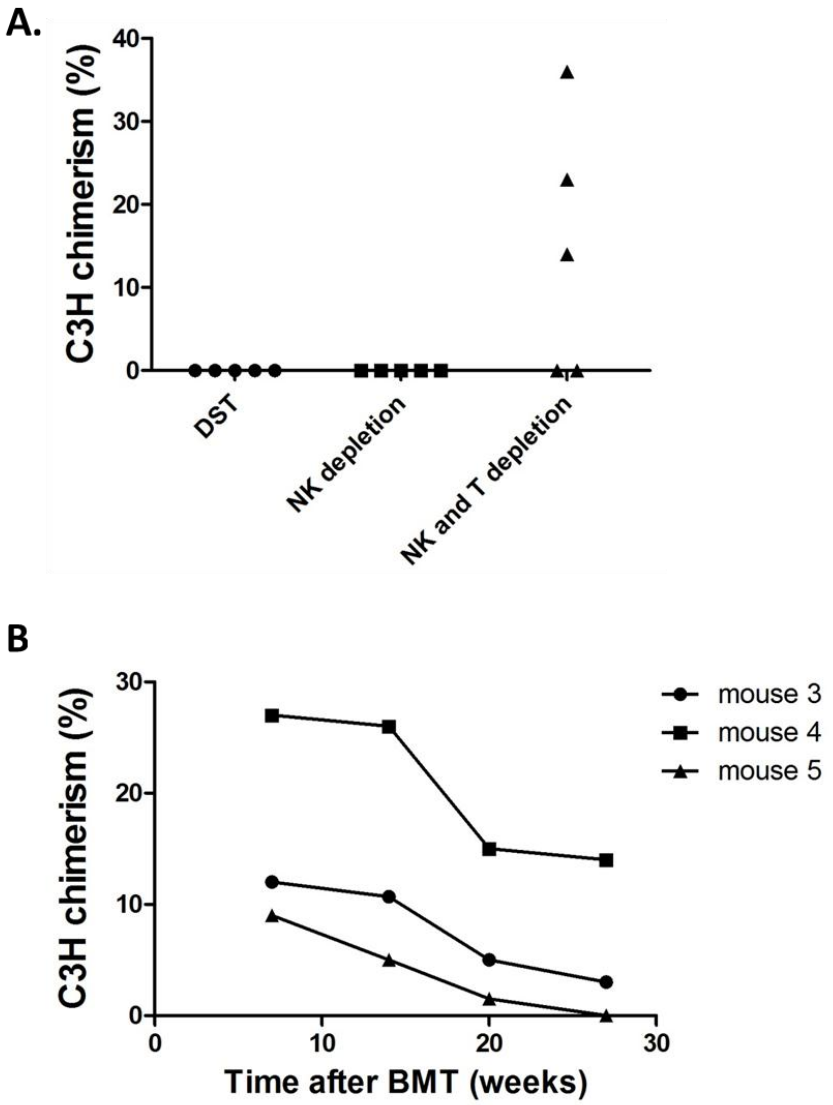

Figure A.1. CY combined with NK and T cell depletion can generate chimerism in NOD mice. Three cohorts of NOD mice receiving fully allogeneic C3H BMCs were conditioned with CY-based protocols 1, 2 or 3 and peripheral blood lymphocyte chimerism was monitored. (A) When NK cells were depleted along with CY (protocol 1), NOD mice could not be made chimeric (0/5).

Similarly, mice treated with NK depletion and a DST combined with CY (protocol 2) could not induce chimerism (0/5). Chimerism could only be generated in mice treated with CY and NK and T cell depletion (3/5). (B) Long-term C3H chimerism levels in each of the three mice conditioned with protocol 3.

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