University of Alberta

Defining the capacity and limits of natural self-tolerance mechanisms, along with the contribution by hematopoietic chimerism, in the immunity/tolerance decision towards an allogeneic islet transplant

by

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"The determined scholar and the man of virtue will not seek to live at the expense of injuring their virtue. They will even sacrifice their lives to preserve their virtue complete."

-Confucius

"Whenever a new discovery is reported to the scientific world, they say first, 'It is probably not true.' Thereafter, when the truth of the new proposition has been demonstrated beyond question, they say, 'Yes, it may be true, but it is not important.' Finally, when sufficient time has elapsed to fully evidence its importance, they say, 'Yes, surely it is important, but it is no longer new.'"

-Michel de Montaigne

ABSTRACT

T cell immunity requires prior antigen-specific activation that is costimulation-dependent; peripheral T cell self-tolerance has been postulated to result from antigen recognition without costimulation. Many experimental strategies developed to induce allogeneic tolerance emphasize costimulation blockade, assuming that T cells recognizing alloantigens without costimulation would be tolerized according to natural tolerance to a peripheral self-antigen. However, it is unclear whether peripheral tolerance to an allogeneic tissue can ever be established naturally; if possible, the histocompatibility limit also needs to be defined. In a mouse model of islet transplantation, I observed natural T cell tolerance to single minor-mismatched islets; consistent tolerance to greater mismatches required systemic chimerism. This suggests that efforts to induce allogeneic islet tolerance with costimulation blockade alone may give limited success. Moreover, there is generally a limited capacity of natural peripheral tolerance mechanisms to induce allogeneic tolerance. However, because systemic chimerism generated robust T cell tolerance towards highly mismatched islet transplants, this suggests islet transplantation tolerance in the relevant nonobese diabetic mouse model may be achieved similarly. Surprisingly, I observed that mixed chimeras of fully allogeneic cells showed considerable split tolerance, as demonstrated by the persistence of T cell but not B cell chimerism, and rejection of donor skin and islet transplants. I determined that a possible mechanism for split tolerance is the differential susceptibility of allogeneic grafts to indirect CD4 immunity. Collectively, my studies clarify the ability of the immune system to naturally become tolerant to an allogeneic tissue, and the contribution of hematopoietic chimerism to generate allogeneic tolerance. Unexpectedly, chimerism induction, which is often considered promising in its clinical applicability, may not be an adequate approach for generating islet transplantation tolerance, due to potentially a strong resistance to tolerance induction from the recipient and the possibility for split tolerance.

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TABLE OF CONTENTS

LIST OF TABLES

Page

Chapter 1

Chapter 2

Chapter 3

Appendices

LIST OF FIGURES

Page

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Appendices

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
k	Kilo (10^3)
m	Milli (10^{-3})
μ	Micro (10^{-6})
°C	Degree(s) Celsius
ADCC	Antibody-mediated cellular cytotoxicity
Aire	Autoimmune regulator
Akita	C57BL/6-*Ins2*^{Akita}
ANOVA	Analysis of variance
APC(s)	Antigen presenting cell(s)
B6	C57BL/6
B6.g7	B6.NOD
B6-RAG	B6.129S7-*Rag1*^{tm1Mom}
B6-RAG-KO	B6.129S7-*Rag1*^{tm1Mom}
B10-RAG/γc	(C57BL/6J × C57BL/10SgSnAi)-[KO]γ*c*-[KO]*Rag2*
B220	CD45R
BM	Bone marrow
bm12	B6.C-*H2-Ab1*^{bm12}
BMT	Bone marrow transplantation
bp	Base pair(s)
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
BUS	Busulfan
C3H-RAG-KO	C3H.129S6(B6)-*Rag2*^{tm1Fwa}
CCAC	Canadian Council on Animal Care
CD40L	CD40 ligand
CFSE	Carboxyfluorescein diacetate succinimidyl ester
Con A	Concanavalin A
CTL(s)	Cytotoxic T lymphocyte(s)
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
DC(s)	Dendritic cell(s)
ddH_2O	Double-distilled water
DN	Double negative
DNA	Deoxyribonucleic acid(s)
DP	Double positive
DST	Donor-specific transfusion
DTH	Delayed-type hypersensitivity
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FL(s)	Fetal liver(s)
FLC(s)	Fetal liver cell(s)
g	Acceleration due to gravity
g	Gram(s)

GVHD	Graft versus host disease
HA	Hemagglutinin
HEL	Hen egg lysozyme
HLA	Human leukocyte antigen(s)
ICOS	Inducible costimulator
IFN	Interferon
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
i.p.	Intraperitoneal(ly)
IP-10	10 kDa IFN-γ-induced protein
i.v.	Intravenous(ly)
KO	Knockout(s)
L	Litre(s)
LPS	Lipopolysaccharide
M	Molar
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma interferon
Minor-H	Minor histocompatibility
MLR	Mixed lymphocyte reaction
mol	Mole(s)
mos.	Months
MR1	Anti-CD40L antibody
NCI-Frederick	National Cancer Institute at Frederick
NIAID	National Institute of Allergy and Infectious Diseases
NK	Natural killer
NOD	Nonobese diabetic
NOD-RAG-KO	NOD.129S7(B6)-*Rag1*^{tm1Mom}
NOD-μMT	B cell-deficient NOD
OVA	Ovalbumin
p(p).	Page(s)
PAMP(s)	Pathogen-associated molecular pattern(s)
PBMC(s)	Peripheral blood mononuclear cell(s)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
pH	Potential of hydrogen
PRR(s)	Pattern recognition receptor(s)
Rad(s)	Radiation absorbed dose
RAG	Recombination activating gene
RAG-KO	B6.129S7-*Rag1*^{tm1Mom}
RAG/γc	(C57BL/6J × C57BL/10SgSnAi)-[KO]γc-[KO]*Rag2*
RAG/γc-KO	(C57BL/6J × C57BL/10SgSnAi)-[KO]γc-[KO]*Rag2*
rpm	Revolution(s) per minute
s.c.	Subcutaneous(ly)

SCID	Severe Combined Immunodeficiency
SD	Standard deviation
SEM	Standard error of the mean
SP	Single positive
SRL	Sirolimus
STZ	Streptozotocin
TAg	SV40 T antigen
TCR(s)	T cell receptor(s)
TCRβ	TCR-beta chain
Tg	Transgenic
Th	T helper
TLR(s)	Toll-like receptor(s)
Treg	Regulatory T
U	Unit (measure of enzyme activity)
WT	Wild type

Chapter 1: Introduction

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1.1. OVERVIEW OF THE IMMUNE SYSTEM

The immune system functions in host defence against pathogenic bacteria, viruses, parasites and other agents (1). It also repairs tissues damaged by injury or infection, thereby facilitating their healing (2), and eradicates tumour cells that may develop into a malignancy (3). Occasionally, however, this highly evolved, versatile machinery can damage the host. Hypersensitivity and allergic reactions (4) do not appear to offer any survival advantage. Unmanaged anaphylactic shock can be rapidly fatal. Autoimmunity that results from the dysregulation and dysfunction of the immune system can be highly destructive (5, 6). In contrast, rejection of a genetically disparate transplant may be considered an unfortunate evolutionary cost for having a host defence mechanism that usually responds to antigens found outside the host (7). All in all, the immune system is not flawless, but in a well functioning immune system undesirable responses are mostly an exception rather than the rule due to a tight regulation on all of the immunological processes.

1.1.1. Features of adaptive immunity

Our immune system can be functionally divided into innate and adaptive components but with overlap (8). The ability to generate adaptive immune responses that are antigen-specific (by T cells and B cells) distinguishes jawless vertebrates and higher organisms from those that are phylogenetically lower (1). There is regulation of the class of immunity; a different type of response can be made to suit each individual challenge (*e.g.* T helper (Th) 1 versus Th2 responses (9), or antibody class switching in B cells (10)). Moreover, immunological memory enables a quicker response to be made towards a previously encountered challenge (11). The subsequent sections will focus on T cell biology.

1.1.2. Antigen-specific activation is a prerequisite for T cell immunity

T cells mount antigen-specific responses due to their expression of antigen-specific T cell receptors (TCRs). However, activation of T cells is required to generate immunity. For functionally competent cells that have never experienced antigen (*i.e.* naïve T cells), activation appears to require at least antigen recognition, by the TCR, on an antigen presenting cell (APC) such as a dendritic cell (DC), in addition to a second signal (12-14) (**Figure 1.1**). Memory cells with a history of antigen-specific stimulation must also be activated to generate a secondary response, but the requirements are generally less (11, 12, 15, 16).

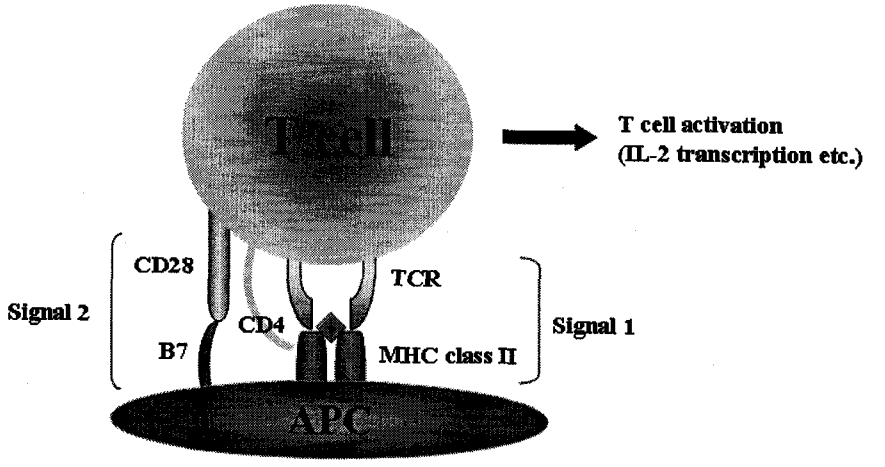

Figure 1.1. Two-signal activation of T cells. To activate a naïve T cell, it is generally thought that the T cell receptor (TCR) must first recognize, in a cognate fashion, a peptide coupled to a major histocompatibility complex (MHC) molecule and expressed on the surface of a professional antigen presenting cell (APC) such as a dendritic cell, as illustrated here with a CD4 T cell that recognizes a peptide on MHC class II (signal 1). The T cell coreceptor, CD4 in this schematic, is an accessory molecule that also binds to the MHC and mediates signal transduction. However, the T cell must receive an additional signal from the APC (signal 2; costimulation), primarily via the interaction between CD28 and B7 expressed on the T cell and the APC, respectively. The outcome is T cell activation, characterized by transcription of the interleukin-2 (IL-2) gene and production of IL-2 that is required for proliferation.

Antigen recognition by T cells is the interaction between their individually unique TCRs with processed peptides presented on major histocompatibility complex (MHC) molecules (17). While the clonal selection theory postulates that individual T cells express only a single specificity of the TCR that binds one unique antigen (18), the reactivity of an individual TCR is likely broader (19, 20). TCRs may bind not only antigens presented on self-MHC but also foreign MHC molecules (20) (*i.e.* alloreactivity; alloreactivity or crossreactivity of T cells is an exception to MHC restriction, wherein a T cell that becomes activated after recognition of an antigen presented by a specific MHC molecule is generally restricted to respond to the same antigen presented by the same but not a different MHC (21, 22)). Thus, the frequency of alloreactive T cells is considerably greater than those that recognize nominal antigens (20, 23).

1.1.3. Antigen specificity, diversity and autoreactivity

The specificity born by a TCR is generated by a random process; this randomness theoretically allows a T cell of virtually any antigenic specificity to be made (known as "diversity") (24). However, this raises the possibility that a TCR specific to a self-antigen may be generated. The absence of a mechanism to

eliminate or control self-reactive cells may lead to pathology that debilitates and even kills the host. Studies by Paul Ehrlich at the turn of the twentieth century, in which he found an absence of autoantibodies to blood antigens following immunization of animals with their own blood, provided an early hint that a mechanism to safeguard against this possibility indeed exists, so as to avoid "horror autotoxicus" (25).

1.1.4. Control of adaptive immunity by T cells

Early studies by Miller (26, 27) led to the conclusion that T cell production occurs in the thymus. Neonatally thymectomized mice showed an inability to reject allogeneic skin grafts and poor development of germinal centers (26), suggesting the importance of T cells in these processes. T cells can either directly respond to an immunological challenge, or recruit other types of immune cells that participate in host defence. CD4 T cells are often the controllers of adaptive responses. They not only function as effectors but govern the reactivity of CD8 T cells and B cells, which often require "help" to respond (28-31).

1.2. IMMUNOLOGICAL TOLERANCE

Adaptive immunity is characterized by selective reactivity, responding strongly to pathogenic agents and generally not to self-tissues. Hence, the T cell repertoire (and similarly for B cells) is generally considered to be self-tolerant. In this thesis, tolerance is defined as an absence of immunity towards what would otherwise be an immunogenic antigen challenge, and results from prior exposure to the specific antigen. Tolerance can therefore be considered a negative response to antigen. The existence of tolerance may either be directly tested by experimental challenge with an immunogen or inferred by the survival or persistence of an otherwise immunogenic antigen in the putatively tolerant host. The discovery of immunological tolerance may be considered a relatively recent event in history and can be attributed to the independent experimental studies of Owen (32), Medawar and colleagues (33), and Hasek (34), along with the postulation of a natural state of immunological tolerance by Burnet and Fenner (35-37).

1.2.1. Central and peripheral tolerance

T cell self-tolerance is attributed to a selection of T cells during or after their development, which involves the physical or functional elimination of self-specific T cells. Historically, central tolerance (also known as

negative selection) has been considered to be largely responsible for shaping a T cell repertoire known for its specificity and its discriminatory nature. Central tolerance is an antigen-specific unresponsiveness due to the recognition of antigen by lymphocyte precursors during their development (**Figure 1.2**). It results from antigen encounter before lymphocytes have matured to the point where they can respond in a positive fashion (immunity) to antigen, and occurs primarily in the thymus for T cells. Together with positive selection, a process in which T cells that can bind self-MHC receive a survival signal for further development, most developing T cells do not survive thymic selection events (38). In negative selection, developing T cells bearing high-avidity TCRs specific for self-antigens are physically eliminated (known as deletion) in the thymus to prevent autoreactivity in the periphery.

The expectation that there would be peripheral self-antigens that are not presented in the thymus to induce negative selection, and experiments that demonstrated a tolerizability of mature T cells raised doubts about the thymic capacity to act as the sole regulator of autoreactivity and led to the idea of peripheral tolerance. Peripheral tolerance is an antigen-specific unresponsiveness due to the recognition of antigen by fully mature lymphocytes (*i.e.* lymphocytes that are functionally competent and have the potential to respond positively to antigen), and not due to antigen encounter during lymphocyte development. This can occur in any tissue, including in the thymus where recirculating mature T cells (39) are able to encounter antigen (**Figure 1.2**).

While studies relating to the autoimmune regulator (*Aire*) gene demonstrating the expression of peripheral tissue-specific antigens in the thymus (40-43) provide renewed support for central tolerance as the sole necessary tolerance mechanism, they do not exclude the contribution by potential mechanisms of peripheral tolerance. The pattern of Aire-driven expression of peripheral tissue-specific antigens by individual thymic medullary epithelial cells is highly heterogeneous, and is restricted both in terms of the frequency of cells that are able to present peripheral antigens and the number of antigens that individual cells express (44). Thymic expression of certain peripheral tissue-specific antigens is not driven by Aire (45). To make peripheral tolerance mechanisms dispensable, Aire and potentially other transcriptional regulators must clearly be able to regulate the expression of all relevant peripheral tissue-specific antigens for which cognate T cells are able to recognize during development, but this remains unknown.

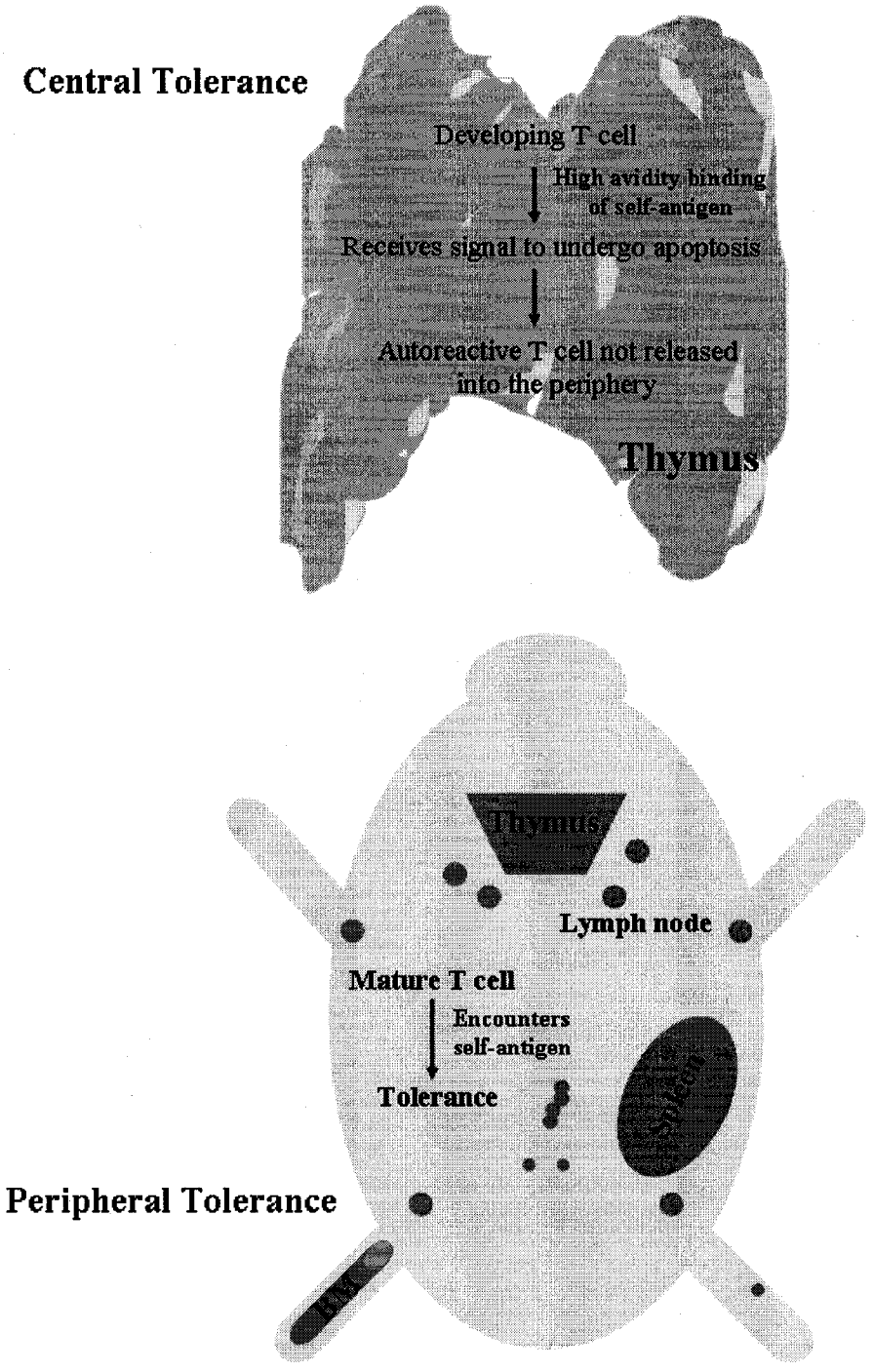

Figure 1.2. Central versus peripheral tolerance. Top: Central tolerance deals with tolerance of developing T cells specific to self-antigens. It occurs in the thymus by physical elimination of self-reactive T cells. Bottom: Peripheral tolerance deals with tolerance of functionally mature T cells specific to self-antigens. It can occur in any tissue (particularly of lymphoid origin) in which T cells circulate and self-specific T cells encounter their cognate antigens. No consensus has been reached regarding the mechanism of peripheral tolerance (single or multiple mechanisms, and which one(s)).

Independent of Aire, there is a large amount of data suggesting the existence of peripheral tolerance mechanisms that contribute to the overall state of self-tolerance. The myriad of studies that demonstrated, in animal transplantation models, tolerance induction towards allogeneic tissues without generating systemic hematopoietic chimerism are suggestive of the involvement of peripheral tolerance mechanisms in facilitating long-term transplant acceptance. Moreover, if central tolerance is an all-inclusive process for self-tolerance, then a testable prediction would be to determine the fate of a peripheral self-tissue that was removed and reintroduced into the same immunocompetent host some time later (*i.e.* if central tolerance alone is sufficient, then the tissue should be perceived by the immune system as self despite its temporary absence) (46). Indeed, in certain models the temporary removal of a peripheral tissue led to loss of tolerance to that tissue, indicating the existence of peripheral tolerance (46). Hence, it may be concluded that tolerization of self-reactive T cells initially occurs during thymocyte maturation in the thymus, and later in the periphery with mature T cells. What is not clear is whether peripheral tolerance mechanisms, in addition to dealing with high-avidity T cells specific to peripheral antigens not present in the thymus, could also be responsible for governing low-avidity T cells (47, 48) that escape central tolerance (despite the presence of their cognate antigens in the thymus). However, low-avidity "escapees" are likely to be maintained in an inactive state by clonal competitive effects (49) rather than by *bona fide* tolerance mechanisms that would have evolved to silence high-avidity tissue-specific T cells.

As indicated, some of the evidence in support of peripheral tolerance comes from transplant studies demonstrating the acceptance of an allogeneic graft given to the recipient as a peripheral tissue. However, the rules that govern the immunity/tolerance decision to a peripheral tissue-specific antigen have not yet been elucidated. Since CD4 T cells control a considerable portion of both B cell and CD8 T cell reactivity (28-31), elucidation of tolerance mechanisms in the CD4 compartment will be crucial to understanding tolerance in general. Peripheral tolerance in CD4 T cells represents a unique problem since these cells are only capable of recognizing antigen presented on the relatively few cell types that express MHC class II (DCs, B cells and endothelial cells) under normal circumstances, and the peptide antigens they recognize are derived largely from proteins taken up from the endocytic pathway, rather than from proteins made within the cell (50, 51). Various models have been generated to explain the general rules determining peripheral CD4 T cell tolerance versus immunity and the specific mechanisms of tolerance

involved. However, limitations not surprisingly exist in these models, but these models have nevertheless helped to refine our understanding of peripheral tolerance.

1.2.2. Tolerance theories

In broad terms, ideas about how T cell selection leads to tolerance of self-tissues have moved from a focus on the timing of antigen exposure in the life of the animal, to the timing of antigen exposure in the life of the lymphocyte together with the regulation of selection by antigen-nonspecific signals. Tolerance due to antigen exposure early in development (fetal or neonatal period) began with the ideas of Burnet, together with the seminal observations of Owen (32) and Hasek (34) and the neonatal tolerance studies of Medawar and colleagues (33). Because of the elegant simplicity of the early tolerance window idea, it has been maintained in a small number of current models (52-54) despite accumulating evidence against this view. Notably, a number of studies have shown that a graft given before the development of the immune system still leads to rejection of that tissue (55-61). This is even true for grafts mismatched for as little as one minor histocompatibility (minor-H) antigen (62), and argues strongly against a tolerance window early in life. Instead the most popular current view is that tolerance of self is primarily established by self-antigen encounter during lymphocyte development (*i.e.* central tolerance, as originally proposed by Lederberg (63)) and secondarily by various mechanisms in the periphery. There are of course proponents of the various extremes, including those viewing self-tolerance as almost completely determined centrally (Aire *etc.*) (41-43) and those proposing tolerance to be largely dealt with in the periphery (64). However, most of the current models of peripheral T cell tolerance (**Table 1.1**) can be divided into three categories: 1. Tolerance is the default pathway in the absence of second signals that are activating; 2) Tolerance results from actively induced signals that inhibit T cell activation; and 3. Most peripheral antigens are ignored, only those widely distributed in lymphoid tissues at high enough doses induce tolerance. These in essence are passively, actively and spatially determined mechanisms of tolerance.

Table 1.1. Selected tolerance models and their characteristics as applied to peripheral CD4 T cell tolerance.

Model	Immunity	Peripheral tolerance	Key determining factor(s)
Associative Recognition	Antigen with T cell help; linked recognition	Antigen without T cell help (default); absence of linked recognition	Timing of antigen exposure
Costimulation: Infectious nonself	Antigen with costimulation	Antigen without costimulation (default)	PAMPs trigger costimulation[1]
Costimulation: Danger	Antigen with costimulation	Antigen without costimulation (default)	Endogenous signals trigger costimulation[2]
Costimulation/ Coinhibition	Antigen without coinhibition; costimulation amplifies	Antigen with induced coinhibitory ligands/signals (feedback/quiescence)	Chronic antigen exposure; others not yet defined
Regulatory T (Treg) cells	Antigen without suppression	Antigen recognition in the presence of effector Treg cells; linked recognition	Timing of antigen exposure[3]
Tuning	Signals above threshold required	Continuous signals that only change slowly	Rate of change in many undefined stimuli
Ignorance	Antigen is present in localized lymphoid tissue	Only when antigen is present in high dose throughout the lymphatic system	Antigen location and dose

[1] PAMPs are pathogen associated molecular patterns that are recognized by recipient Toll-like receptors on antigen presenting cells (APCs).
[2] Stressed or damaged cells, but not healthy or apoptotic cells, release molecules that trigger the activation of APCs.
[3] This determining factor is taken from the model of Coutinho and colleagues (65), and does not necessarily represent a consensus view in the field of Treg cells.

1.2.2.1. Tolerance due to antigen encounter in absence of an activating signal

1.2.2.1.1. Associative antigen recognition (linked T cell help)

The cornerstone of this model is the proposal that all lymphocytes acting in a conventional adaptive immune response (CD4 and CD8 T cells, and B cells) require two antigen-specific signals for its induction (53, 66, 67). Consistent with experimental data (12, 13), the first signal comes from antigen engagement by the antigen-specific receptor expressed on a T or B cell. The second signal, however, comes from an effector helper T cell that is also recognizing a linked epitope of the antigen (hence associative antigen recognition) (68). Without a signal from an effector helper T cell, antigen engagement leads to tolerance (by deletion). Thus, in this model key to self-tolerance is the induction of tolerance in self-specific helper

T cells due to the absence of second signals from effector helper T cells recognizing self-antigen in a linked fashion. The absence of effector helper T cells when the immune system is first generated early in life provides an early window of time in which antigen encounter can only lead to tolerance and not immunity. The tolerance established early due to deletion of self-specific helper T cells is maintained throughout life as each new helper T cell that is generated enters a peripheral environment lacking self-specific help. The postulate that helper T cells themselves need help generates a chicken and egg conundrum: where did the first effector helper T cell come from? Cohn and Langman proposed that there is a slow time-dependent spontaneous generation of effector helper T cells, and that self-specific helper T cells would encounter their self-antigen prior to differentiating into an effector cell and therefore would be killed.

This model is supported by studies showing that B cells and CD8 T cells often require linked T cell help for their differentiation into effectors (69-71), and these cells can be tolerized when encountering antigen without help (72, 73). There are other studies suggesting a requirement for T cell help in generating helper T cell responses (74), although these studies and the concepts therein have not impacted on the current design of experiments in immunology. There are downsides to the associative recognition model in explaining self-tolerance. These include the requirement for additional assumptions to explain helper-independent lymphocyte responses and the seemingly unstoppable autoimmunity that would occur each time a newly generated (not yet tolerized) self-specific helper T cell recognizes its antigen on an APC that is also presenting peptides of a pathogen to an effector anti-pathogen helper T cell. We would suggest that the linked recognition of epitopes intrinsic to T cell help and the associative antigen recognition model are perfectly suited to control the effector class (including memory (75)) and magnitude of immune responses, and probably play little if any role in determining somatic selection of the baseline repertoire. Because effector molecules of one class can inhibit the function of another, it is critical that the class of immune response is coherent. This can only be achieved by having class regulated through a cellular communication, and determined on an antigen-by-antigen basis. In contrast, tolerance can be achieved on an epitope-by-epitope basis. Furthermore, when B cells, CD4 T cells or CD8 T cells encounter antigen in the absence of a collaborating cell type (*e.g.* in B cell-deficient mice, or upon CD4 T cell depletion), the responding cell no longer is capable of receiving or inducing the appropriate signals from the collaborating cell when it is returned to the system (76). In this way the factors controlling the class of the initial

response are maintained upon subsequent antigen encounters. In this view the experiments interpreted as demonstrating tolerance due to the absence of T cell help may instead reflect the presence only of a primary response in a class that was not measured, and a lack of memory induction (75). The lack of a cell intrinsic capacity to generate secondary responses probably arose only after this function evolved to be under the control of helper T cells, long after the primary tolerance mechanism(s) had been established.

1.2.2.1.2. Costimulation-based models

While associative recognition has helped in understanding B cell activation, the difficulties in explaining the generation of the first effector helper T cell and other considerations gave rise to costimulation-based models. The idea that antigen-nonspecific signals from APCs provide costimulatory signals for T cell activation originated with Lafferty and Cunningham's model that developed from an explanation for the stronger responses to allogeneic compared to xenogeneic cells (77). They hypothesized that stimulator cells (*i.e.* APCs) present antigens to cognate lymphocytes (signal 1) in the presence of a second (costimulatory) signal supplied by the APCs. Recognition of signal 1 alone by lymphocytes resulted in their inactivation. Because of a lack of experimental support, however, their model was largely ignored and did not gain popularity for many years until a surprising observation was noted by Jenkins and Schwartz (78). Using chemically treated splenocytes as stimulators, they found the responding T cells to become unresponsive, suggesting that the chemical treatment impaired the ability of APCs to provide additional signals to responding T cells that had recognized cognate peptide/MHC complexes. This finding was crucial to the further experimental development of costimulation-based models.

A key weakness of the early costimulation-based models was the lack of control over the expression of costimulatory molecules by APCs. Since APCs are equally capable of presenting host as well as foreign antigens, it is unclear how an APC can distinguish between the two types of antigens and express costimulatory molecules appropriately to activate cognate T cells. To solve this problem, Janeway and later Matzinger offered alternative solutions. Janeway proposed that APCs expressed receptors recognizing evolutionarily conserved molecular motifs found on pathogens (now known as pattern recognition receptors or PRRs), and the recognition of these structures would result in APC activation and expression of costimulatory molecules (79, 80). Hence, self/nonself discrimination was based on self being

noninfectious while nonself antigens included only those antigens with an infectious component. Indeed, experimental support of his model arrived with cloning a few years later of the first Toll-like receptor (TLR), a PRR (81), and since then other TLRs specific for various pathogen-associated molecular patterns (PAMPs) have also been identified (82). In contrast, Matzinger proposed a costimulation-based model known as the Danger model (83) that abandons self/nonself discrimination as the key factor in the immunity/tolerance decision (84, 85). She hypothesized that injured or stressed cells would release endogenous "danger" signals that could activate APCs to become costimulatory, thereby allowing antigens presented on APCs to be recognized by cognate T cells in an immunogenic context. Importantly, her model offered reasons as to why established tumours and organ transplants could be tolerated or rejected by the host immune system (*i.e.* tumours that have been established would not release APC activating signals, while surgical trauma during organ transplantation would lead to their release from injured cells). Importantly, experimental data in support of the Danger model have arisen over the years (62, 86-92).

1.2.2.2. Tolerance due to antigen encounter in presence of an inhibitory signal

1.2.2.2.1. Coinhibition

The proposal that tolerance results from antigen encounter without an activating signal means antigen receptor engagement alone is a negative signal (passive tolerance). Sinclair developed an alternative model in which the antigen receptor signal is positive. In this model costimulation amplifies an immune response rather than reverses an antigen receptor negative signal (consistent with recent *in vitro* studies (93)); costimulation is counterbalanced by receptors that mediate negative "coinhibitory" signals (active tolerance). Coinhibition is a negative signal mediated by an antigen-nonspecific receptor working in concert with an antigen-specific signal through the antigen receptor. Under quiescent conditions, where costimulatory signals are limited, coinhibition may predominate (*e.g.* due to the higher affinity of B7 for Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) compared to CD28). The concept of coinhibitory receptors was derived from ideas about the mechanisms of antibody feedback (94) a number of years before costimulation arrived on the scene. It originated with Sinclair's proposal (95) that B cells have Fc receptors and that coaggregation of the antigen receptor and the Fc receptor shuts off the B cell. The B cell Fc

receptor (FcγRIIB) was thus the first coinhibitory receptor described, and it was found to have a motif in its intracytoplasmic domain common to many subsequently described coinhibitory receptors (96, 97). Coinhibition was expanded to be part of a general model of immune regulation in T cells and B cells (98-100) involving the balance between costimulation and coinhibition by numerous receptors (*e.g.* CTLA-4, Fas, programmed death-1 (PD-1), CD5, CD22, CD72 and interferon-gamma (IFN-γ) receptor). Thus, coinhibitory signals can be delivered either by antibodies, cytokines, or receptor/ligand interactions.

There are now many studies that seem consistent with a role for coinhibitory receptors in peripheral CD4 T cell tolerance, as demonstrated by blocking the coinhibitor or eliminating it by gene knockout (KO) (101-105). Coinhibition has seemingly become the paradigm for CD4 T cell tolerance and tolerance in general (106), and the signal 1 alone models are clearly antiquated. However, there needs to be developed a clear definition of what controls coinhibition if we are to fully understand how it contributes to the immunity/tolerance decision or other potential functions it may have (107). While there are some clear and simple hypotheses for the control of costimulation (control by "danger" signals or PAMPs), there is not yet a corresponding concept for the control of coinhibition, beyond the postulate that widely distributed antigens (leading to chronic antigen encounter) favour coinhibition. Nevertheless, augmenting coinhibitory signals already shows potential as a strategy for tolerance induction in transplantation (108-110).

1.2.2.2.2. Regulatory T cells

Suppression or regulation could be considered a specific subset of coinhibitory signals, as suppression may induce tolerance via antigen recognition in the presence of tolerogenic antigen-nonspecific cosignals. Models that attribute peripheral tolerance to regulatory T (Treg) cells simply restrict the delivery of coinhibitory signals to a specialized T cell subset. While the suppressive mechanism may be antigen-nonspecific (*i.e.* suppression by cytokines such as transforming growth factor-beta or IL-10) (111, 112), Treg cells add an additional dimension in that the coinhibitory signals are thought to be delivered by antigen-specific cells (113). Treg cells, like helper T cells, may act through linked recognition (114, 115). If triggering of Treg cells is antigen-specific, the obvious goal would be to devise a model whereby Treg cells suppress self-reactive but not foreign reactive T cells. Surprisingly, few had even made an attempt, with the only clear model being that proposed by Coutinho and colleagues (65). This model suggests that

newly generated T cells from the thymus are only sensitive to suppression by Treg cells for a short period. In this way most foreign reactive T cells would proceed to the stage where they cannot be suppressed, while self-reactive T cells would encounter their cognate self-antigen and Treg cells during the phase of sensitivity to suppression. Unfortunately this model is not supported by the existing data (85). Moreover, there is accumulating evidence that Treg cells suppress anti-microbial responses just as effectively as anti-self responses (116, 117). TLR ligands (lipopolysaccharide (LPS) and CpG) triggering DCs could overcome suppression by Treg cells (118). Thus, Treg cells have little preference for suppressing self-reactive cells as opposed to foreign reactive ones. It therefore appears that Treg cells do not contribute to the decision making process (self versus foreign/pathogen) that is based on somatic selection of lymphocyte clones. The data suggest that even in the presence of Treg cells this decision seems largely to be governed by the activation of APCs. However, other data seem to indicate an opposing role for TLR ligands when at a higher concentration. It was recently shown that Treg cells express a number of TLRs, one of which is the LPS receptor TLR4, and in the presence of LPS the Treg cells could become activated to control inflammatory responses (119). This would suggest that during an immune response against an invading bacterial pathogen, for instance, Treg cells are important in regulating the magnitude of the anti-pathogen response such that it remains protective in terms of ridding the pathogen but not so strong that there is excessive bystander damage to host tissues (119, 120). In this way, Treg cells may be part of a negative feedback mechanism that limits the overall magnitude of any response, whether it is self- or pathogen-specific. They may also play a role in the maintenance of memory (121).

While previous failures in identifying the genetic loci corresponding to Treg cell function led to the downfall of the Gershon and Kondo initiated concept of Treg cell (or suppressor T cells as used during that time period) existence (122, 123), the recent discovery of Foxp3 as a key transcriptional factor for Treg cell development (124) arguably provides the strongest evidence yet that Treg cells exist as a critical differentiation stage or even as a distinct cell type in the overall T cell repertoire. This discovery offers strong support to previous studies demonstrating the production of Treg cells via thymic selection (125), as well as sheds light on studies that argue for Treg cell generation by multiple distinct pathways (126). Defects in Foxp3 lead to lymphoproliferation and autoimmunity (5, 6). However, similar to defects in CTLA-4 and Fas, it is not clear whether these autoimmune diseases truly represent a breakdown in

peripheral tolerance to tissues-specific antigens or simply a generalized defect in lymphocyte control such that nonspecific, tissue damaging inflammatory events ensue.

1.2.2.3. T cell tuning

While the theory of tuning activation thresholds was developed many years ago (127, 128), it has only been experimentally examined recently in the context of peripheral CD4 T cell tolerance. It was postulated that T cells are able to constantly "tune" (adjust) their signaling based on the ambient level of signals present in the environment. This tuning sets the threshold level of signals required to activate a T cell upon subsequent encounters with cognate antigen. In the face of changing levels of antigen the T cell retunes and resets the threshold. In this view, the trigger for a response is the rapid change in the levels of signals (including antigen levels) such that the T cell does not have sufficient time to readjust the threshold needed for activation. Evidence that CD4 T cells within a monoclonal repertoire can make such adaptations was recently provided (129-131). That the level of tolerance attained may vary depending on the level of antigen thus appears likely. However, this appears more to be a detail of mechanism rather than the basis of an explanation for the negative selection of self-reactive T cells and the maintenance of pathogen-specific cells. Since rapid changes in the level of antigen alone are not sufficient to trigger a response, it seems more likely that it is the change in the level of other signals (*e.g.* costimulatory signals) that is key.

1.2.2.4. Ignorance

In terms of self-tolerance, the ignorance model of Zinkernagel and colleagues suggests that tolerance to self is induced when any antigen is presented for a sufficient time and dose throughout lymphoid tissue (132). Thus all self-antigens that normally are found within the central and peripheral lymphoid tissues will induce tolerance. The ignorance model provides a novel explanation for the regulation of immunity in the periphery. It suggests that peripheral tissue-specific antigens are ignored because they reside in parenchymal tissues and are not expressed or presented by migratory cells in lymph nodes at sufficient levels. Induction of a productive T cell response only occurs when antigen reaches a localized lymphoid tissue in sufficient amounts for a sufficient period of time. Encounter with antigen outside lymphoid tissue leads neither to immunity nor tolerance (*i.e.* ignorant T cells remain "naïve").

Although ignorance of peripheral antigens has been suggested experimentally (133-137), it may only exist for some peripheral self-antigens. It remains unclear whether ignorance is a generalizable phenomenon or a rare exception. However, the current evidence appears to favour tolerance rather than ignorance of most transgenically or naturally expressed peripheral self-antigens presented in the context of both class I (138) and class II (139). The proposition that CD4 T cells are ignorant of tissue-restricted peripheral self-antigens is perhaps the most dubious aspect of Zinkernagel's model. If self-reactive T cells are ignorant rather than tolerant, each time a peripheral tissue is infected the self-antigens of that tissue will be taken up by local APCs and presented in the draining lymph node resulting in immunity. Since CD4 T cells, in the absence of a CD8 response, are capable of significant tissue destruction, autoimmune disease would be extremely frequent in an immune system governed by ignorance.

1.2.3. Tolerance models

It has been difficult to study peripheral tolerance in CD4 T cells *in vivo*, therefore much of the early studies were done *in vitro* with T cell clones. As predicted by the two-signal model of Lafferty and Cunningham (77), these data suggested that helper T cell activation requires a signal triggered by specific antigen (peptide/MHC) binding to the TCR and antigen-nonspecific signal(s), termed costimulation, delivered by an APC. In the absence of costimulation, the T cell clones instead became unresponsive (78) (anergy; defined as an intrinsic state of functional inactivation of a T cell following antigen encounter, which is associated with persistence of the T cell in a hyporesponsive state (140)). However, it is not clear that the *in vitro* models reflect tolerance mechanisms occurring *in vivo*. In contrast to the many elegant studies of helper T cell tolerance to ubiquitous self-antigens (141, 142) and breakdown in tolerance (143-147), there have been few studies of the successful establishment of peripheral tolerance in CD4 T cells. The study of *in vivo* CD4 T cell tolerance (**Table 1.2**) to peripheral tissue-specific antigens has been faced with two major hurdles: 1. The lack of clearly identified tissue-specific antigens; and 2. The absence of methods to follow the antigen-specific T cells in the sea of T cells specific to other antigens. Survival of viral superantigen reactive T cells in bone marrow chimeric mice provided a system to study the tolerant state in peripheral CD4 T cells (148). However, since the antigens were also encountered in the thymus, this model did not allow the study of tolerance to uniquely peripheral antigens. By blocking thymocyte encounter with

superantigens using antibodies to I-E, and releasing the autoreactive cells into the periphery, Jones and colleagues studied the establishment of peripheral tolerance once antibody treatment had been terminated (149). Although they observed a dramatic peripheral deletion of the relevant Vβ-expressing CD4 T cells and CD8 T cells, the widespread distribution of the deleting antigen does not reflect the distribution or dose that is likely for normal tissue-specific peripheral antigens. Furthermore, in other studies the "absence of antigen" problem was overcome by simply injecting an antigen into an animal. If unresponsiveness is induced, it was assumed that the mechanisms involved reflect those that would normally occur during tolerance to tissue-specific peripheral self-antigens (150-152). However, this is a large assumption as the distribution of an injected antigen, timing of its presence in ontogeny, quantity, and cells presenting the antigen, are all likely to be different from the natural situation. In the case of intravenous injection of soluble antigens/peptides, the resultant short half-life of antigen is also unlikely to mimic most true peripheral self-antigens. An approach that removed many hidden assumptions was the development of antigen transgenic (Tg) mice using tissue-specific promoters. However, this approach has not always been successful in generating a truly peripheral antigen, as thymic expression often occurs, making it difficult to analyze peripheral tolerance independent from previous antigen encounter in the thymus (153-155).

Another approach to study peripheral tolerance, one that arrived serendipitously rather than through planned tolerance studies, is the use of certain gene KO and spontaneous mutant mice that appear to develop autoimmune disease. Examples include the spontaneous *lpr* or *gld* mutant (156) or CTLA-4-KO mice (157). Studies in these systems have provided some experimental support for the view that coinhibitory molecules are critical for peripheral tolerance. However, these knockouts display generalized defects, including in some cases a loss of tolerance to ubiquitous nuclear autoantigens. They have not been shown to have a particular loss of tolerance to natural tissue-specific antigens that would lead to tissue-specific autoimmune diseases such as type 1 diabetes. Thus, it is not fully clear whether these molecules are involved in the establishment of tolerance to tissue-specific antigens in the periphery, or alternatively play a role in homeostatic mechanisms or regulation of effector class. More recently, knockouts for other molecules, such as PD-1 (103), have revealed coinhibitors that may more directly play a role in tolerance to tissue-specific peripheral antigens.

Table 1.2. Summary of different *in vivo* models to study peripheral tolerance.

Model	Antigen	Pros	Cons
Bone marrow chimera	Endogenous viral superantigen	Antigen-reactive T cells can be tracked by specific Vβ expression	Widespread distribution of antigen, including in the thymus where central tolerance could occur
Systemic injection of antigen	Any	Wide selection of model antigens to study tolerance induction	Distribution (antigen can reach the thymus to induce central tolerance), timing of presence, half-life and quantity of antigen do not mimic a natural peripheral antigen
Antigen transgenic	Allogeneic MHC (tissue-specific promoter)	Antigen-reactive T cells can be tracked by specific Vβ expression	1. MHC molecules are not one but hundreds of antigens (high frequency of antigen-reactive cells) 2. Non-tissue-specific expression can occur (antigen in thymus) 3. In tolerance or immunity, T cells restricted to direct allorecognition would not be capable of indirect allorecognition (and vice versa)
TCR transgenic cells into antigen transgenic hosts	Non-MHC antigen (*e.g.* hemagglutinin)	1. System can be manipulated to reduce frequency of antigen-reactive T cells 2. TCR transgenic cells can be tracked by clonotypic antibodies	1. TCR transgenic cells not on a RAG-KO background (expression of a second TCR may alter fate of T cell after antigen encounter) 2. Restricted presentation of transgenic antigen (*e.g.* B cells only) 3. Non-physiological level of antigen being expressed or non-tissue-specific expression
Transplantation	Single minor, multiple minor, or MHC plus minor antigens	1. Physiological level of antigen expression 2. Frequency of antigen-reactive T cells can approach that to a nominal antigen (*i.e.* single minor mismatch)	1. T cells restricted to direct allorecognition would not be capable of indirect allorecognition (and vice versa); problem with APC depletion 2. Costimulatory blockade studies: if immunity, this may be due to provision of costimulatory signals that have not been blocked by the antibodies used 3. Graft healing: a need for empirical determination of healing time that is sufficient for tolerance

1.2.3.1. Transgenics

Initially MHC molecules were the antigens of choice for peripheral Tg expression. This is because a high frequency of cells can react to an allogeneic MHC (23) and T cells reactive with certain MHC molecules can be tracked because they express a specific Vβ. Lo and colleagues expressed MHC class II molecules on beta cells or acinar cells of the pancreas (158-160) and studied the TCR Vβ family that reacted with the MHC class II. While they found that the T cells were not deleted but instead became anergic (160, 161), others who generated similar models did not find anergy (162-164). Thymic expression of the transgene may have been responsible for the anergic state; however, the discrepancies between the various studies raise a difficulty in interpreting MHC Tg mouse models. Unlike normal peripheral self-antigens, Tg MHC molecules on a peripheral tissue are in effect hundreds of antigens because there is no central tolerance to the Tg MHC and its multitude of associated peptides. Another major drawback of MHC Tg studies relates to the MHC-restricted nature of antigen recognition by T cells (21, 22). T cells specific for intact Tg MHC molecules (direct pathway of allorecognition) are not able to recognize the processed peptides of the Tg MHC that are presented by APCs expressing host MHC (indirect pathway of allorecognition). However, host APCs could either pick up and present peptides of the Tg MHC on their own MHC class II, or alternatively, on MHC class I (*i.e.* crosspresentation). While crosspresentation had been seen many years ago (165), evidence has only appeared recently that it may play an important role in peripheral tolerance to tissue-restricted antigens (139, 166-169). Thus, MHC Tg models miss any tolerogenic effects via antigen presentation on host MHC, a process that allows tissue APCs to pick up and present tissue-specific antigens. However, initial studies using mice with Tg expression of the lymphocytic choriomeningitis virus glycoprotein in the pancreas, where both pathways of presentation could occur, appeared to show that T cells were ignorant rather than tolerant (133). The reason for ignorance was unclear, but may reflect an important role for antigen expression level, as tolerance has been observed in other systems (170).

Difficulties in visualizing tolerance mechanisms can be overcome by using mice with a monoclonal T cell population specific for a self-antigen. This has been achieved with TCR Tg mice that are either on the Severe Combined Immunodeficiency (SCID) background or lacking a functional recombination activating gene (RAG) protein in order to prevent endogenous TCR gene rearrangements. However, few studies have used such mice to study peripheral tolerance in CD4 T cells. In some studies

TCR Tg mice specific for hemagglutinin (HA) in I-E^d were combined with viral HA antigen Tg mice, expressing HA on hematopoietic cells or numerous tissues (139, 171, 172). Antigen expression in the thymus could be detected thus necessitating transfer of TCR Tg cells into antigen-expressing hosts. In both studies hyporesponsiveness *in vitro* (anergy) was observed. However, these studies are difficult to interpret for a number of reasons. The TCR Tg mouse employed in one set of studies was not on a SCID or RAG-KO background (139, 172), and therefore some of the T cells would have expressed endogenous TCRs as well as the Tg TCR. Expression of a second TCR is likely to alter the outcome of antigen encounter (173), as has already been shown for antigen encounter within the thymus (174). Indeed, using the same Tg TCR along with different HA antigen transgenics (125, 175), escape from negative selection in the thymus by T cells expressing endogenous TCR-alpha chains was found (176). In these studies it could not be established whether the tolerant state in the periphery was true peripheral tolerance or a state induced during nondeletional encounter with antigen in the thymus. Another difficulty lies in the site of expression of the target antigen. It is becoming clear that the outcome of antigen presentation (tolerance or immunity) is greatly influenced by the type of cell presenting the antigen (177-181). In the Lanoue *et al.* study, the vast majority of cells presenting the self-antigen were B cells that would not be expected to present naturally occurring peripheral self-antigens. The quantity and wide distribution of antigen in all of these studies are likely to have influenced the outcome. Most truly peripheral (not present in the thymus) self-antigens would not have a wide distribution but instead would be found within a particular organ/tissue or the lymph node draining that tissue (166, 182). Similar difficulties due to antigen/tissue distribution and/or endogenous TCR gene rearrangements also apply in earlier work with systemically injected peptides (151) and in other recent studies of CD4 T cell tolerance (129, 183). However, such studies may have relevance for understanding tolerance to a small subset of systemic self-antigens that putatively only arise later in life after the full development of the T cell repertoire (151). A study by Forster *et al.* may provide the best hints about peripheral CD4 T cell tolerance. Using Tg mice expressing the SV40 T antigen (TAg) in the pancreas and Tg CD4 T cells specific to TAg (184), they found that the T cells were not deleted in the thymus early in life but were either deleted or partially anergic in the periphery. The tolerance was only partial, as insulitis without diabetes was present and tolerance did not occur when the T cell population was monoclonal (endogenous TCRs excluded by crossing the host to a RAG-KO background). Thus, Forster's

study was the first to eliminate the confounding variable of endogenous TCR expression when analyzing CD4 T cell tolerance using TCR Tg cells. However, since interactions between T cells of different specificities greatly influence the outcome, it is not clear that such studies with a monoclonal repertoire will reflect the outcomes/mechanisms that occur under the physiologic conditions of a polyclonal repertoire. Clarification of these issues will require seeding small numbers of monoclonal TCR Tg T cells of the RAG-KO background into a normal repertoire and tracking their fate.

Combined use of TCR and antigen transgenics has also been employed to test what signals are involved in self-tolerance: Does tolerance result from antigen receptor engagement alone (signal 1) or are coinhibitory receptors necessary to maintain self-tolerance? While the lymphoproliferation and systemic autoimmune-like phenotypes in CTLA-4-KO and Fas-deficient mice suggest they play a critical role in self-tolerance, there has been little in the way of direct evidence for a preferential loss of peripheral tolerance in these animals (versus nonspecific heightened responsiveness). Abbas and colleagues has begun to address this issue by transferring wild type (WT) versus CTLA-4-KO CD4 anti-hen egg lysozyme (HEL) TCR Tg T cells (not on RAG-KO background) into mice expressing HEL in pancreatic beta cells with subsequent immunization using HEL in various adjuvants (185). They compared T cell activity at the site draining the neo-self-antigen and at the site where HEL was introduced in immunizing form. CTLA-4 deficiency led to increased accumulation of Tg T cells in the lymph node draining the pancreas but not draining the site of immunization, and this accumulation was associated with ensuing autoimmune diabetes. However, diabetes could be induced even with WT Tg T cells if stronger adjuvants were used. These data suggest the possibility that CTLA-4 may function to allow self-tolerance to be maintained in the face of concomitant immunity by T cells crossreactive to pathogen-associated and self-epitopes. Thus, the balance between costimulation and coinhibition can be tipped towards immunity/autoimmunity with sufficiently strong adjuvants such as complete Freund's adjuvant. Further experiments are needed to fully determine if coinhibitory signals from CTLA-4 or other coinhibitors act constitutively or are regulated such that they act predominantly to prevent self- but not pathogen-specific reactivity. The critical question is whether the immunity/tolerance decision is determined by constitutive coinhibition together with inducible costimulation (*i.e.* costimulation alone is the determining factor since coinhibition is always active) or by a system where both coinhibition and costimulation are inducible and both can play a determining role.

One common feature to all *in vivo* tolerance studies using TCR Tg mice thus far is that the T cells that are eventually deemed to be tolerant (whether anergized or deleted) arrive at this state after having made what appears to be a conventional immune response, including both proliferation and differentiation (at least in terms of changes in surface markers). This is not what one would predict if peripheral tolerance were simply about deleting self-reactive specificities from the repertoire (*i.e.* why waste energy proliferating?). It instead suggests that the peripheral immunity/tolerance decision is one of determining the magnitude of response, and not a question of whether there will be a response. The response versus no response decision may instead be determined by the presence or absence of a given specificity in the repertoire, a property largely if not completely determined during thymocyte selection. We cannot however rule out the possibility that peripheral deletion does substantially shape the repertoire of T cell specificities. If this is the case, proliferation prior to deletion (tolerance induction) may indicate that the gap that evolved between tolerogenic and immunogenic presentation is quite narrow. In the context of a costimulation-based model this would mean that it is not a black-and-white situation of self-antigens presented on resting APCs and pathogen-associated antigens on activated (costimulatory) APCs. Instead, naïve T cells may have to "add" up the sum of a number of encounters with an APC presenting the cognate antigen before a final decision between tolerance and immunity can be made. In this way, for example, the summation of encounter with 8 resting and 2 activated APCs presenting antigen will lead to some cell division but eventual tolerance, while encounter with 5 resting and 5 activated APCs may lead to sustained immunity. There may also be a summation in signals from separate sequential costimulatory signals that prevents premature termination of the response (186). We do not yet have a handle on how wide the divide is between tolerogenic and immunogenic presentation. Elucidation of these quantitative aspects will increase the precision of our description of immunity versus tolerance, but whether they will also force a change in overall conceptual models remains to be seen (14).

1.2.3.2. Transplantation models

Expression of antigens under the control of tissue-specific promoters has permitted peripheral tolerance to a defined antigen to be studied without knowledge of natural tissue-specific antigens. The approach however is not without some drawbacks. It is not always clear that the antigens are expressed at physiologic levels

or in appropriate sites and the forced expression of an antigen not normally expressed by a particular cell type may have adverse affects on the function/viability of the cell independent of any immune response (158, 164). Another less traveled path to generate a model peripheral antigen that may overcome some of these deficiencies is to graft a tissue naturally expressing its own histocompatibility antigens. The obvious difficulty with using grafts as the source of a model peripheral antigen is that grafts normally induce immunity rather than tolerance. However, they provide an excellent model with which to test our ideas of peripheral tolerance mechanisms, for if we truly understand what it is that makes grafts but not self-tissues trigger an immune response, it should be feasible to block the trigger and generate tolerance. The most successful method of inducing transplantation tolerance is through the generation of systemic chimerism with donor cells; tolerance induced is predominantly central rather than peripheral. In contrast, approaches that have focused on blocking costimulation, including donor APC depletion, blockade of costimulatory receptors/ligands by antibodies/fusion proteins, and removing the signals that lead to APC activation and upregulation of costimulatory molecules, would be more relevant for understanding peripheral tolerance.

Donor leukocytes have long been considered important in stimulating an anti-graft response. One primary difference between a graft and a normal peripheral self-tissue that may contribute to the different ways these two entities are treated by the immune system is the presence of APCs in the graft (but not the self-tissue) that express antigens to which the immune system of the recipient is not centrally tolerant. In contrast, the parenchymal tissue of both the graft and a peripheral self-tissue expresses antigens to which recipient T cells are not centrally tolerant. Thus, simply eliminating the donor APCs should put the graft and the self-tissue on a more equal footing. Depletion of donor APCs from transplants leaves only parenchymal tissue, and not resident APCs, expressing the graft antigens. Thus, the distribution of cells expressing graft antigens becomes the same as for cells expressing a peripheral self-antigen. However, donor APC depletion is unlikely to fully mimic natural peripheral tolerance. It leads to graft acceptance with many MHC-mismatched grafts (187-195) but not reliably with MHC-matched, minor-H-mismatched grafts (196). Similar to the difficulties encountered with MHC Tg studies, donor APC depletion does not take into account the natural role of antigen presentation by host APCs (with or without crosspresentation). When the MHC is matched, both donor and recipient APCs are able to present donor minor-H antigens in the same MHC haplotype. Therefore, removing the donor APCs would not fully block presentation of

donor antigens to T cells capable of directly recognizing the graft. Antigen presentation by host APCs would still occur and would be capable of activating T cells that can directly recognize donor cells and their peptide/MHC complexes. In contrast, with MHC-mismatched grafts, removal of APCs leads to a loss of function because T cells with direct specificity/restriction for donor MHC molecules cannot be primed by APCs presenting donor antigens on recipient MHC; conversely, T cells capable of recognizing donor antigens in recipient MHC molecules are not able to directly recognize the donor tissue and reject it (46). In the long-term, antigen presentation by host APCs may be tolerogenic, but as before, T cells that directly recognize the mismatched donor MHC antigens cannot recognize the donor antigens when presented on recipient MHC molecules. Thus, MHC-mismatched grafts that are depleted of donor APCs do not induce tolerance in T cells that directly recognize graft antigens (195).

A way to tackle the contribution of both donor and recipient APCs is to block their ability to send costimulatory signals. The view that inducible costimulatory signals provided by APCs are necessary for a functional immune response has become the leading paradigm (79, 197-201). Blockade of some of the costimulatory pathways (CD28/B7 and CD40/CD40 ligand) has achieved some success in various transplant models (202-208). The mechanism of graft acceptance/tolerance achieved by this method may involve both deletion of reactive cells and class regulation (immune deviation) of the remaining cells (201, 206, 209-213). However, this method has not been fully successful in inducing long-term donor-specific tolerance (as defined by challenge with second donor grafts) under the most stringent conditions. This may reflect the possibility of other costimulatory pathways not blocked by the treatment taking effect (214, 215) or that the mechanisms of peripheral tolerance do not have the capacity to deal with the large repertoire of responding cells in the alloreactive population. The synergistic effects of recipient lymphocyte depletion and costimulation blockade support the latter possibility. One of the most promising recent approaches combines lymphocyte depletion and blockade of T cell growth factor signals generated by IL-2 and IL-15 using lytic receptor agonist (IL-2) and antagonist (IL-15) fusion proteins in combination with sirolimus to block IL-2-induced proliferative signals, while maintaining the pro-apoptotic activity of IL-2 (216). This method indicates that blockade of signals downstream of costimulation can also help to achieve tolerance.

Another approach to make peripheral graft antigens be treated like self-antigens is to reduce the signals that generate the costimulation that triggers graft rejection. This approach is based on the view that

costimulatory signals are stimulated by one of two general sources. Costimulatory signals may be triggered either by TLR recognition of PAMPs or by endogenous signals from stressed or damaged cells. In the latter situation graft rejection could be explained by the surgical tissue damage that is likely to trigger costimulation in both donor and recipient APCs. However, since the repertoire of T cells responding to a fully allogeneic graft includes memory cells, it is likely that these cells were triggered by crossreactive antigens associated with inducers of costimulation. Thus, in this case it does not seem possible to block all the relevant costimulation inducers since some of the costimulatory activation of T cells had occurred before transplantation. To generate a model where all potential inducers of costimulation-triggered rejection can be controlled it seems necessary to use a graft that has only a small number of minor-H mismatches. In this way the response of a naïve anti-graft repertoire may be analyzed for conditions that lead to peripheral tolerance. We recently took this approach and asked whether allowing a single minor-H-mismatched graft to heal into an immunodeficient recipient (the male antigen H-Y in a female host), so that tissue damage and its associated APC activating signals had time to dissipate, would allow the graft to be treated as self. We found that even long-healed grafts were rejected after the recipient immune system was reconstituted (61, 62). Thus, surprisingly the graft antigens were not treated like self-antigens despite the small mismatch and despite allowing the graft to heal in prior to allowing the immune system to encounter it. Rejection may have been due to some long-term abnormalities in the grafted tissue (transcriptional analysis of the grafted tissue provided some support for this possibility (62)), or the origin of the grafted tissue. We and others had chosen skin grafts for these types of experiments and it may be difficult to eliminate all inducers of costimulation from an external tissue such as skin, given its continuous exposure to microbes and potential tissue damage (scratching *etc.*). A recent study supports this latter possibility as female recipient mice were unable to reject male skin grafts from donors defective in TLR signaling (217). Thus, a more critical test will require allowing an internally placed graft (*e.g.* heart or islets) to heal in and then see if it could induce peripheral tolerance in a newly generated immune system.

Waldmann and colleagues observed dominant peripheral CD4 T cell tolerance to multiple minor-mismatched skin grafts that were transplanted onto recipients given a tolerance inducing antibody regimen. The involvement of a dominant mechanism mediated by linked suppression was supported, in one study, by long-term acceptance of (donor × third party)F1 skin grafts (114) and by transfer of the tolerant state to a

naïve host in a second study (218). In a third study, they showed that tolerated skin grafts carried passenger T cells capable of preventing rejection (219). While the combination of these studies demonstrated robust dominant tolerance to multiple minor-mismatched grafts, it is not yet clear why the antibody treatments induced such a state or whether this state in any way mimics natural peripheral self-tolerance. Nevertheless, application of infectious tolerance would be ideal for clinical tolerance protocols in transplantation and autoimmune disease, as these mechanisms alleviate the necessity of knowing all the target antigens involved; tolerance should spread to any unrecognized donor antigens or autoantigens.

1.2.3.3. Relevance of CD8 T cell tolerance studies on CD4 T cell tolerance

Some studies of CD8 T cell tolerance suggested ignorance of peripheral self-antigens (133, 134, 220). In another study, the transfer of Tg T cells specific to a widely distributed antigen in the recipient (anti-H-Y CD8 T cells into a male mouse) resulted in their expansion and then deletion (221). However, deletion was not complete and did not result in reduced T cell numbers below that originally injected; the remaining cells appeared anergic. In contrast, TCR Tg CD8 T cells specific to K^b expressed on liver were not ignorant or anergic but instead induced autoimmune tissue destruction and underwent deletion (222). Evidence of tissue destruction and deletion was also observed when sufficiently high numbers of TCR Tg T cells recognizing ovalbumin (OVA) on MHC class I were transferred into hosts that transgenically expressed OVA in islets (138, 223). It is not clear whether all aspects of these studies may be generalized to CD4 T cell tolerance. However, it is likely that increased frequencies of autoreactive cells will lead to tissue destruction when CD4 T cells are employed just as in the above CD8 T cell experiments. In other studies, CD8 T cell peripheral tolerance was associated with downregulation of the CD8 coreceptor (224).

While tolerance has been discussed thus far in simplified terms as an "all-or-none" phenomenon, the reality is that there will be a threshold of avidity for tolerance; self-specific cells of sufficiently low avidity will escape the tolerance mechanisms. The functional relevance of such cells is highlighted by studies of Sherman and colleagues (225). They described an aspect of CD8 T cell tolerance that likely applies to CD4 T cells. Tg mice with HA expressed in the pancreas were functionally tolerant of HA in that they accepted an HA-expressing tumour while non-Tg mice did not. However, immunization with virus expressing HA led to tumour rejection even in HA Tg mice. Rejection was associated with the

production of low-avidity cytotoxic T cells specific to HA that presumably eliminated the tumour and yet did not cause destruction of pancreatic beta cells expressing HA. This suggests that tolerance versus immunity is not simply a consequence of clonal selection of receptor specificities resulting in the physical or functional elimination of self-reactive cells. Self-reactive cells can be functional at eliminating an invader (in this case a tumour) without significant destruction of self-tissues. Thus, the maintenance of such low-avidity self-reactive T cells if crossreactive to an epitope of a pathogen may provide a survival benefit. Tolerance of self-tissue may be maintained either because of its "healthy" state (no APC activating signals), or because of a lower expression of the relevant epitope.

Existing models of CD8 T cell tolerance not only provide potential clues as to the mechanisms that may be operational in CD4 T cell tolerance but they also suggest new ways to study CD4 T cell tolerance. Pregnancy as a model for examining peripheral tolerance is one such example and it is arguably a very natural (less manipulated) system for studying peripheral tolerance mechanisms. Since the appearance of fetal antigens only occurs long after the generation of the maternal T cell repertoire, central tolerance cannot play a primary role in the acceptance of the fetus. It is therefore possible that mechanisms of tolerance to the fetus may be similar to mechanisms involved in tolerance to certain self-antigens that have been hypothesized to appear only late in ontogeny (226). MHC class I-restricted TCR Tg mice have made it possible to monitor anti-paternal CD8 T cells during pregnancy. These studies found that maternal tolerance to fetal alloantigens was transient involving a temporary reduction/deletion of cognate T cells (227), and induction of unresponsiveness in the undeleted T cells (228).

1.2.4. Discussion

The ideal system for studying mechanisms of peripheral CD4 T cell tolerance should have the following characteristics: 1. Expression/presentation of a defined peptide antigen (not allogeneic MHC) restricted to a single peripheral tissue, and whose timing of expression in the life of the animal can be controlled; 2. Methods of studying MHC class II-restricted T cell effector responses to the peripheral antigen (not just proliferation); 3. Methods of following the fate of the antigen-specific T cells expressing a single TCR (a condition not fulfilled by the TCR transgenics that allow endogenous TCR expression); and 4. Defined conditions under which the antigen induces tolerance versus immunity. Most of the studies reviewed above

demonstrate some but not all aspects of the ideal model. Consequently, the generalizability of the conclusions drawn from each model remains in doubt. Did the immune system evolve multiple mechanisms of peripheral tolerance (deletion, anergy, regulation *etc.*), or is it simply our variable experimental systems that lead to the variable answers while the natural course included only a single chosen mechanism? In terms of the physical or functional selection of the T cell repertoire into the good clones (anti-pathogen) and the bad clones (anti-self), no compelling argument has been put forward to account for the multiple mechanisms observed experimentally. Once the first peripheral selection mechanism to arise was in place there would seem to be little selective pressure for additional mechanisms. Hence, the additional mechanisms apparent in various models may have a different function, perhaps in controlling the expansion/contraction or differentiation of clones from the already determined steady state repertoire (67). This control of the magnitude and class of response could equally well be important in maintaining host integrity, and defects in these mechanisms could lead to self-destruction. Thus, it may be possible to define the mechanism(s) involved in the baseline repertoire selection, and separate mechanisms that control the use of the clones within that repertoire. It is still controversial, however, whether the baseline peripheral repertoire is largely if not completely shaped by central selection events or if peripheral selection also plays a large role. Recent data have revealed a causal relationship between the absence of expression of a single, defined peripheral antigen due to thymic Aire deficiency, and the induction of autoimmunity specific to the same antigen (45, 229, 230). This suggests that at least for some peripheral self-antigens, thymic negative selection of self-specific T cells would be necessary and sufficient to prevent autoimmunity without peripheral selection of the repertoire. Moreover, breakdown in self-tolerance due to Aire deficiency can synergize with other tolerance defects to exacerbate autoimmunity (231).

If shaping of the repertoire does not occur peripherally, then control of expansion/contraction and differentiation of the established baseline repertoire is the sole means of preventing peripheral autoimmunity. The conventional view instead would be that both central and peripheral tolerance determines clonal specificity of the baseline repertoire. Which view is correct can only be answered by determining whether or not the frequency of autoreactive cells is higher in the pool of recent thymic emigrants when compared to long established peripheral T cells. Besides recessive tolerance, dominant tolerance induced by natural and/or induced Treg cells remains a popular alternative (232). Since their

suppressive function can apply to immunity or tolerance (116, 117, 121), the evolutionary significance of Treg cells in repertoire selection remains in doubt. The importance of addressing this issue has been persistently overshadowed by the quest to employ Treg cells in such areas as transplantation tolerance, based on a large body of promising experimental data (233). A challenging area for the future will be to define the function of each costimulatory or coinhibitory molecule. It is unlikely that they function redundantly (234), and elucidation of each of their specific functions and the hierarchy of their effects is likely to give us a much clearer picture of both peripheral tolerance and class regulation.

1.3. APPLYING TOLERANCE IN ISLET TRANSPLANTATION

The road to transplantation as a means to treat disease spans more than a century worth of effort, beginning with experimental kidney transplantation in dogs in 1902, followed by the first successful human kidney transplantation between identical twins over 50 years later (235). Transplantation has become increasingly prevalent since then. Many different types of grafts from either cadaveric or living, allogeneic or syngeneic donors can be given (236). With allogeneic transplants, graft survival at 1 year is consistently high across the different types of transplants but more variable at 5 years and progressive worse at 10 years (236, 237). This is in spite of the concomitant use of immunosuppression (237, 238).

Transplantation has also become an important treatment option for type 1 diabetics who have lost their insulin producing pancreatic beta cells via autoimmunity. Islet transplantation was shown to provide stable insulin independence in humans (239) after the success observed in smaller animals (240). Indeed, islet transplantation has become a global phenomenon (241, 242). However, the high rate of graft survival and insulin independence at 1 year is not sustained at 5 years potentially due to immunological rejection and/or other mechanisms (243), and the use of multiple islet donors for transplantation has led to a high degree of allogeneic sensitization (244). Given the success of islet transplantation even in the short term, in combination with the early success in nonhuman primate studies in which xenogeneic islets were tested as an alternative tissue source (245, 246), the consensus view from an immunological perspective appears to favour tolerance induction in the recipient (240, 247, 248) so as to prevent islet rejection.

While type 1 diabetes is an autoimmune disease, rejection of allogeneic islets is likely the combined result of autoimmunity and alloimmunity (249), thus creating a greater challenge to tolerance

induction. Alloimmunity, or allogeneic resistance, is the immunological resistance mounted by one organism against antigens expressed by a genetically disparate organism of the same species that largely depends on adaptive immunity evolved to exist in jawed and even jawless vertebrates (1). Experimental models for testing the effectiveness of a given tolerance induction strategy often begin in nonautoimmune animals to assess its ability to block alloimmunity, and if successful, in autoimmune hosts in which alloimmunity and islet-specific autoimmunity must be abrogated. The nonobese diabetic (NOD) mouse (250) is often the model of choice in this latter assessment. NOD mice spontaneously develop hyperglycemia due to islet-specific autoimmunity mounted by T cells that destroys pancreatic beta cells; the incidence of diabetes is dependent on age, gender and environmental conditions (251). Importantly, NOD mice have been preferred to understand human autoimmune diabetes due to many similarities shared between mice and humans in the disease each species develops (252, 253). Similar to the human disease, islet-specific autoimmunity in the NOD model is a multi-factorial process.

Interestingly, while we have suggested that presentation of peripheral self-antigens to induce natural tolerance is unlikely the role of B cells, B cells appear to play an important role as APCs in the development of autoimmune diabetes (254-256), but its requirement is controversial (257). Moreover, B cells alone may not be sufficient to mediate a breakdown in self-tolerance in NOD mice (258). Nevertheless, even though they could develop a reduced level of insulitis (259, 260), B cell-deficient NOD mice either do not manifest hyperglycemia (259, 261) or are overtly diabetic at a reduced rate (260). The ability of NOD B cells to disrupt self-tolerance may be related to the presence of a defective B cell pool that activates autoreactive T cells (258, 262). NOD B cells are potentially better able to activate T cells due to their dysregulated expression of costimulatory molecules (263). Additionally, NOD mice have been found to have a multitude of other defects in their immune system (264). Hence, unlike nonautoimmune mouse strains, induction of long-term graft acceptance in NOD mice is unusually challenging because of the combined problems of alloimmunity, autoimmunity, and various other immunological defects. However, the inherent difficulty in tolerizing the NOD immune system may be considered advantageous, since arriving at a successful solution for inducing robust tolerance in NOD mice offers considerable promise to generate islet transplantation tolerance clinically.

1.4. OVERVIEW OF MY THESIS

Different mechanisms have been proposed and observed for inducing peripheral self-tolerance, but it remains unknown whether there exists one prevailing mechanism of peripheral tolerance or multiple mechanisms are required. Studying the host response towards a tissue transplant placed prior to the generation of lymphocytes is a powerful approach for modeling tolerance induction to a peripheral antigen during immune system development. Not only does such a model provide a better understanding of self-tolerance but this knowledge is potentially applicable to transplantation tolerance. By varying the degree of mismatch between donor and recipient, it is possible to define the capacity and limit of peripheral tolerance in generating tolerance to a peripheral tissue. In many previous studies of host response to transplant alloantigens, the importance of chimerism in allogeneic tolerance has often been observed, but it is unclear whether chimerism is in fact necessary and sufficient, or whether peripheral tolerance can alone deal with alloantigens. In my studies, the immunity/tolerance decision on an islet transplant is examined to gain insight into both peripheral self-tolerance and allogeneic tolerance. The data in this thesis are presented in three themes: 1. Testing for tolerance induction to an allogeneic islet transplant in a model of natural tolerance and the requirement for systemic chimerism; 2. Determining whether systemic hematopoietic chimeras of the NOD background generated with a clinically relevant regimen are robustly tolerant or split tolerant to an allogeneic islet transplant; and 3. Testing whether hematopoietic cells and solid tissue transplants are differentially susceptible to indirect CD4 alloimmunity leading to split tolerance.

First, I employed a pre-immunocompetence transplant model in mice to study tolerance (**Figure 1.3** and **Chapter 2**). Allogeneic islets were transplanted into adult recipients genetically deficient in adaptive immunity, which was subsequently restored by hematopoietic stem cell reconstitution (therefore the transplant was present pre-immunocompetence). In this system, T cell development occurs in the presence of endogenous self-antigens, and importantly alloantigens that would be presented in the absence of costimulation due to graft healing, hence tolerance mechanisms that act on self-reactive T cells would also be available to alloantigen-specific T cells. Thus, the process by which a transplant could be treated as a self-tissue would be natural tolerance, which is defined as tolerance that is induced solely by naturally evolved mechanisms involved in generating self-tolerance, and not including the manipulation of immunity by the introduction of pharmacologic agents or antibodies that may invoke other distinct mechanisms. I

provide evidence that a pre-existing transplant is capable of tolerizing newly developed T cells specific to alloantigens when the degree of mismatch is a single minor-H antigen. Increasing the mismatch to contain multiple minor-H differences requires systemic chimerism to induce robust islet tolerance. This indicates the capacity of natural tolerance mechanisms to induce allogeneic tolerance in the setting of islet transplantation is rather limited.

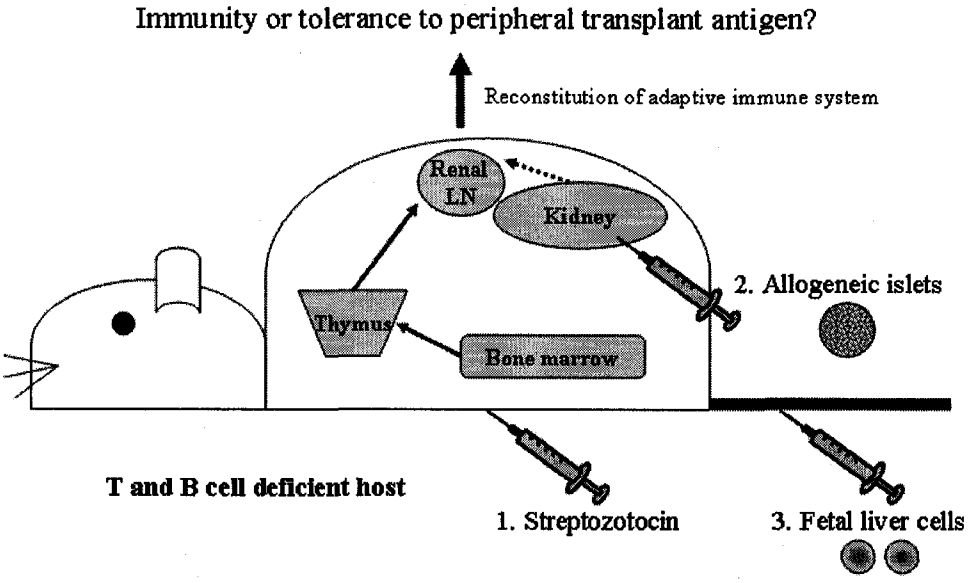

Figure 1.3. Testing the capacity and limits of natural peripheral self-tolerance using an allogeneic islet transplant. A schematic of my experimental plan to study peripheral tolerance induction to an allogeneic transplant placed prior to development of the adaptive immune system. A host lacking T cells and B cells and therefore unable to mount adaptive immune responses is made diabetic using streptozotocin (1). Allogeneic islets of a determined mismatch are then placed into one of the host kidneys as a transplant site and are allowed to heal in to reduce the presence of APC activating signals released after surgical trauma (2). To restore the adaptive immune system, fetal liver cells containing hematopoietic stem cells are injected into the host (3). These stem cells initially migrate to the bone marrow. T cell precursors differentiated from stem cells then migrate from the bone marrow to the thymus to undergo further development. Mature T cells leaving from the thymus to enter the periphery are able to circulate in various secondary lymphoid tissues, including the renal lymph node (LN) draining the islet bearing kidney. In the LN, T cells survey antigens presented by APCs, including the transplant antigens that have been picked up by APCs in the kidney and delivered to the draining LN. Under these conditions, the following questions are asked: 1. Do T cells specific to the transplant undergo peripheral tolerance naturally? 2. By varying the mismatch to increase or decrease the frequency of antigen-specific T cells, what size of a mismatch can be present for T cell tolerance to be induced peripherally? 3. If the transplant contains passenger lymphocytes that generate systemic chimerism, does T cell encounter of antigen in the thymus lead to central tolerance and thereby natural tolerance to the transplant?

Having defined this limit and the importance of chimerism for tolerance towards an islet transplant, my second study explored the ability of hematopoietic chimerism to generate tolerance in the NOD model involving a complete mismatch in the donor/recipient combination (**Figure 1.4** and **Chapter**

3). I generated stable NOD mixed bone marrow chimeras using an irradiation-free protocol involving costimulatory blockade. Interestingly, these chimeras developed a split tolerant phenotype, in which the composition of donor hematopoietic cells transformed from being multilineage to eventually T cells exclusively (B cells and other non-T cell lineages were rejected), and there was rejection of donor skin and islet transplants given in the presence of chimerism. In comparison, chimeras generated with a nonautoimmune-prone recipient strain demonstrated robust tolerance. These data indicate that in spite of the importance of chimerism in allogeneic tolerance, a limited scope of tolerance (split tolerance, or tolerance to one type of graft but not one or more other types of grafts from the same allogeneic donor) involving donor islet rejection could be generated when the host demonstrates considerable tolerance resistance. However, I was able to uncover an unrecognized benefit of chimerism, the presence of which could generate humoral tolerance to not only donor but also third party alloantigens.

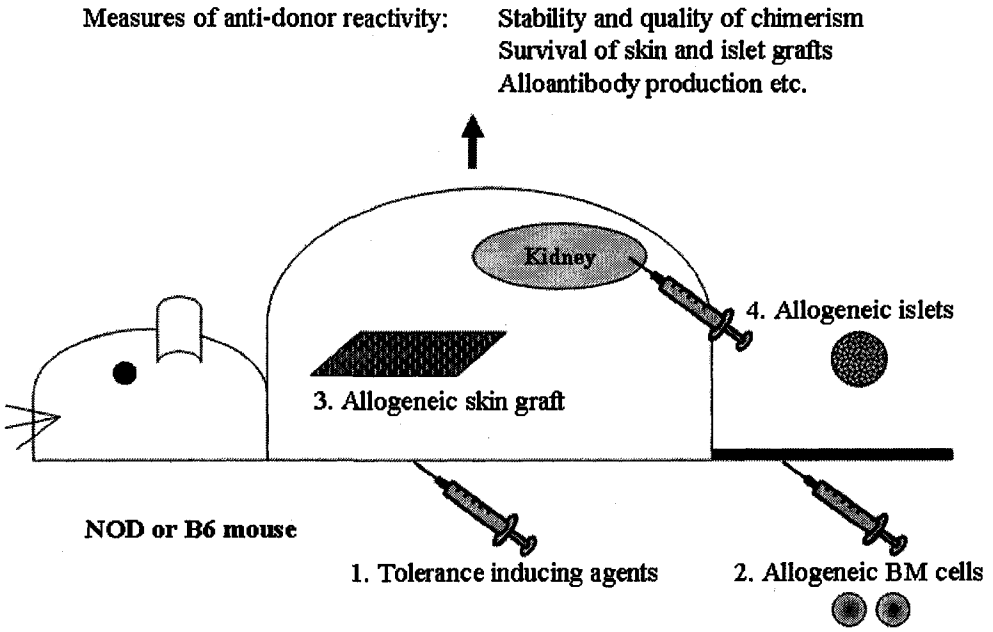

Figure 1.4. Testing the ability of mixed hematopoietic chimerism to induce allogeneic tolerance in the NOD mouse. Autoimmune-prone NOD mice and nonautoimmune B6 mice are given fully allogeneic bone marrow (BM) cells under the cover of tolerance inducing agents. At time of BM infusion or after chimerism induction, a donor skin or islet graft is given. To test for tolerance to alloantigens, chimerism is monitored in terms of its persistence, stability and composition. Donor skin or islet graft survival and the production of alloantibodies are also monitored.

In the third study, I investigated one of the potential mechanisms for split tolerance in NOD chimeras, hypothesizing that the persistence of donor T cells and the loss of other donor hematopoietic cells

as well as solid transplants reflect a differential susceptibility to indirect CD4 alloimmunity, due to the predominance of this type of reactivity in NOD mice (**Figure 1.5** and **Chapter 4**). This was tested in a TCR Tg system in which only monoclonal CD4 T cells were present, and manipulation of the donor/recipient combination allowed the indirect alloimmunity alone to be studied. When allogeneic islet transplants were given to these recipients, I observed that a state of hematopoietic chimerism was established due to the migration of passenger lymphocytes resident in an islet transplant, in spite of the recipient's ability to mount an immune response sufficient to vigorously reject the islet transplant solely by indirect alloimmunity. In a second model, recipients were challenged with allogeneic hematopoietic stem cells and also given skin transplants that could only be rejected indirectly. In spite of an immune response that led to skin rejection, the recipients became chimeric through the generation of mature cells from donor stem cells; however, the chimerism consisted of T cells but not B cells. These data indicate that it is indeed possible for differential susceptibility to indirect CD4 alloimmunity to give rise to a split tolerant phenotype and offer a plausible explanation for split tolerance in NOD chimeras. Interestingly, however, when recipients that were unable to reject allogeneic T cells by the indirect pathway earlier were challenged with skin transplants, long-term graft acceptance was observed indicating tolerance induction.

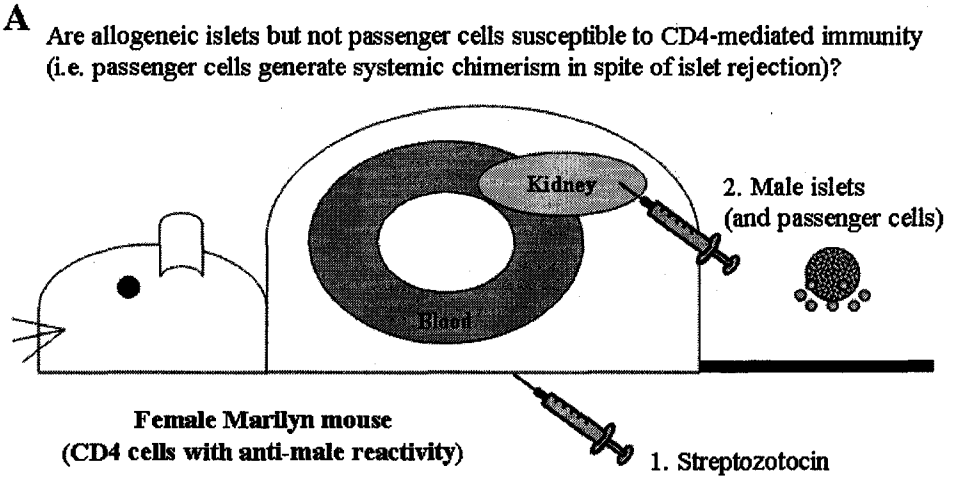

Figure 1.5. Testing the susceptibility of different allogeneic grafts to indirect CD4 immunity. The TCR Tg Marilyn mouse contains a monoclonal population of CD4 T cells that recognize the male antigen in MHC class II but lacks all other T cells and B cells. It rejects male but not female grafts. Depending on whether cells of the graft express the cognate or non-cognate class II, rejection can occur either by direct or indirect alloimmunity, respectively. (*A*) Marilyn mice are made diabetic and then given allogeneic male islets containing male passenger lymphocytes, all of which can only be rejected indirectly by recipient T cells. To determine whether each component of the graft is susceptible to indirect CD4 alloimmunity, survival of the male islets is monitored by blood glucose, while male passenger lymphocytes are detected by flow cytometry.

B Is an allogeneic skin graft differentially susceptible to CD4-mediated immunity compared to hematopoietic cells?

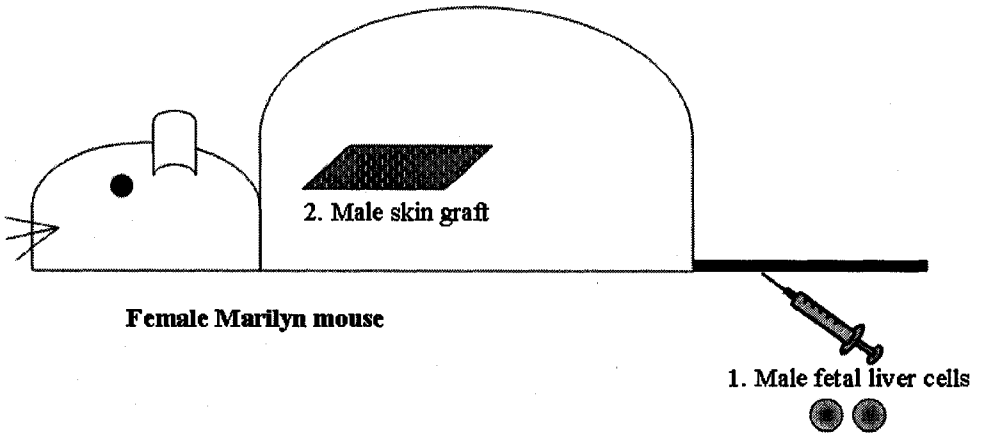

Figure 1.5 (continued). (*B*) Marilyn mice are challenged with male fetal liver cells and male skin grafts from the same type of allogeneic donor that can only be rejected indirectly. Survival of male skin graft is monitored by visual inspection, while male fetal liver cells are monitored by flow cytometric detection of T cells and B cells generated from these stem cells.

Collectively, these studies clarify the ability of natural tolerance mechanisms to be applied to allogeneic islet tolerance, and the contribution of hematopoietic chimerism to tolerance towards an allogeneic islet transplant. In the relevant NOD model of autoimmune diabetes, chimerism induction may not necessarily result in tolerance towards allogeneic islets but is potentially associated with a highly split tolerant phenotype due to differential susceptibility of different allogeneic grafts to indirect CD4 alloimmunity.

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Chapter 2: Natural tolerance to allogeneic islets

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2.1. INTRODUCTION

Allogeneic transplantation before the development of recipient immunocompetence theoretically provides the greatest opportunity to achieve donor-specific tolerance. All of the tolerance processes that occur for self-reactive T cells are potentially available for donor reactive T cells, and in most cases the transplant has time to heal in prior to encountering the recipient's immune system, potentially eliminating or reducing the activating signals for host APCs that are released from damaged tissues (1-3). The observations of Owen and Medawar and colleagues (4-6), together with the theories of Burnet and Fenner (7), led to the generally held view that allogeneic cells or tissues given before immunocompetence should be treated as self-tissue and induce tolerance (8-11). However, over the last 30 years the data have almost been equally divided between support and rejection of this view. For example, Owen described a natural situation where dizygotic cattle twins possessed blood cells of their fraternal twin and remained lifelong chimeras, appearing to treat the foreign cells as self (4). Experiments by Medawar's group (5), and also those of Hasek (12), showed that experimentally introducing foreign blood cells early in life (before full immunocompetence) could lead to tolerance of donor antigens. The B cell tolerance of ABO incompatibilities in infant cardiac transplantation is a dramatically successful recent application of the pre-immunocompetence graft concept (11, 13). In contrast, studies by Le Douarin and colleagues showed that xenogeneic and allogeneic limb buds grafted into embryos were not treated as self by the newly generating immune system (14, 15). Similarly, McCullagh showed that pre-immunocompetence allogeneic fetal skin grafts given to fetal sheep could trigger immunity rather than tolerance (16). More recently other natural tolerance models have been examined. Grafts were given pre-immunocompetence by using genetically immunodeficient adult recipients and allowing T cells to develop *de novo* in the presence of the transplant. Like the studies of Le Douarin and McCullagh, these studies showed that allogeneic grafts (skin, heart or islets) given pre-immunocompetence were not treated as self but instead triggered immunity (2, 3, 17-20). We hypothesize that the paradox of immunity or tolerance with pre-immunocompetence grafts is most simply explained by the nature and distribution of the tissues involved (*i.e.* donor tissue and host site), and the length of time the grafts are established before immunocompetence sets in. Experiments that showed tolerance used hematopoietic cells as the donor tissue while those showing immunity used solid grafts. Hematopoietic cells can migrate systemically raising the possibility that solid tissue grafts fail because their

antigens remain localized in the periphery and the natural peripheral tolerance mechanisms are unable to act on the alloreactive T cell repertoire encountering antigens that are not widely distributed. Consistent with this hypothesis, we found that pre-immunocompetence skin grafts mismatched for a single minor-H antigen only induced tolerance if they contained passenger lymphocytes capable of generating systemic chimerism (20). However, at least two important questions remain unanswered. 1. Are natural tolerance mechanisms ever able to successfully induce tolerance to antigens of allogeneic tissue without systemic lymphocyte chimerism and if so what determines this outcome? 2. Are hematopoietic cells better able to take advantage of natural tolerance mechanisms because they migrate systemically or would they also be better at inducing tolerance when present in a locally restricted fashion? Answering these questions is critical to understanding the capacity and limits of natural tolerance to act on allogeneic tissue. Herein we tested whether the degree of antigenic mismatch, the type of donor tissue and its distribution determine the ability of natural tolerance to be established to alloantigens.

2.2. MATERIALS AND METHODS

2.2.1. Animals. Adult WT C57BL/6 (B6; H-2^b), BALB/c (H-2^d) and DBA/2 (H-2^d) mice, immunodeficient BALB/c mice bearing the *scid* mutation (BALB/c-SCID), and day 13 of gestation pregnant B6 and BALB/c mice were obtained from the National Cancer Institute at Frederick (NCI-Frederick; Frederick, MD). Immunodeficient B10.D2 mice with a disruption of the recombination activating gene 2 (B10.D2 RAG; H-2^d) (21) were obtained from Taconic Farms (Germantown, NY). (C57BL/6J × C57BL/10SgSnAi)-[KO]*γc*-[KO]*Rag2* (RAG/γc-KO or RAG/γc; H-2^b) mice that are deficient in T cells, B cells and natural killer (NK) cells were obtained through the National Institute of Allergy and Infectious Diseases (NIAID) Exchange Program. WT B10.D2 and BALB.B (H-2^b) mice were obtained from Jackson Laboratory (Bar Harbor, ME). B6.129S7-*Rag1*^{tm1Mom} mice (hereafter called B6 RAG, RAG-KO or RAG; H-2^b), originally from Jackson Laboratory, were bred at the University of Alberta. Where indicated, body weight of mice was measured by a portable balance (Model CS 200, capacity 200 g × 0.1 g, Ohaus Corporation, Pine Brook, NJ). All protocols on care and handling of animals were carried out in facilities accredited by the Canadian Council on Animal Care (CCAC).

2.2.2. Diabetes induction, islet transplantation and nephrectomy. Islet recipients were made diabetic chemically by a single intraperitoneal (i.p.) injection of streptozotocin (STZ; 200 mg/kg for B6 RAG and 210 mg/kg for BALB/c-SCID) dissolved in acetate buffer (see **Appendices**, **A.1.4.1** and **A.1.4.2**, for protocols; p.160). Diabetes was confirmed by measuring blood glucose (>15.0 mmol/L; most recipients were >20.0 mmol/L), using a ONE TOUCH® Ultra Blood Glucose Monitoring System (LifeScan Canada, Burnaby, BC). Approximately 500 donor islets (see **Appendices**, **A.1.7**, for islet isolation protocol; p.164) were placed into the renal subcapsular space of diabetic, and, in a few cases in single minor-H-mismatched transplants, nondiabetic recipients (to exclude any confounding effects on tolerance or immunity from STZ and/or diabetes-related immunosuppression (22); also see **Appendices**, **A.3**, p.168). Where indicated, second islet transplants were performed in recipients with an intact first islet transplant (acceptance >120 days), in the contralateral kidney. Nephrectomy was done at least 10 days post second transplant to remove the kidney bearing first set islets and assess second set islet survival. Graft rejection was defined as two consecutive blood glucose measurements >15.0 mmol/L taken on two different days.

2.2.3. Immune reconstitution of immunodeficient recipients of transplants. Fetal livers (FLs; a source of hematopoietic stem cells that can generate mature T cells and B cells (3, 20) were extracted from the fetuses of pregnant mice (day 14 or 15 of gestation for B6; day 14-16 of gestation for BALB/c), pooled and homogenized into a single cell suspension, counted and resuspended in phosphate-buffered saline (PBS) before injection into immunodeficient recipients. For reconstitution of BALB/c-SCID and B6 RAG mice, 20-40 $\times$ 10^6 and 15-30 $\times$ 10^6 cells, respectively, were injected intravenously (i.v.) in most cases (some were given i.p. with a similar degree of reconstitution). Some FL-reconstituted BALB/c-SCID recipients were given 2 $\times$ 10^6 unmodified DBA/2 spleen cells i.v. one day earlier where indicated. For H-Y-mismatched experiments, reconstitution was done in female RAG mice using female fetal liver cells (FLCs) only (pooled from multiple fetuses; each FL contains approximately 20 $\times$ 10^6 cells). Fetuses were sex-determined by polymerase chain reaction (PCR). To do this, deoxyribonucleic acids (DNA) were first extracted from individual FLs using a commercial kit (DNeasy® Tissue Kit, QIAGEN, Mississauga, ON; following the manufacturer's instructions for total DNA purification from cultured animal cells). Detection of a 278-base-pair (bp) DNA of the *Sry* gene found on the Y chromosome (forward primer sequence: 5'-

AGACAAGTTTTGGGACTGGTGAC-3'; reverse primer sequence: 5'-AGCCCTCCGATGAGGCTGATA-3'), along with a housekeeping gene (a 217-bp DNA within the mouse nicotinic acetylcholine receptor beta subunit gene; forward primer sequence: 5'-CCTCTGCCTTCACTGCCTCTCAG-3'; reverse primer sequence: 5'-GTGGGGTGAGCGATGATGCAG-3'), was performed. The PCR mixture contained 10 μL of DNA and 40 μL of reaction mix (1.2 mM magnesium chloride, 0.2 mM of each deoxyribonucleotide triphosphate, 0.2-0.4 μM of each primer and 1 U of recombinant *Thermus aquaticus* DNA polymerase, in a 1× reaction buffer; Invitrogen, Carlsbad, CA). PCR conditions consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, and a final extension step at 72°C for 7 minutes. PCR products were electrophoresed and visualized on a 2% agarose gel containing ethidium bromide (**Figure 2.1**) (see **Appendices, A.1.8.1-A.1.8.3**, for general molecular biology protocols; p.166).

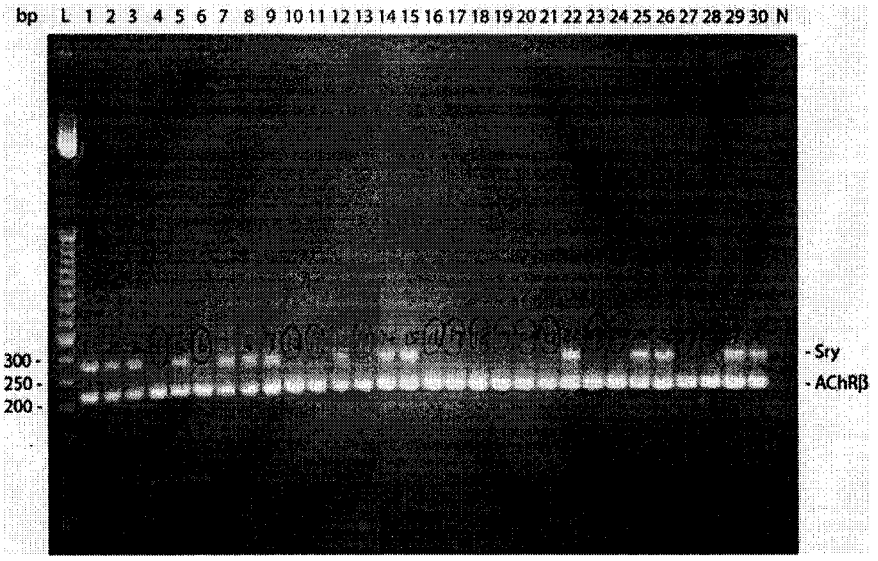

Figure 2.1. PCR detection of H-Y in mouse fetal liver. To determine the sex of fetuses from which FLs were harvested, DNA were extracted from FLCs and amplified for the *Sry* gene (Sry) found in males. As an internal control, the mouse nicotinic acetylcholine receptor beta subunit gene (AChRβ) was amplified in the same reaction. Two bands indicate a male. One band indicates a female. Shown is one gel from a single experiment on B6 FLs. A DNA ladder (L) was run alongside and the relevant bands for reference are marked. Numbers above the gel (1-30) denote individual FLs. A negative control without the addition of DNA (N) was also tested.

2.2.4. Flow cytometry and 5-bromo-2'-deoxyuridine labelling. For flow cytometry, peripheral blood samples were obtained by tail bleeding. Blood was mixed with sodium heparin at a 1:1 ratio. Spleen and thymus were harvested and homogenized in PBS by pressing the tissue between frosted glass slides. Bone marrow (BM) cells were obtained by harvesting the femur and tibia and flushing the marrow with PBS

using a syringe capped with a 27-gauge needle. To analyze cells in the peritoneum, a peritoneal lavage was performed by flushing the peritoneal cavity with PBS and recovering the fluid using a Pasteur pipette. Fluorescent anti-mouse TCR-beta chain (TCRβ; H57-597), immunoglobulin M (IgM; II/41), CD8α (53-6.7), CD19 (6D5), CD25 (PC61), CD44 (IM7) and CD49b (DX5) antibodies were purchased from eBioscience (San Diego, CA), anti-mouse CD4 (RM4-5), CD45R (B220; RA3-6B2) and CD62L (MEL-14) antibodies from Caltag (Burlingame, CA), and anti-mouse Ly-9.1 (30C7), CD5.1 (H11-86.1), H-2D^d (34-2-12), H-2D^b (KH95), BrdU (3D4), Vβ5.1/5.2 (MR9-4), Vβ6 (RR4-7), Vβ8 (F23.1) and Vβ11 (RR3-15) antibodies from BD Pharmingen (San Diego, CA). Cells were first incubated with a cocktail consisting of anti-CD16/32 antibody (2.4G2; Bio Express, West Lebanon, NH), and mouse, rat and hamster sera to prevent binding via the Fc portion. Four-color antibody staining was then carried out by incubating cells with the appropriate antibodies simultaneously (see **Appendices, A.1.5.1-A.1.5.6**, for complete staining protocol and protocols for preparing flow cytometry reagents; pp.161-162). Where biotinylated antibodies were used, secondary incubation with allophycocyanin-conjugated streptavidin (eBioscience) was performed and followed by a second washing before analysis. A FACSCalibur™ flow cytometer (BD Biosciences) equipped with CellQuest™ Pro software was used for data acquisition and analysis. To assess cell cycling based on the uptake of the nucleotide analog 5-bromo-2'-deoxyuridine (BrdU) during DNA synthesis, BALB/c-SCID recipients of islet transplants and naïve WT BALB/c mice were given BrdU (Sigma-Aldrich) in their drinking water *ad libitum* (0.8 mg/mL; water protected in aluminum foil) for 5 days, after which BrdU incorporation was measured using a commercial kit, following the manufacturer's instructions (BrdU Flow Kit, BD Pharmingen, San Diego, CA).

2.2.5. *In vivo* depletion of NK cells, *ex vivo* depletion of T cells and adoptive transfer. NK cells were depleted from B6 RAG mice by i.p. injection of 35 μl of anti-asialo GM1 antibody (Wako Chemicals USA, Richmond, VA) 10 and 3 days before injection of donor cells. NK cell depletion was assessed by staining with antibody to NK1.1 (PK136; eBioscience). T cells were depleted from peritoneal lavage cell preparations using anti-CD90 microbeads and MACS® cell separation columns (Miltenyi Biotec, Auburn, CA). As measured by flow cytometry, the peritoneal cell preparations contained approximately 1% T cells after depletion; 2×10^6 donor peritoneal cells were injected i.v. or i.p.

2.2.6. Immunization, *in vitro* cytotoxic T lymphocyte and mixed lymphocyte reaction assays. Three months after FLC injection (*i.e.* after immune system generation), female RAG recipients of male islet transplants were immunized i.p. with 5×10^6 gamma-irradiated (1500 rads; ^{137}Cs irradiator, Gammacell 40, Atomic Energy of Canada, Ottawa, ON) WT male B6 spleen cells in PBS. Three months later, all animals were immunized i.p. with 5×10^6 irradiated or non-irradiated WT BALB.B spleen cells in PBS. After 3-5 weeks, *in vitro* cytotoxic T lymphocyte (CTL) responses were assayed by the JAM Test as described (23) (see **Appendices, A.1.3.1-A.1.3.3**; p.158). Briefly, 6×10^6 recipient splenocyte responders were stimulated for 6 days with 2×10^6 irradiated donor or third party splenocytes. Targets were ^{3}H-thymidine-labelled Concanavalin A (Con A; Sigma-Aldrich Canada, Oakville, ON) stimulated syngeneic, donor and third party splenocyte blasts. Immunization with male cells and third party cells was done sequentially to avoid the immunodominance of the anti-BALB.B response over the anti-H-Y response (24). To set up the mixed lymphocyte reaction (MLR) assay, recipient spleen cells containing 2×10^5 T cells were cultured with titrated numbers of irradiated stimulators (beginning at 1×10^6 spleen cells) for 72 hours, then pulsed with ^{3}H-thymidine at 1 μCi/well and incubated for an additional 16-18 hours. Cells were then harvested onto glass fibre filters (Wallac, Turku, Finland) using a Harvester 96® Mach IIIM cell harvester (Tomtec Inc., Hamden, CT). Counting of radioactive incorporation (one minute counts) was performed by a MicroBeta® TriLux luminescence counter (PerkinElmer, Waltham, MA).

2.2.7. Histology. Tissues harvested from various locations in FLC-reconstituted mice were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (see **Appendices, A.1.6.1**, p.163). To detect insulin, pancreas sections were first stained for insulin by immunohistochemistry (25) (see **Appendices, A.1.6.2**, p.163), then counterstained with hematoxylin and eosin. Slides were examined by light microscopy.

2.2.8. Statistical analysis. Means are reported along with the standard deviation (SD) or the standard error of the mean (SEM). Statistical analyses included the Kaplan-Meier method and the log rank test for graft survival data, the Kruskal-Wallis test followed by Dunn's multiple comparison test for CTL responses to heart grafts and donor peritoneal cell chimerism, linear regression for CTL responses to H-Y-mismatched

islet grafts, and one-way analysis of variance (ANOVA) and Tukey's multiple comparison test for TCR Vβ expression (GraphPad Prism Software, San Diego, CA).

2.3. RESULTS

2.3.1. Natural tolerance can be established to minimally mismatched internal transplants

Contrary to the expectations of theories postulating an early tolerance window, solid tissue transplants given pre-immunocompetence generally trigger immunity rather than tolerance, even in the case of skin grafts with the minimal H-Y mismatch (26). These skin transplants only induced tolerance if they carried passenger T cells capable of generating systemic chimerism (20). We examined whether the inability to induce natural peripheral tolerance to an allogeneic tissue was absolute or if instead tolerance might be established to weakly mismatched internal transplants; the exposure of skin grafts to the external environment or other factors may reduce their ability to establish peripheral tolerance. Since an H-Y mismatch alone is not sufficient to trigger consistent rejection of most tissues other than skin grafts (27-29), our question was instead whether the pre-immunocompetence male internal transplant would be immunologically ignored (30) or induce tolerance (examined by testing for recipient CTL response to H-Y *in vitro*, after immunization with male spleen cells). The general experimental design was to give female B6 RAG immunodeficient recipients, lacking T cells and B cells, male islet transplants prior to generation of the recipient's adaptive immune system *de novo*. Immune reconstitution was achieved by injecting WT female FLCs containing hematopoietic stem cells. Mouse FLCs generally lack pre-existing mature T cells and B cells until day 17 of gestation (**Figure 2.2**); hence the source of all T cells and B cells for reconstituting the adaptive immune system of the immunodeficient recipients should originate from the differentiation of hematopoietic stem cells contained in the FLCs given at the gestational age that we had chosen. Importantly, all new T cells and B cells that are produced as such would undergo the process of tolerance. We used male RAG mice as islet donors to eliminate systemic chimerism by passenger lymphocytes (20). After immune reconstitution, recipients were immunized with male spleen cells and tested for the generation of an anti-H-Y CTL response. Immunization allowed us to test whether the recipients were ignorant or had become tolerant of the graft antigen, due to its presence during immune

development. **Figure 2.3** shows that T cells that developed in these recipients were indeed specifically tolerant rather than ignorant of H-Y, as they demonstrated a poor CTL response to male targets but not third party targets, while control mice made strong responses to both.

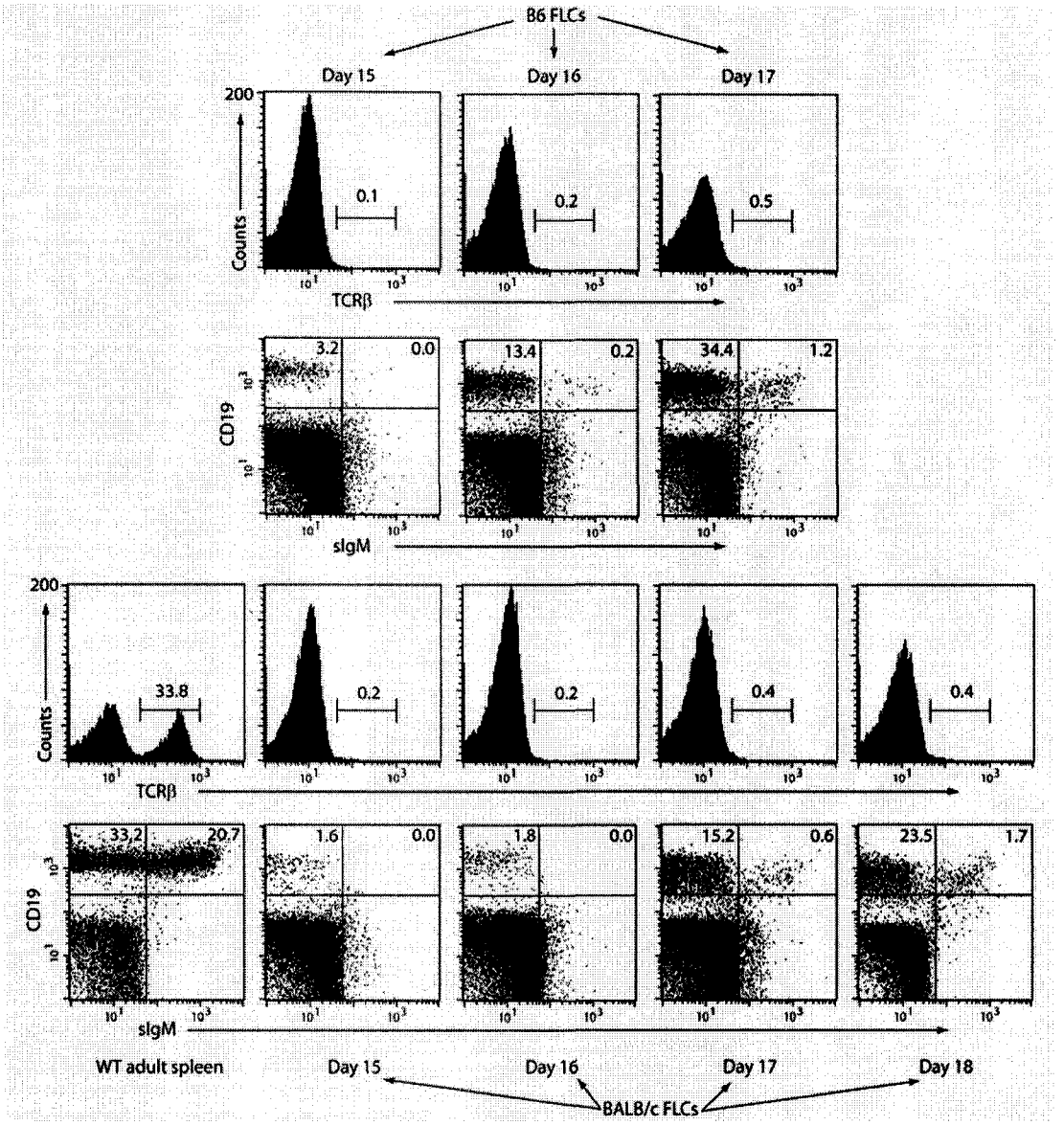

Figure 2.2. Detection of mature T cells and B cells in FLCs. FLs of B6 (days 15-17 of gestation; top 2 rows) and BALB/c (days 15-18 of gestation; bottom 2 rows) fetuses were harvested (n=3-4 per gestational age) and their cells analyzed by flow cytometry. Shown are representative plots of one fetus at each of the indicated day of gestation and compared to spleen cells from an immunocompetent adult mouse. First and third rows: Detection of $TCR\beta^+$ T cells. Mature T cells were generally absent in days 15-17 of gestation FLs as indicated by the percentage of gated cells. Second and fourth rows: Detection of $CD19^+$ B cells along with expression of surface immunoglobulin. B cell precursors expressing CD19 were detected in days 15-18 of gestation FLCs but mature B cells expressing surface IgM (sIgM) were not detected until day 16 for B6 and day 17 for BALB/c. The relevant quadrant percentages are shown.

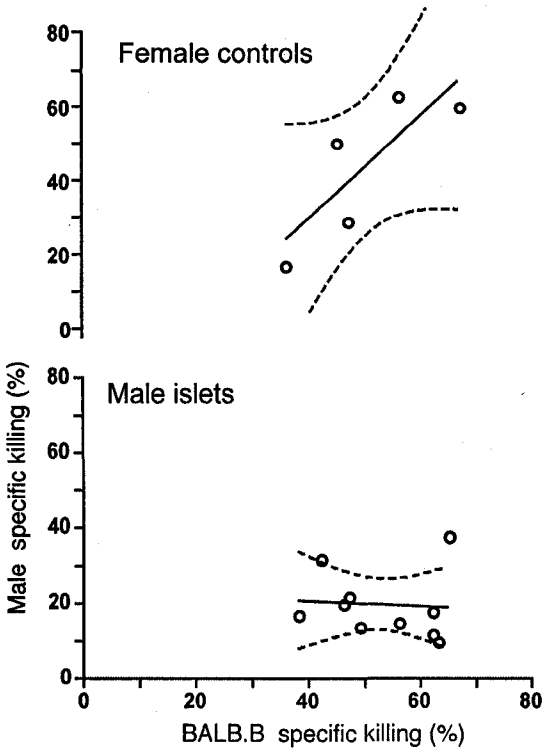

Figure 2.3. Pre-immunocompetence single minor-H-mismatched islet transplants are not ignored but instead induce tolerance in a newly generated adaptive immune system. STZ-induced diabetic B6 RAG female recipients were given B6 RAG male islet transplants, followed 2 days (n=3) or 8-10 weeks later (n=7) by an injection of female B6 FLCs. Data from these two cohorts were grouped together as no difference in the CTL response of the islet transplant recipients was observed between these different healing times. Three months after FLC injection (after immune system generation in the recipients) the mice were immunized with WT male B6 spleen cells. A further 3 months later, all animals were immunized with WT BALB.B spleen cells to test for a third party response. Three to 5 weeks later, recipient splenocytes were assayed for the killing of B6 male versus female, and BALB.B targets. For comparison of H-Y-specific and BALB.B-specific killing, the maximum percentage of killing after subtraction of killing of syngeneic targets is shown for individual animals, along with regression lines and 95% confidence intervals. Recipients of male islet transplants appeared specifically hyporesponsive to H-Y, but killed BALB.B control targets ($r = -0.082$), in contrast to control mice lacking male islet transplants that demonstrated a correlation between the ability to kill both BALB.B and H-Y targets ($r = 0.823$). Controls included recipients with a female islet transplant and non-transplanted B6 females.

Consistent with these data, experiments involving male donor hearts given as non-vascularized transplants in different internal locations were performed and showed similar results (**Appendices, A.2,** p.167). Almost all recipients of a male heart graft under the kidney capsule were not primed *in vivo* by the transplant; after immunization, the majority of recipients did not respond or responded weakly (control recipients of female grafts responded well to immunization), indicating the lack of *in vivo* CTL priming was due to the long-term presence of the un-rejected graft and tolerance induction to H-Y rather than ignorance of the graft antigen. Interestingly, recipients that showed a weak response had a male graft with a short healing time. In contrast, recipients of a male heart graft under the ear skin were primed *in vivo* by the graft and made anti-H-Y CTL response that was detected *in vitro* without immunization (control female grafts did not stimulate a CTL response to H-Y). Collectively, these data suggest that an internal weakly mismatched transplant is not ignored by a newly generating immune system, instead the location of the established graft in addition to its degree of mismatch determine whether the graft triggers CTL priming or tolerance.

2.3.2. Low frequency of natural tolerance with multiple minor-H mismatches; requirements for chimerism

Having provided evidence that natural tolerance can be established to antigens in well-healed minimally mismatched allogeneic tissue, we next determined if this could be extended to greater mismatches. We examined whether natural tolerance could be induced to islet transplants with multiple minor-H mismatches and the effect of passenger lymphocyte-derived chimerism on tolerance induction. We compared islet transplants from WT versus RAG donors on the B10.D2 background given to BALB/c-SCID recipients, which were later reconstituted with WT FLCs. In this model, reconstitution by FLCs generated an adaptive immune system comparable to that of WT mice (**Figure 2.4**). Control syngeneic BALB/c islets survived long-term post immune reconstitution (data not shown), as did all established WT B10.D2 donor islets; all these recipients universally accepted a second donor islet transplant (**Figure 2.5, *A***). In contrast, half of the recipients of B10.D2 RAG islets rejected the islet transplant (including grafts healed in for 7 months) and those that did not were often able to reject a second donor transplant. We also assessed the fate of B6 RAG islets fully mismatched for the MHC, which were rejected significantly less often than those that differed only by multiple minor-H antigens (**Figure 2.5, *A***). However, the majority of recipients rejected a second donor transplant. Thus, consistent tolerance to multiple minor-H mismatches was only achieved with WT donors that were able to generate lymphocyte chimerism in the recipients (**Figure 2.5, *B***). This chimerism was exclusively of T cells as detected in the blood (**Figure 2.5, *B***), similar to what we had seen previously with skin grafts (20).

By flow cytometry, we further characterized these passenger lymphocytes. First, we examined their distribution beyond the peripheral blood. **Figure 2.6, *A*** shows that the passenger cells had a broad distribution, as they were readily detected in the thymus, spleen and peritoneum. Based on the expression of CD44 and CD62L, the passenger T cells were predominantly of a memory phenotype (**Figure 2.6, *B***). They also appeared to be dividing rapidly, based on a greater proportion of T cells that incorporated BrdU in pulsing experiments, compared to T cells in WT mice (**Figure 2.6, *C***). However, in spite of this predominance of T cells migrating out into the recipient's circulation and expanding, we did not observe any decrease in recipient body weight post islet transplantation (besides a drop early on due to diabetes induction) that would suggest graft versus host disease (GVHD) (**Figure 2.6, *D***). Post FLC reconstitution,

a state of stable mixed chimerism was established (**Figure 2.5, *B*** and **Figure 2.6, *E***). This chimerism was maintained at a low level (3.2 ± 0.6% at 30 weeks post reconstitution). Moreover, the donor T cells consisted of both the CD4 and CD8 subsets (**Figure 2.6, *F***).

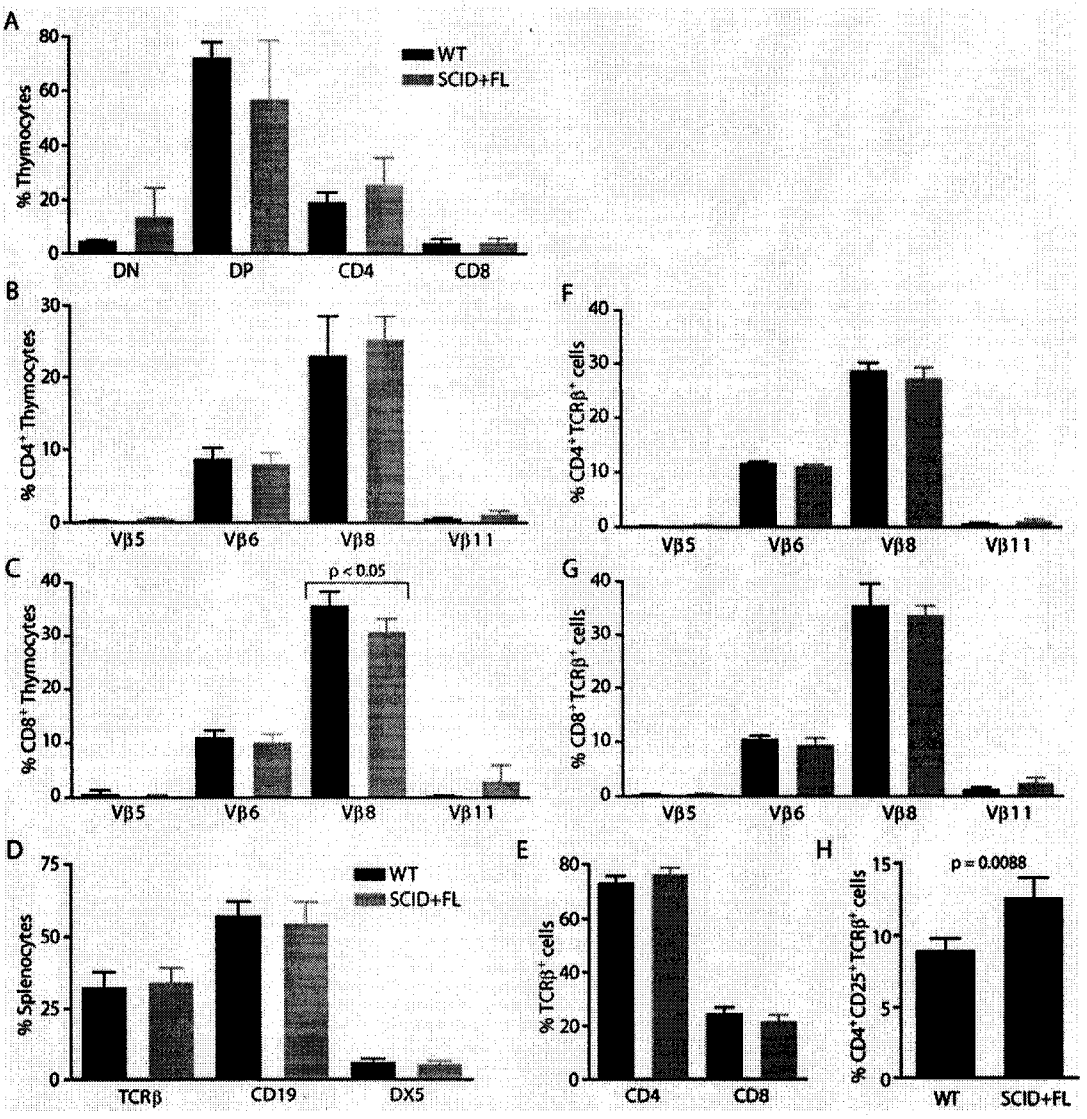

Figure 2.4. Immune reconstitution of BALB/c-SCID mice by FLCs. BALB/c-SCID mice were given 20 × 10^6 WT BALB/c FLCs (SCID+FL; n=4). More than 36 weeks later, flow cytometric analysis of the level of immune reconstitution was performed on their thymi (*A-C*) and spleens (*D-H*), and compared to WT BALB/c mice (WT; n=3). (*A*) Overall composition of the thymus, sorted into cells that are double negative (DN) for CD4 and CD8α, double positive (DP), or CD4 or CD8α single positive (SP). (*B*) Frequency of expression of different Vβs examined in CD4 SP thymocytes. Vβ5 and Vβ11 are clonally eliminated during development due to expression of the corresponding superantigen in the BALB/c background. (*C*) Vβ expression in CD8 SP thymocytes. (*D*) Composition of the spleen as related to T cells ($TCR\beta^+$), B cells ($CD19^+$) and NK cells ($DX5^+TCR\beta^-$). (*E*) Distribution of splenic T cells into the CD4 versus CD8α subset. (*F*) Vβ expression in CD4 T cells. (*G*) Vβ expression in CD8 T cells. (*H*) Frequency of all T cells expressing CD4 and CD25 (potential Treg cells). All bars depict mean and SD. No statistically significant differences were detected in any of the comparisons made, except in *C* and *H*.

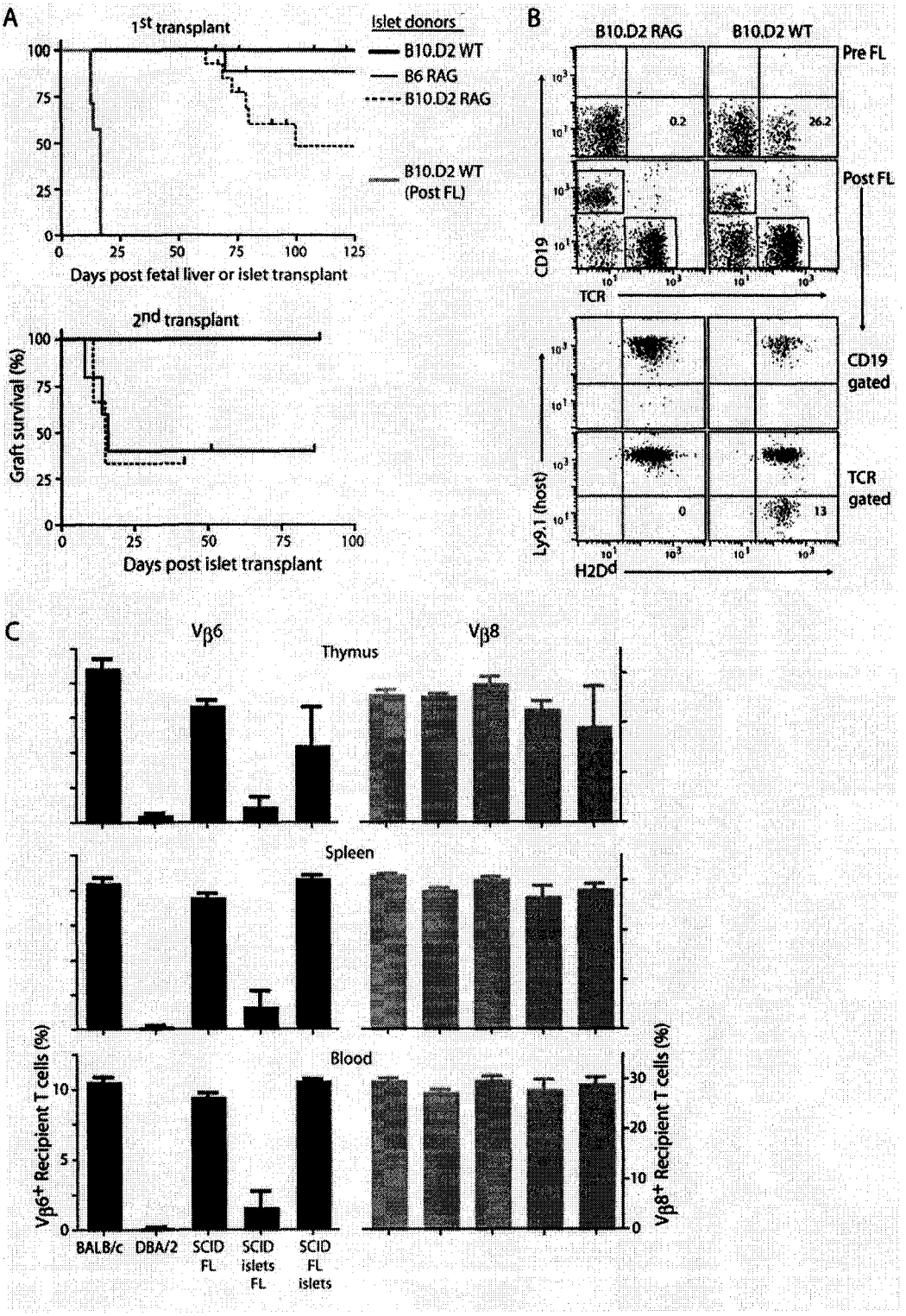

Figure 2.5. Reduced natural tolerance to increasingly mismatched internal transplants lacking passenger lymphocytes: requirements for systemic chimerism. (*A*) Top survival graph, first transplant: Graft survival in days post FLC injection is shown for STZ-induced diabetic BALB/c-SCID mice that received B10.D2 WT versus B10.D2 RAG islets (n=11 and 14, respectively; p=0.009) or B6 RAG islets (n=10; p=0.01 versus B10.D2 RAG) 4-7 months pre-FL; control syngeneic BALB/c islets (not shown) survived long-term post FLC reconstitution (>131, >185x3 days). Also shown is graft survival in days post islet transplant of B10.D2 WT islets (n=7) given >13 weeks post-FL (thick grey line). Some recipients

with functioning grafts beyond 100 days were given a second donor islet transplant (lower survival graph; B10.D2 WT, n=8, versus B10.D2 RAG, n=3, p=0.011; B6 RAG, n=5; not shown, BALB/c, >35x3 days). (*B*) Representative flow cytometry analyzing the presence and phenotype of donor cells from the peripheral blood of mice shown in *A*. T cells of the donor (Ly9.1$^-$) are present in recipients of WT B10.D2 but not B10.D2 RAG islets (lower right versus left dot plots, respectively). All 11 recipients were T cell chimeras, with 3.2 ± 1.6% of T cells being of donor origin (Ly9.1$^-$TCR$^+$) in 7 mice analyzed at 30 weeks post FLC injection. (*C*) Frequency of donor reactive (Vβ6) and control (Vβ8) T cells in the thymus (gated on host CD4 SP cells), spleen and blood (gated on host TCR$^+$ cells) of normal BALB/c and DBA/2 mice, and BALB/c-SCID mice given BALB/c FLCs alone or DBA/2 islets before or after FLC injection (analysis at >33 weeks after FLC injection; mean and SEM; n=2-5 mice per group). DBA/2 islets given after FLC injection were rejected approximately 4 days prior to the Vβ analysis. Chimerism with islet derived donor T cells (CD5.1$^+$) in the spleen of the recipients with islets given pre-FL ranged from 1.4-3.9% of all T cells. Percent Vβ6 comparing SCID FL versus SCID islets FL: p<0.001 for thymus, blood, and spleen; comparing SCID islets FL to SCID FL islets: thymus, p>0.05, spleen and blood, p<0.001.

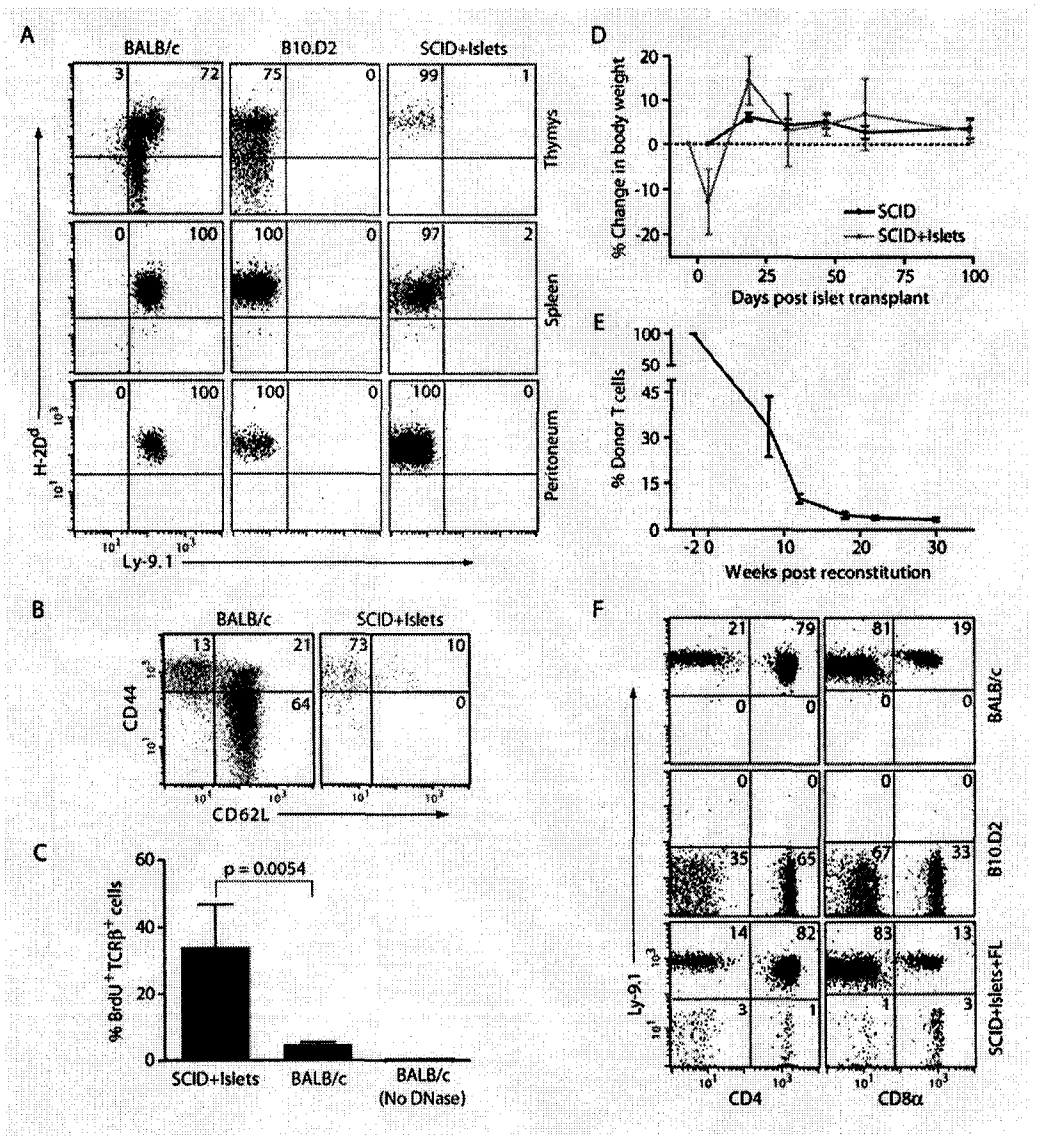

Figure 2.6. Characterization of passenger cells from healed-in WT islet transplants. (*A*) Approximately 15 weeks post transplant, BALB/c-SCID mice given WT B10.D2 islets (SCID+Islets; n=3) were sacrificed to analyze for the presence of chimerism generating passenger cells in the indicated

locations. Staining of naïve BALB/c and B10.D2 mice is shown as controls. Donor cells express H-2D^d but not Ly-9.1. Plots shown are lymphoid cells gated on TCRβ^+ cells (donor CD19$^+$ cells were not detected; not shown). (*B*) Islet recipients from *A* were analyzed for the expression of CD44 and CD62L on passenger T cells detected in the peripheral blood. A naive WT B10.D2 mouse is shown for comparison. Upper left quadrant (CD44^{hi}CD62L^{lo}) contains memory T cells. Lower right quadrant (CD44^{lo}CD62L^{hi}) contains naive T cells. Relevant quadrant percentages are presented. The majority of passenger T cells (72.8 ± 10.2%; mean and SD) were of a memory phenotype. (*C*) BrdU was added to the drinking water of transplanted mice in *A* as well as naive WT BALB/c mice (n=4) for 5 days, after which splenic T cells were analyzed for BrdU incorporation. As a negative control, spleen cells from BrdU pulsed BALB/c mice (n=2) were stained for BrdU without the addition of DNase in the staining procedure. In the SCID+Islets group, BrdU incorporation by T cells of donor origin was examined. All bars depict mean and SD. (*D*) Body weights of BALB/c-SCID mice with (SCID+Islets) or without (SCID) DBA/2 islet transplants (n=4 and 5, respectively) were obtained at various times up to approximately 100 days post transplant. Data are expressed as the percentage of change in body weight (mean and SD) compared to the previous measurement (initial measurement set at 0% change). Early drop of body weight in the SCID+Islets group was due to diabetes induction on day -3. (*E*) Time course of donor T cell chimerism, expressed as a percentage of all T cells, in the peripheral blood of BALB/c-SCID mice given WT B10.D2 islets, pre and post FLC reconstitution. Shown at each time point is mean and SEM of 7 or 8 mice analyzed. (*F*) BALB/c-SCID mice given WT B10.D2 islets were analyzed at 30 weeks post FLC reconstitution (SCID+Islets+FL; n=7) for the composition of passenger T cells (CD4 versus CD8) in the peripheral blood. Recipient but not donor cells express Ly-9.1. WT BALB/c and B10.D2 mice were used as negative and positive controls for donor cells, respectively. Quadrant percentages are provided in each plot.

Due to the presence of donor cells in the thymus (**Figure 2.6, *A***), we wanted to assess whether chimerism from islet-derived passenger lymphocytes induced tolerance via deletion of newly generated recipient T cells. To do so, we employed a surrogate model in which we transplanted DBA/2 islets into BALB/c-SCID mice, followed by FLC reconstitution. In this donor/recipient combination that is also multiple minor-mismatched, the frequency of V$\beta6^+$ recipient T cells that are reactive to the donor superantigen encoded by *Mtv-7* can be assessed. Recipient V$\beta6^+$ but not control V$\beta8^+$ T cells were greatly reduced in frequency in recipients of DBA/2 islets compared to untransplanted controls. The loss of V$\beta6^+$ T cells was evident not only in the periphery (blood and spleen) but also in the thymus and occurred when the islets were given before but not after immune reconstitution (**Figure 2.5, *C***). Furthermore, deletion of V$\beta6^+$ T cells occurred in both subsets of TCRβ^+ T cells (**Table 2.1** and **Table 2.2**) but not those expressing V$\beta8$ (**Table 2.3**). The possibility that recipient V$\beta6^+$ cells were sequestered in another location was unlikely, as in addition to their reduction in the thymus, spleen and blood, a decreased level was also observed in the BM and the peritoneum that was dependent on the presence of donor islets (**Table 2.1** and **Table 2.2**). In further support of a deletional mechanism, immune reconstituted recipients of donor islets, compared to those without a transplant, showed antigen-specific, reduced T cell proliferation *in vitro* when stimulated by donor cells (**Figure 2.7**).

Table 2.1. Vβ6 expression on recipient CD4 T cells in immune reconstituted BALB/c-SCID mice with or without a healed-in DBA/2 islet transplant.

	% $CD4^+V\beta6^+CD5.1^-$ cells[1]			
Location	**SCID+FL[2]**	**SCID+Islets+FL[3]**	**DBA/2[3]**	**p[4]**
Spleen	11.2 ± 0.5[5]	2.4 ± 1.0	0.2 ± 0.1	0.0001
BM	10.1 ± 2.3	3.6 ± 0.7	0.6 ± 0.1	0.0209
Peritoneum	12.2 ± 1.3	1.6 ± 0.6	0.0 ± 0.0	0.0005
Thymus	12.0 ± 0.4	7.1 ± 2.3	0.9 ± 0.5	0.0098
Blood	11.3 ± 0.4	1.4 ± 0.8	0.1[6]	0.0003

[1] BALB/c recipient T cells but not DBA/2 donor T cells express the allelic CD5.1 marker.
[2] Three to four animals analyzed.
[3] Two animals analyzed.
[4] Comparison between SCID+FL and SCID+Islets+FL.
[5] Mean and SD.
[6] Analysis done on one animal.

Table 2.2. Vβ6 expression on recipient CD8 T cells in immune reconstituted BALB/c-SCID mice with or without a healed-in DBA/2 islets transplant.

	% $CD8\alpha^+V\beta6^+CD5.1^-$ cells[1]			
Location	**SCID+FL[2]**	**SCID+Islets+FL[3]**	**DBA/2[3]**	**p[4]**
Spleen	10.4 ± 1.2[5]	2.8 ± 1.3	0.6 ± 0.4	0.0021
BM	7.8 ± 2.6	2.7 ± 1.8	0.0 ± 0.0	NS[6]
Peritoneum	12.0 ± 2.7	2.2 ± 1.5	0.0 ± 0.0	0.0094
Thymus	10.2 ± 2.3	2.5 ± 0.7	1.0 ± 0.9	0.0118
Blood	9.8 ± 0.3	2.1 ± 1.9	0.1[7]	0.0048

[1] BALB/c recipient T cells but not DBA/2 donor T cells express the allelic CD5.1 marker.
[2] Three to four animals analyzed.
[3] Two animals analyzed.
[4] Comparison between SCID-FL and SCID-Islets-FL.
[5] Mean and SD.
[6] Not statistically significant.
[7] Analysis done on one animal.

Table 2.3. Vβ8 expression in immune reconstituted BALB/c-SCID mice with or without a healed-in DBA/2 islet transplant.

	% $CD4^+V\beta8^+CD5.1^-$ cells[1]			% $CD8\alpha^+V\beta8^+CD5.1^-$ cells		
Location	**SCID+FL[2]**	**SCID+Islets+FL[3]**	**p**	**SCID+FL**	**SCID+Islets+FL**	**P**
Spleen	22.0 ± 2.6[4]	18.1 ± 4.6	NS[5]	38.3 ± 2.8	35.3 ± 0.6	NS
BM	15.1 ± 3.0	11.0 ± 5.8	NS	33.8 ± 5.6	28.2 ± 0.7	NS
Peritoneum	21.1 ± 1.3	19.8 ± 3.7	NS	35.0 ± 4.5	32.9 ± 8.8	NS
Thymus	24.5 ± 1.3	25.6 ± 0.2	NS	36.2 ± 1.2	33.7 ± 1.9	NS
Blood	22.6 ± 0.8	16.8 ± 7.3	NS	37.4 ± 1.3	33.2 ± 4.2	NS

[1] BALB/c recipient T cells but not DBA/2 donor T cells express the allelic CD5.1 marker.
[2] Three to four animals analyzed.
[3] Two animals analyzed.
[4] Mean and SD.
[5] Not statistically significant.

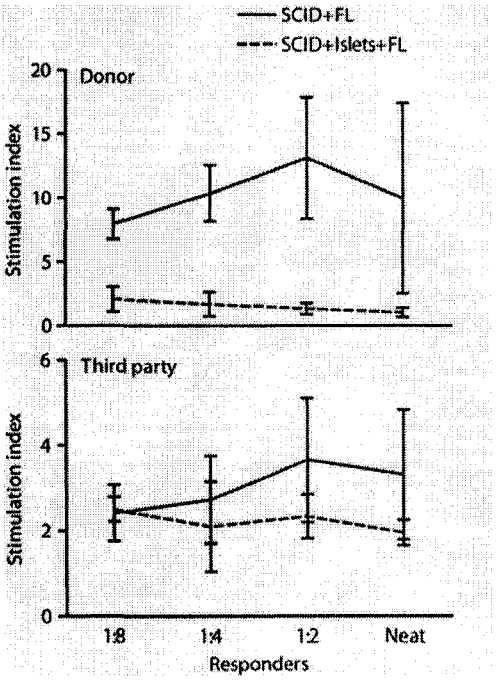

Figure 2.7. *In vitro* assessment of tolerance. More than 20 weeks post FLC reconstitution, BALB/c-SCID mice with (SCID+Islets+FL; n=2) or without (SCID+FL; n=4) prior transplantation of DBA/2 islets were assessed for their response to donor (top) and third party (bottom; B6) alloantigens *in vitro* by the MLR assay. Recipient spleen cells were cultured with stimulators serially diluted 2-fold (neat to 1:8 dilution). Data shown are mean and SD at each dilution of responders expressed as a stimulation index (the counts per minute from donor or third party stimulation divided by the counts per minute from syngeneic BALB/c stimulation).

The tolerogenicity of passenger lymphocytes in the multiple minor-H-mismatched model was an intrinsic property of the cells themselves. BALB/c-SCID mice were given WT FLCs with or without prior injection of DBA/2 spleen cells. This led to chimerism only in FLC recipients that also received donor spleen cells and was associated with the loss of $V\beta6^+$ cells (**Figure 2.8, *A***). Moreover, acute rejection of

donor islet transplants occurred only in recipients of FLCs alone, as long-term acceptance was observed in recipients of donor spleen cells and FLCs (**Figure 2.8, *B***). These data indicate that the prior presence of donor hematopoietic cells that were not passenger lymphocytes of donor islets were by themselves sufficient to induce tolerance, and that there was an absence of islet-specific antigens (*i.e.* DBA/2 spleen cells and islets share an identical set of alloantigens) against which recipient T cells must be tolerized.

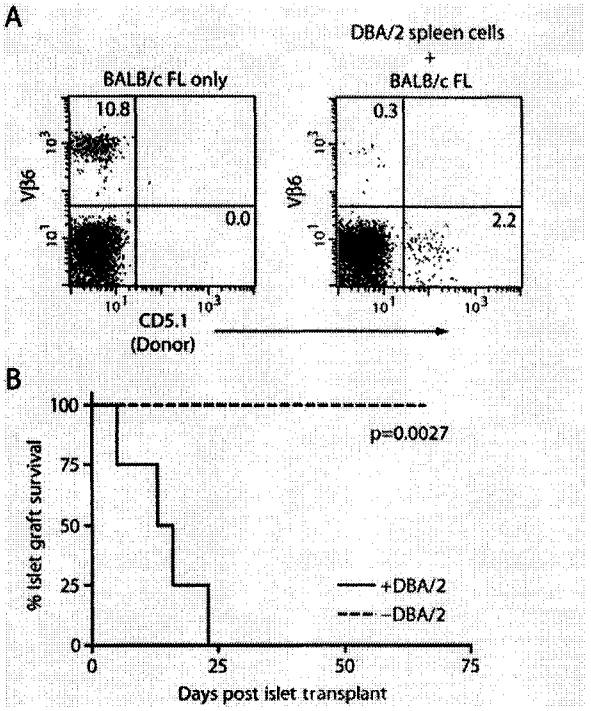

Figure 2.8. Intrinsic tolerogenicity of passenger lymphocytes. (*A*) Naïve BALB/c-SCID mice were given WT FLCs with or without injection of 2×10^6 DBA/2 spleen cells one day earlier (n=4-5). Approximately 9 weeks later, their peripheral blood was analyzed by flow cytometry. $TCR\beta^+$ cells were gated to examine CD5.1 expression (donor marker) versus Vβ6. Relevant quadrant percentages are provided in each representative plot. FLC-reconstituted mice contained a normal level of recipient ($CD5.1^-$) $V\beta6^+$ cells and were devoid of donor cells. However, mice given donor spleen cells in addition to FLCs were chimeric and contained a reduced level of recipient $V\beta6^+$ cells. (*B*) Recipients of FLCs only (–DBA/2) and those given FLCs and donor spleen cells (+DBA/2) were made diabetic and given DBA/2 islet transplants. Survival curve is shown along with p value.

Our previous studies showed that recapitulating the development of an adaptive immune system by FLC reconstitution of adult immunodeficient B6 recipients did not result in any destructive autoimmunity (3), indicating that newly developed T cells underwent proper selection to generate a self-tolerant repertoire. This was similarly suggested in BALB/c-SCID recipients of WT FLCs based on the lack of rejection of syngeneic islets given after reconstitution (data not shown). To distinguish between *bona fide* self-tolerance towards recipient type islets versus suppression of immunity due to diabetes induction prior to islet transplantation (22), we monitored the blood glucose of BALB/c-SCID mice given FLCs beginning around the time of reconstitution to >35 weeks later and observed normoglycemia throughout this period (**Figure 2.9, *A***). Moreover, histology performed on a number of recipient tissues including the pancreas showed an absence of a mononuclear infiltrate (**Figure 2.9, *B-E***), suggesting the

absence of organ-specific autoimmunity. Thus, similar to the B6 strain (3), BALB/c-SCID recipients of WT FLCs also showed the proper establishment of a self-tolerant adaptive immune repertoire.

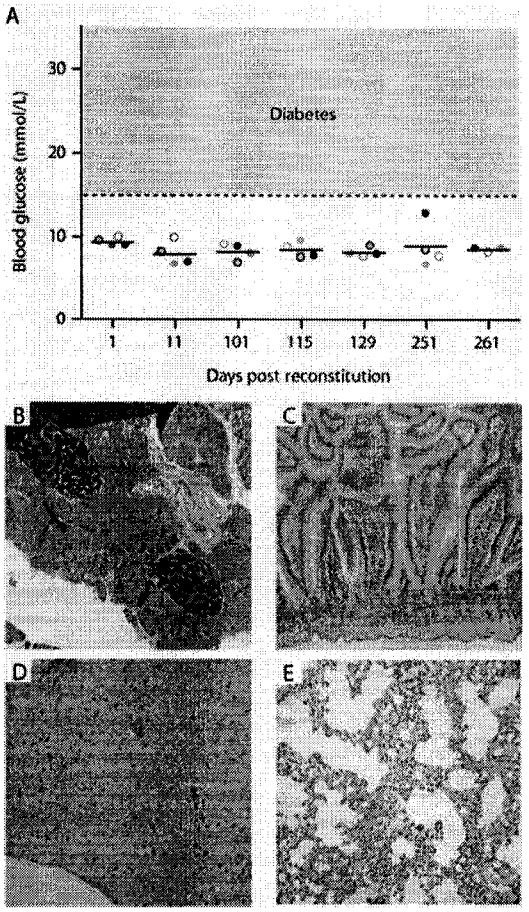

Figure 2.9. Absence of destructive autoimmunity following immune reconstitution of BALB/c-SCID mice by FLCs. BALB/c-SCID mice (n=4) given WT FLCs were examined for signs of autoimmunity following reconstitution of their adaptive immune system. (*A*) Blood glucose monitoring in individual mice at various times post reconstitution. Each mouse is represented by a different symbol. Shaded area above the horizontal dotted line (≥15.0 mmol/L) represents the lower limit for which animals are considered to be diabetic. (*B-E*) Tissue samples were harvested from each mouse, stained with hematoxylin and eosin and analyzed at 100× magnification. Shown are sections of pancreas in *B* (insulin stained brown by immunohistochemistry), duodenum in *C*, liver in *D*, and lung in *E* from one representative animal. Other tissues examined included heart, kidney, skin and thyroid (data not shown).

2.3.3. Recipient NK cells restrict donor cell chimerism to the peritoneal cavity: natural tolerance of systemic but not localized donor cells

Having established that natural tolerance to donor cells/tissues does not readily occur in the absence of passenger lymphocytes, except notably with minimal minor-H mismatches, a question arose as to why donor lymphocytes were better tolerogens than solid donor tissues or cell aggregates (*e.g.* islets). A major difference between such solid tissues and donor lymphocytes is the localized versus systemic nature of donor antigen respectively (donor lymphocytes migrate). This difference is often assumed to be critical in explaining the tolerizing ability of donor lymphocyte infusions; to our knowledge this assumption has never been tested. We set up a system where donor lymphocytes, present before recipient immune system generation, were restricted in location versus systemic and asked if they were equally capable of inducing

tolerance (see experimental design in **Figure 2.10**, ***A***). We made use of a serendipitous observation that MHC-mismatched lymphocytes cannot escape or survive outside the peritoneal cavity when the recipient has functional NK cells. We chose peritoneal cells, depleted of most T cells (to avoid GVHD), as a source of donor lymphocytes because they contain a large population of self-sustaining B-1 cells ($C19^+CD43^+CD5^+$) (31), and because of the tolerance observed in recipients with long lasting passenger B cell chimerism (32). BALB/c peritoneal cells were injected into NK cell-sufficient B6 RAG or NK cell-deficient RAG/γc-KO mice. As anticipated, the allogeneic donor lymphocytes injected either i.v. or i.p. into RAG/γc-KO mice survived long-term and were present at high frequency in the spleen and peritoneum (**Figure 2.10**, ***B*** and ***C***). In sharp contrast, donor cells did not survive when injected i.v. into B6 RAG mice. However, when donor cells were injected i.p. into B6 RAG mice they were able to survive in the peritoneum but were absent in lymphoid tissues outside the peritoneum, including the spleen, lymph nodes, BM and thymus (**Figure 2.10**, ***B*** and ***C*** and data not shown). These differences between B6 RAG versus RAG/γc-KO recipients were also observed by assessing donor cells in peripheral blood; donor cells were present at low levels in all RAG/γc-KO mice but in none of the B6 RAG recipients (**Figure 2.10, *E***). These data suggested that, once in circulation, the donor cells were susceptible to NK cell killing and that the peritoneum is instead either a "protected zone" where the donor cells can either resist killing or perhaps do not stimulate NK cell activity, or that NK cells exhibit poorer function. To further examine whether NK cells were at the root of the differing results in B6 RAG versus RAG/γc-KO mice, we compared the ability to establish donor cell chimerism in RAG mice and RAG mice depleted of NK cells. **Figure 2.10, *D*** shows that NK cell depletion of RAG mice allowed chimerism to be established using the i.v. route of injection, and allowed donor cells to migrate and survive outside the peritoneum (in the spleen) when injected i.p.

Having established localized versus systemic donor lymphocyte chimerism, we asked whether a newly generated immune system tolerates or rejects donor cells when restricted locally to the peritoneum (B6 RAG recipients) versus when they are present more systemically (RAG/γc-KO recipients). **Figure 2.11** shows that donor cells persisted in both the spleen and peritoneum of most FLC-reconstituted RAG/γc-KO mice (though at a lower frequency once the FLCs established an immune system). In contrast, any localized chimerism established in the peritoneum of RAG mice failed to persist following immune reconstitution, as donor cells were undetectable in all reconstituted B6 RAG recipients.

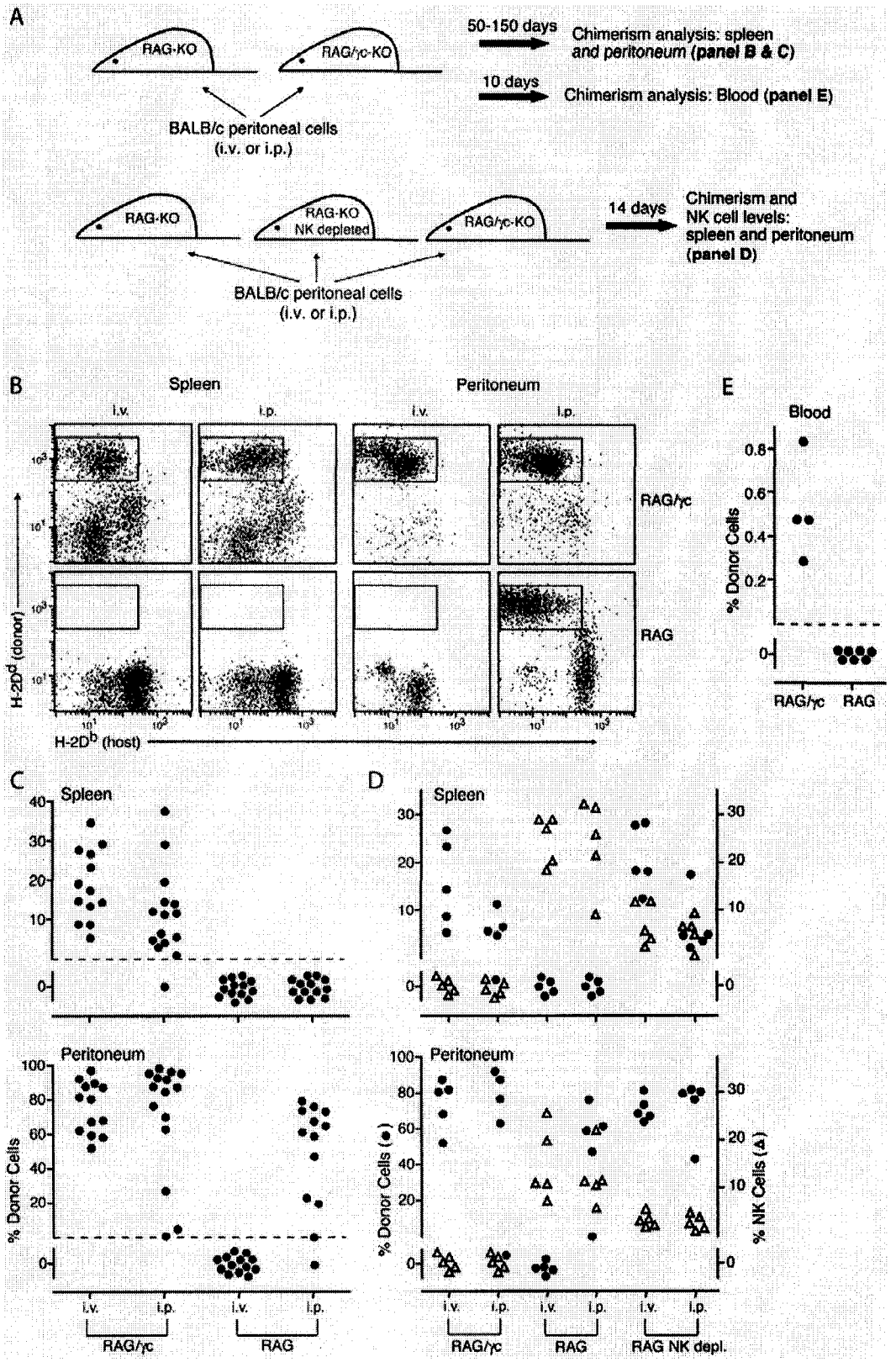

Figure 2.10. Donor lymphocytes are restricted to the peritoneal cavity in NK cell-sufficient but not NK cell-deficient recipients. Experimental design is shown in *A*. In *B* and *C*, chimerism was assessed

from 50-150 days post i.v. versus i.p. injection of donor BALB/c peritoneal cells into RAG-KO versus RAG/γc-KO mice. (*B*) Representative flow cytometry data assessing donor cell chimerism in the lymphocyte gate of recipient spleen and peritoneal cells. Donor cells are defined by the rectangular box in each dot plot. (*C*) The percentage of donor cells in the recipient spleen and peritoneum. Each symbol represents values for an individual mouse. (*D*) The percentage of donor cells and NK cells in the spleen and peritoneum of RAG/γc, RAG, and anti-asialo GM1 treated RAG mice 2 weeks post injection, i.v. or i.p., of BALB/c peritoneal cells. (*E*) Chimerism in peripheral blood of individual recipients assessed 10 days post i.p. injection of donor BALB/c peritoneal cells into RAG versus RAG/γc mice.

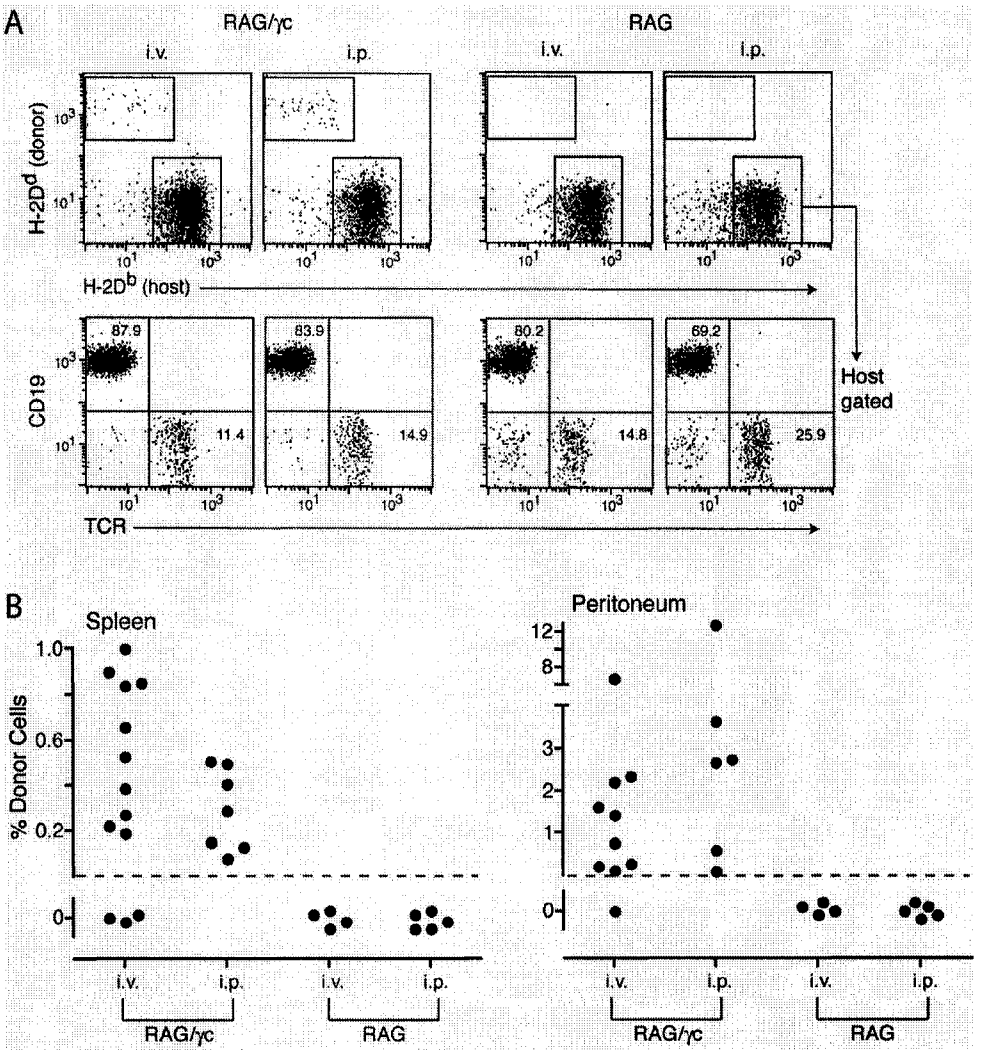

Figure 2.11. Natural tolerance of donor lymphocytes when present systemically but not when restricted to a peripheral site. RAG versus RAG/γc mice previously injected i.v. versus i.p. with donor BALB/c peritoneal cells were reconstituted with B6 FLCs and assessed for donor cell chimerism 50-150 days post immune reconstitution (as described in **Figure 2.10**). (*A*) Representative flow cytometry data assessing donor cell chimerism amongst the peritoneal cells analyzed. Donor and recipient cells are defined by the upper left and lower right rectangular box in each top row dot plot respectively. Lower dot plots show recipient B cell (CD19) and T cell (TCR) reconstitution after gating on recipient cells. (*B*) The percentage of donor cells in the spleen and peritoneum of individual recipients. Comparing RAG/γc versus RAG mice, $p<0.01$ for i.v. injection of peritoneal cells (combined data of spleen plus peritoneum), and $p<0.001$ for i.p. injection.

2.4. DISCUSSION

Self-tolerance due to encounter of self- but not foreign antigens as the immune system develops has long been considered the primary factor that controls immunity versus tolerance (often termed self/nonself discrimination); however, despite its appeal in explaining transplant rejection, this parsimonious solution to the immunity/tolerance problem has encountered its strongest challenge from data in the transplantation field itself (reviewed in (26)). Pre-immunocompetence transplants should theoretically have the same opportunity to trigger natural tolerance mechanisms as do peripheral self-antigens and therefore pharmacologic immunosuppression may not be needed under these conditions. We therefore refer to pre-immunocompetence transplants as natural tolerance models. Given these considerations, it has been surprising that pre-immunocompetence solid tissue transplants have almost universally generated immunity (2, 14-20) or putative ignorance (3) rather than tolerance. However, almost all of these studies were done with fully MHC- and minor-H-mismatched transplants of skin, a strong stimulus that is apparently too strong for natural peripheral tolerance mechanisms to cope with. Nevertheless, reducing the mismatch to as little as the single minor-H antigen H-Y was not sufficient to establish natural tolerance to a pre-immunocompetence skin graft, despite the apparent establishment of full self-tolerance in this model (3). We therefore examined whether the ability of natural tolerance to take hold with allogeneic tissue may be determined not only by the degree of antigenic mismatch but also by the type of tissue or its location. The data herein indicate that natural tolerance is established to minimally mismatched islet tissue transplanted internally (under the kidney capsule) (**Figure 2.3**). The ability of graft location to determine immunity versus natural tolerance to the graft is unlikely to be related to the phenomenon of immune privilege (33), as the renal subcapsular location is not an immune privileged site. Furthermore, when the mismatch was increased to multiple minor-H or MHC mismatches, such pre-immunocompetence transplants in the kidney were able to induce tolerance only in a small fraction of recipients unless they carried passenger lymphocytes that generated systemic chimerism (**Figure 2.5**).

Using a surrogate model that also involves a multiple minor-H-mismatched donor/recipient combination but in which donor reactive T cells could be followed by the expression of a specific Vβ, we examined the mechanism by which passenger lymphocytes induced T cell tolerance. The systemic chimeras demonstrated a strong loss of donor superantigen reactive T cells expressing Vβ6, which likely

occurred via presentation of the superantigen on host APCs to T cells (34), due to the absence of I-E expression on passenger T cells. Thus, consistent with the presence of passenger cells in the thymus (**Figure 2.6**, ***A***), central deletion was likely an important mechanism that generated T cell tolerance towards multiple minor-H-mismatched donor islets. In contrast, the tolerance observed in our single minor-H-mismatched model even in the absence of passenger lymphocytes would suggest peripherally induced tolerance. Peripheral deletion of H-Y-specific T cells following their encounter, in the draining lymph node, with cognate antigen presented by APCs that lack costimulatory signals (due to the presence of a healed-in graft) is a candidate mechanism. However, future studies to confirm this hypothesis are warranted.

Since there was a strong association between the loss of recipient $V\beta6^+$ cells and tolerance induction, we wanted to test whether BALB/c $V\beta6^+$ cells were required in the rejection of DBA/2 islets in this specific donor/recipient combination. To do this, we examined whether the absence of $V\beta6^+$ T cells in the WT BALB/c T cell repertoire would prevent the rejection of DBA/2 islets (conversely, whether the presence of non-$V\beta6^+$ T cells alone could induce rejection). BALB/c-SCID mice were made diabetic, given DBA/2 islet transplants, adoptively transferred with WT BALB/c spleen cells that were either unmodified or depleted of $V\beta6^+$ cells and monitored for graft rejection. Our preliminary data were inconclusive, as only one of the four recipients of WT cells but none of those given Vβ6-depleted cells underwent rejection (graft acceptance >100 days; see **Appendices, A.4**, p.188). If $V\beta6^+$ cells alone were responsible for islet rejection, they could either recognize on target cells the donor superantigen (any T cell expressing Vβ6 could be involved), or epitopes derived from the minor-H differences (only those $V\beta6^+$ T cells specific for donor minor-H antigens would be involved), and induce target killing. However, early studies in which BALB/c mice were given skin or heart grafts from donors expressing the DBA/2 superantigen in addition to minor-H differences showed no accelerated rejection compared to grafts expressing the minor-H differences alone (35, 36), suggesting a lack of contribution by the superantigen in a histocompatibility response. Although a minority of BALB/c recipients rejected skin grafts from congenic BALB/c donors expressing the DBA/2 superantigen (37), it was difficult to ascertain the histocompatibility effect of the superantigen due to subsequent demonstration that these congenic donors differed from the recipient strain in at least one other non-H-2 histocompatibility locus (38). Moreover,

upon encounter with an endogenous viral superantigen presented by lymphoid cells, T cells have generally been observed to undergo expansion followed by deletion or induction of anergy (39-41). In these studies, it is unclear whether the expanded T cells acquired effector function transiently prior to their elimination or inactivation. However, *in vitro* studies have indicated that both CD4 and CD8 T cells primed by superantigen bearing cells do not demonstrate cytotoxicity; only IFN-γ production by CD8 T cells was observed (42). Thus, in the donor/recipient combination that we studied, the donor superantigen is an unlikely target of host immunity, but this cannot be ruled out. Tolerance in recipient T cells capable of recognizing the multiple minor-H differences would also contribute to the consistent long-term graft acceptance observed. We surmised that the migration of passenger cells into recipient thymus could lead to deletion of not only recipient $V\beta6^+$ cells (via recognition of donor superantigen), but provide an opportunity for recipient non-Vβ6 T cells specific for donor minor-H antigens to also be deleted. This was not testable in our model because the minor-H antigen responding T cells could not be tracked. Nevertheless, the loss of these T cells, either physically or functionally, likely contributed to tolerance.

We found the passenger lymphocytes that were the source of systemic chimeric cells to mainly consist of memory cells that appeared to be cycling actively (**Figure 2.6**). Due to the presence of an "empty space" into which these cells migrated from the transplant, it is likely that they were undergoing homeostatic proliferation in the host (43), consistent with the increased BrdU incorporation that we detected. Indeed, the phenotypic changes associated with homeostatic proliferation (44, 45) potentially mediated their entry into the thymus to induce tolerance in donor reactive recipient T cells (46, 47). As demonstrated in a recent study, either CD4 T cells or CD8 T cells could be tolerogenic upon thymic entry (46).

The most obvious conclusion from our studies is that the capacity to establish natural tolerance to allogeneic tissue is quite limited without chimerism, consistent with the potential role of chimerism observed in clinical organ transplant recipients (48). This may not be entirely surprising in hindsight, since the adaptive immune system shows an evolutionary predominance of non-self-reactive T cells, a considerable proportion of which consists of pre-existing memory T cells arisen from their reactivity to environmental antigens. Having such an immune repertoire should clearly stand against favour of any potential capacity for natural self-tolerance mechanisms to tolerize host T cells specific for alloantigens.

However, until these studies here in combination with our previous studies (3, 20), this has not been a well tested prediction.

The identification of the peritoneum as a site where chimeric cells can resist killing by host NK cells allowed us to test whether the differing outcome between pre-immunocompetence hematopoietic and solid tissue transplants is related to the systemic versus localized nature respectively of the donor cells. While we have not yet identified the reasons behind the protection of donor peritoneal cells from NK cell killing in the peritoneum, our data indicate that donor chimerism restricted to this peripheral site does not induce tolerance in a newly generated immune system. In contrast, pre-immunocompetence systemic chimerism achieved in NK cell-deficient recipients survives long after development of the recipient's immune system (**Figure 2.11**). These data suggest that, in addition to tolerance in the newly generated host T cell compartment, host NK cells generated from FLCs also become tolerant of donor cells. We expect this NK cell tolerance will involve a regulation of activating/inhibitory receptor expression during NK cell development (49), a process that does not readily occur in the already mature NK cell repertoire of RAG-KO recipients. The mechanism of resistance to NK cell killing by donor cells in the peritoneum could include differences in peritoneal NK cell function or number relative to other sites, or an absence of the stress-induced changes that trigger NK cells (50-52) when donor peritoneal cells are placed in their natural environment. However, in a model of tumour immunity by NK cells, a lower activity was observed in NK cells endogenous to the peritoneal activity, consistent with possible differences in NK cell function depending on its residence (53).

Together with recent studies (54-56), that have shown an increased scope of central tolerance (57) to self-antigens, our data suggest that the mechanism(s) of natural peripheral tolerance did not evolve the capacity to easily deal with a tissue expressing a multitude of new antigens. We suggest that assessment of tolerance versus immunity to pre-immunocompetence transplants with graded degrees of antigenic mismatch could provide a detailed insight into the number of self-antigens peripheral tolerance evolved to handle. While the studies herein suggest that the number lies somewhere between the number of antigens in H-Y-mismatched and multiple minor-H-mismatched tissue, a more precise definition could be achieved by the stepwise addition of single minor-H mismatches.

The limited capacity of natural peripheral tolerance has important implications for strategies aimed at achieving transplantation tolerance in the clinic. Most basic research towards this goal has been focused on achieving donor-specific tolerance by blocking putative "costimulatory" signals. The rationale behind this approach comes from the view that natural self-tolerance involves encounter of self-antigen in the absence of costimulatory signals, and that therefore blocking costimulatory signals during transplantation should allow natural tolerance mechanisms to take hold. However, our studies with long-healed transplants in immunodeficient recipients, where inflammatory molecules and costimulatory triggers are likely to be minimal (2, 3, 58), suggest that costimulation blockade should not work well with strongly mismatched tissue if it really reflects the action of a natural tolerance mechanism. Therefore, since some models using antibody targeting of costimulatory molecules, such as CD40 ligand (CD40L), can induce tolerance to strongly mismatched tissue (59), we suggest that such therapies invoke pathways beyond the natural self-tolerance mechanisms. This view is supported by recent data showing that the ability of targeting CD40L to induce tolerance of donor tissue depends on host mast cells (60). A similar requirement for NK cells has also been shown (61, 62). Since it would seem unlikely that mast cells or NK cells are required for natural self-tolerance, the data suggest that targeting CD40L not only blocks costimulatory signals but also triggers additional regulatory mechanisms unrelated to natural self-tolerance. However, we cannot fully rule out the possibility that long-healed transplants continue to send as yet unrecognized signals that trigger costimulation. The data with minor-H mismatches or greater were suggestive of an association between increased graft healing time and natural tolerance. A small fraction of animals with either MHC- or multiple minor-H-mismatched islets in our study appeared tolerant, and this may be due to the increased graft healing time compared to previous studies where tolerance was not observed (19). More detailed studies will be required to determine the precise contribution of tissue "health" or homeostasis (2, 3, 63, 64) in any tolerance generated with pre-immunocompetence transplants. Interestingly, we found greater acceptance of MHC-mismatched compared to multiple minor-H-mismatched pre-immunocompetence islet transplants; a result that is consistent with an increased importance for the indirect pathway in triggering the anti-donor response to long-healed transplants where a paucity of donor APCs is expected (26, 65, 66). Without donor APCs the direct response is expected to be greatly reduced, leaving T cells triggered via the

indirect pathway that are only able to engage and attack cells of the donor transplant if they are MHC-matched.

In summary, our studies provide an explanation for the opposing outcomes in studies of pre-immunocompetence transplants and suggest that systemic donor chimerism is required to obtain consistent natural tolerance to multiple minor-H-mismatched or MHC-mismatched solid donor tissues, as is the case with islet transplants. However, natural self-tolerance can indeed be applied to an allogeneic tissue but this capability is rather limited.

2.5. AUTHOR'S CONTRIBUTIONS

WFC designed and performed the islet transplantation studies, analyzed data and wrote the paper. Data in Figure 2.10, *E* were generated and analyzed by HR, who also revised the paper (remaining data in Figure 2.10 and all data in Figure 2.11 were generated by Carl Tan). CCA conceived and designed the study, analyzed data and wrote the paper.

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Chapter 3: Generating tolerance to allogeneic islets by mixed chimerism

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3.1. INTRODUCTION

Establishing chimerism by bone marrow transplantation (BMT) has been thought to induce the most robust form of immunological tolerance to alloantigens (1-4) because it takes advantage of central tolerance, consistent with the way the immune system has likely evolved to eliminate most of the potentially destructive anti-self responses (5). In our recently published studies (6) (also refer to **Chapter 2**) we found that natural self-tolerance mechanisms can better be applied to an allogeneic tissue when the tissue contains chimerism-generating passenger lymphocytes, further supporting the importance of hematopoietic chimerism in allogeneic tolerance induction. However, it is clear that even with chimerism, whether induced naturally or otherwise (7-12), it is possible for donor tissues to be rejected (known as split tolerance, since the host appears to be tolerant to one type of donor graft but not a second type from the same donor). We recently confirmed and extended this observation to murine chimeras generated by more than one clinically relevant protocol (13). Importantly, split tolerance appears to be possible in mixed chimeras (11-13) that are more clinically favoured than full chimeras. Most past studies demonstrated split tolerance in chimeras that maintained donor hematopoietic cells but rejected donor skin transplants, the cause of which was likely immunity towards polymorphic tissue-specific antigens expressed by donor skin but absent from their BM cells (11, 13-19). While split tolerance involving tissues other than skin has not often been reported in murine chimeras, in a canine model split tolerance was observed in which chimeric recipients rejected donor hearts (20). Furthermore, pancreatic isoantigens were identified in rabbits (21). These findings suggest the possibility that split tolerance involving non-skin antigens may occur. In addition, potential host resistance to tolerance induction, or a general defect in self-tolerance, could lead to a state of split tolerance with or without involvement of donor tissue-specific antigens. We investigated whether this might be the case with NOD mice, whose background possesses the genetically dissociable traits of tolerance resistance (22-24) and autoimmune mediated beta cell destruction (25). Unlike nonautoimmune hosts, NOD mice are capable of allogeneic islet rejection by the combined effects of autoimmunity and alloimmunity (26). Since chimerism is viewed to be a potential approach towards islet transplantation tolerance in type 1 diabetics, the potential for increased split tolerance in the NOD mouse model of human type 1 diabetes therefore requires full evaluation. Surprisingly, we found that NOD chimeras lacking beta cell autoimmunity, unlike chimeric B6 controls, developed multiple levels of split

tolerance such that donor T cells survived, but other donor hematopoietic cells including B cells, as well as donor skin and islet transplants were rejected. Split tolerance was mediated by non-MHC genes of the NOD genetic background. We also identified that, in mixed chimeras, humoral tolerance to donor alloantigens could be extended to third party alloantigens.

3.2. MATERIALS AND METHODS

3.2.1. Animals. Adult C3H (H-2^k), B6 (H-2^b) and DBA/2 (H-2^d) mice were purchased from NCI-Frederick (Frederick, MD). FVB (H-2^q), B6.NOD (B6.g7; H-2^{g7}), NOD.B10 (H-2^b) and NOD (H-2^{g7}) mice were purchased from Jackson Laboratory (Bar Harbor, ME). BALB/c (H-2^d) mice were purchased locally from the Health Sciences Laboratory Animal Services. C3H.129S6(B6)-*Rag2*^{tm1Fwa} (C3H-RAG-KO) mice were purchased from Taconic Farms (Germantown, NY). B6.129S7-*Rag1*^{tm1Mom} (B6-RAG-KO) and NOD.129S7(B6)-*Rag1*^{tm1Mom} (NOD-RAG-KO) mice, as well as the F1 progeny of NOD and B6 ((NOD×B6)F1) or NOD and BALB/c ((NOD×BALB/c)F1) crosses were bred at the University of Alberta. B cell-deficient NOD (NOD-μMT) mice (27) were kindly provided by Dr. David Serreze and bred on-site. Nondiabetic female NOD mice were used at 7-12 weeks of age. In indicated experiments, pregnant C3H and NOD mice were generated and their fetuses used at day 15 of gestation as a source of FLCs. All care and handling of animals were carried out in accordance with the guidelines of the CCAC.

3.2.2. Chimerism induction protocol and BMT. To induce chimerism in NOD or B6 mice, nondiabetic recipients were given a donor-specific transfusion (DST) of 20×10^6 unmodified C3H whole spleen cells administered i.p. on day -7, anti-CD40L antibody (MR1; Bio Express, West Lebanon, NH) and CTLA-4-Ig (binds B7; Bio Express) at 0.25 mg i.p. each on days -7, -5, -3, 0, 2, 4 and 6, busulfan (BUS; either kindly provided by Orphan Medical Inc., Minneapolis, MN, or purchased from the Cross Cancer Institute Pharmacy, Edmonton, AB) at 20 mg/kg i.p. on day -1, and sirolimus (SRL; Wyeth Canada, Montreal, QC) at 3 mg/kg i.p. and daily from days 0-28 (**Figure 3.1**). BUS tablets (2 mg) were dissolved in 0.111 mL of N',N-dimethylacetamide, 0.214 mL of polyethylene glycol and 0.675 mL of normal saline per tablet. SRL (1 mg/mL solution) was diluted in PBS prior to injection. Twenty or 40×10^6 unmodified C3H BM cells

harvested from the femur and tibia of donor mice were injected in PBS i.v. on day 0. To assess the short-term effects of BUS on the immune system, NOD mice were either treated with vehicle or BUS at 20 mg/kg i.p., and their spleens and thymi were harvested 1, 9 or 20 days after treatment for analysis.

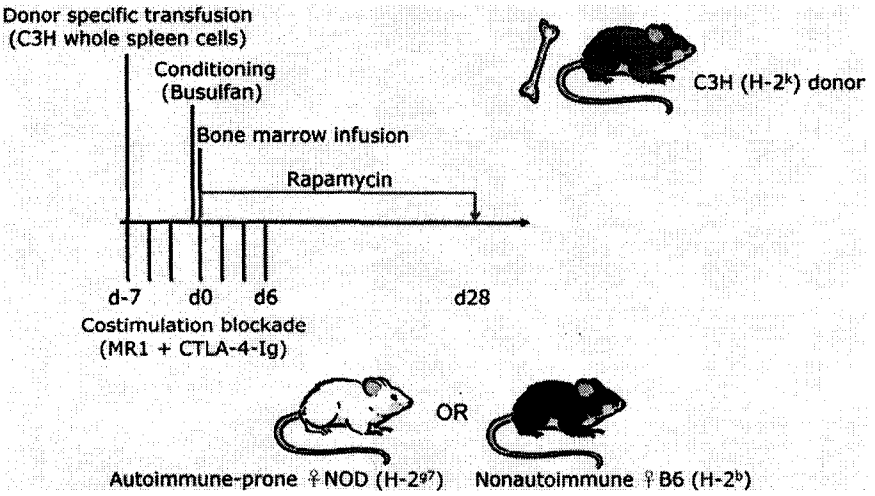

Figure 3.1. Chimerism induction protocol. See Materials and methods for complete details.

3.2.3. Skin transplantation. Full thickness tail skin grafts were transplanted onto the lateral thoracic wall of anaesthetized recipients. Grafts were secured with sutures and protected with gauze and bandage for a minimum of 7 days. Health of donor skin was monitored by visual and tactile inspection. The day of skin rejection was defined as graft necrosis of approximately 100%.

3.2.4. Islet isolation and transplantation, nephrectomy and glucose monitoring. Islet isolation was carried out as previously described (28). NOD and B6 mice were made diabetic by a single i.p. injection of STZ (Sigma-Aldrich Canada, Oakville, ON) at 200-225 mg/kg. Diabetes was confirmed by a blood glucose measurement of >20.0 mmol/L. Five hundred islets were transplanted into recipient renal subcapsular space. Blood glucose was monitored to detect rejection (>15.0 mmol/L on two consecutive readings on different days). Some recipients that rejected the islets were given second transplants to the contralateral kidney. Some recipients that showed long-term acceptance of donor islets (>100 days post transplant) were assessed for their dependence on the islet grafts to maintain normoglycemia by undergoing recovery nephrectomy. To monitor the rate of diabetes development, we obtained weekly blood glucose

measurements from untreated and treated NOD mice until disease onset (>15.0 mmol/L in at least two readings from separate days) or up to 43 weeks of age.

3.2.5. Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were obtained by tail bleeding. Spleen, thymus and lymph nodes were homogenized in PBS. To analyze cells in the peritoneum, a peritoneal lavage was performed by flushing the peritoneal cavity with PBS. Cells prepared for antibody staining were incubated with a combination of anti-CD16/CD32 antibody (2.4G2), and mouse, rat and hamster sera before the addition of primary antibody cocktails. Chimerism in the NOD model was detected by staining for donor MHC molecules, either H-2K^k-expressing (C3H donor; 36-7-5) or H-2D^q/L^q-expressing (FVB donor; KH117) cells that were distinguished from NOD cells expressing H-2D^b (KH95). Other antibodies used for chimerism analysis included TCRβ (H57-597), CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70.15), CD11c (HL3), CD19 (6D5), CD25 (PC61.5) and CD49b (DX5). For Vβ analysis, anti-Vβ6 (RR4-7), Vβ10^b (B21.5) and Vβ17^a (KJ23) antibodies were used. Staining of CD4 T cells expressing Foxp3 (FJK-16s; eBioscience) was performed according to the manufacturer's instructions. Additional antibodies used for flow cytometry included CD44 (IM7), CD62L (MEL-14), CD122 (5H4), and the DC-specific antibody clone 33D1 as indicated. Biotinylated antibodies were detected using Tricolor- or allophycocyanin-conjugated streptavidin. Antibodies were purchased from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA) and Caltag (Burlingame, CA). Data were acquired using a two-laser FACSCalibur™ (Becton Dickson, Sunnyvale, CA) and analyzed with CellQuest™.

3.2.6. NK cell depletion. NK cells were depleted *in vivo* by injecting 30-40 μL of rabbit anti-mouse/rat asialo GM1 antibody (Wako Chemicals USA, Richmond, VA) in PBS i.p. 3 days before experiment. Some recipients were further treated with BUS at 20 mg/kg i.p. 1 day before experiment. On the day of experiment (day 0), BUS-treated mice with or without NK cell depletion were given a mixture of 15×10^6 C3H and 15×10^6 NOD FLCs in PBS i.v.

3.2.7. Adoptive transfer of B cells. C3H spleens were purified for B cells by a two-step magnetic sorting procedure. Whole spleen cells were first labelled with anti-CD90.2, anti-CD4 and anti-CD49b (with or

without anti-CD8α) microbeads, and negative selection was performed using the LD MACS® separation column (Miltenyi Biotec, Auburn, CA). The unlabeled fraction was then incubated with anti-CD19 microbeads, and positive selection was performed using the LS column. Five million magnetically sorted B cells (mean purity >99% from three independent experiments) were injected in PBS i.v. into NOD-RAG-KO mice (day 0) that were either left untreated, given SRL at 3 mg/kg i.p. on days 0-2, or previously depleted of NK cells by anti-asialo GM1 treatment. On day 3, recipient spleens were harvested for manual cell count (to calculate the absolute number of donor cells present) and flow cytometric analysis.

3.2.8. *In vivo* proliferation assay. An *in vivo* proliferation assay was used to assess donor-specific tolerance. Splenocytes from control naïve or chimeric NOD mice were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) at 5 μM (see **Appendices**, **A.1.5.4**; p.161). Twenty million cells per i.v. injection were given to anti-asialo GM1 treated NOD-RAG-KO, C3H-RAG-KO and B6-RAG-KO mice representing syngeneic, donor type and third party recipients respectively. Four days later, recipient spleens were harvested and CFSE dilution in the transferred cells was examined by flow cytometry gating on $TCR\beta^+$ cells. We did not observe any significant signs of graft versus host reaction other than splenomegaly in our recipients. Total splenocyte numbers were also determined, by counting on a hemacytometer, to calculate the absolute number of T cells recovered.

3.2.9. Serum alloantibody detection. To detect alloantibodies in recipient serum, at greater than 14 weeks post BMT we immunized NOD and B6 chimeras as well as control nonchimeric NOD and B6 mice with 10×10^6 gamma-irradiated (1500 rads; ^{137}Cs irradiator, Gammacell 40, Atomic Energy of Canada, Ottawa, ON) or non-irradiated C3H whole spleen cells i.p. More than 3 weeks later, we secondarily immunized them with 10×10^6 irradiated FVB whole spleen cells i.p. Three to 4 weeks after each immunization, animals were tail-bled and sera harvested. Sera from immunized NOD or B6 chimeras, immunized nonchimeric controls, or unimmunized naïve controls were incubated with syngeneic, donor type and third party spleen cells. Binding of serum immunoglobulin G (IgG) antibodies to spleen cells was detected using fluorescein isothiocyanate (FITC)-conjugated Fc_γ-specific rabbit anti-mouse IgG $F(ab')_2$ fragments (Jackson ImmunoResearch, West Grove, PA) and analyzed by flow cytometry.

3.2.10. Statistical analysis. Means are reported along with SD or SEM as indicated. Two-tailed Student's *t*-test was used for comparison of means between two groups. Log-rank test was used to compare survival curves. One-way ANOVA and Tukey's multiple comparison test were used to compare three or more means. All statistical analyses were done using Prism 4 (GraphPad Software, San Diego, CA) with statistical significance defined as $p<0.05$.

3.3. RESULTS

3.3.1. Split tolerance towards donor hematopoietic lineages develops in tolerance resistant NOD mice; effect of SRL on host NK cell function

Using an irradiation-free, costimulation blockade-based nonmyeloablative protocol, we induced mixed hematopoietic chimerism in NOD mice using fully mismatched BM and tested for subsequent tolerance induction to donor tissues. For comparison, we also induced chimerism in B6 mice that are not known for tolerance resistance or autoimmunity. We chose the alkylating, chemotherapeutic drug BUS as the conditioning agent to generate hematopoietic "space" because it is already widely used in clinical treatment of cancer, and the level of chimerism achieved in animal studies has shown that it can be adjusted according to dose (29). Thus, it has considerable potential in clinical chimerism approaches. Aside from its likely targeting of myeloid cells (30) and early hematopoietic stem cells (31-35), we first examined the effect of BUS specifically on the NOD immune system. We investigated whether BUS affects the cellularity and/or composition of the NOD thymus and spleen, which is unknown. We injected NOD mice with a single dose of BUS alone and examined their thymus and spleen on different days post treatment, compared to vehicle injected mice. At 20 mg/kg, which is between 4-7 fold less than its Lethal Dose 50% depending on the recipient strain (29, 36), BUS administered in a single injection has been shown previously to induce mild myelosuppressive effects (29, 37). We found that at this dose, BUS had no significant effect on the cellularity or composition of the thymus and spleen of NOD mice at any of the days examined (**Figure 3.2**, ***A-E***). Consistent with early studies on other rodents (30, 38), a single dose of BUS has no acute immunosuppressive effects on the NOD immune system, as it does not induce peripheral lymphopenia or alter the thymus.

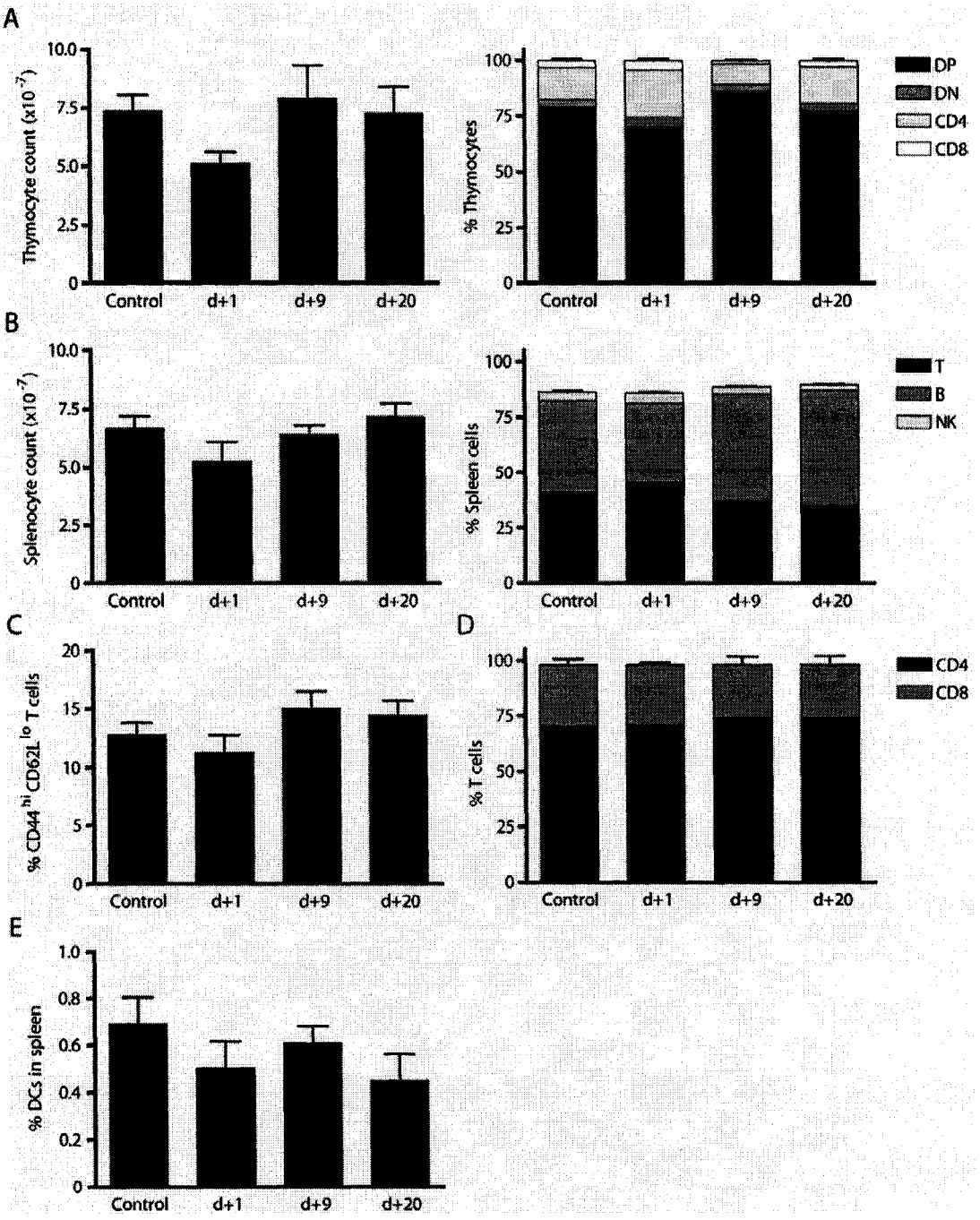

Figure 3.2. BUS does not affect the cellularity or composition of the NOD thymus and spleen. Naïve female NOD mice were either given vehicle (Control) or BUS at 20 mg/kg i.p. and analyzed 1 (d+1), 9 (d+9) or 20 (d+20) days later. (*A*) Left: Thymocyte numbers. Right: Composition of thymocytes assessed by flow cytometry. (*B*) Left: Splenocyte numbers. Right: Proportion of T cells ($TCR\beta^+$), B cells ($CD19^+$) and NK cells ($TCR\beta^-DX5^+CD122^+$) in the spleen, as determined by flow cytometry. (*C*) Proportion of memory T cells present in spleen based on expression of CD44 and CD62L and expressed as a percentage of all T cells. (*D*) Proportion of CD4 versus CD8 T cells in spleen expressed as a percentage of all T cells. (*E*) Proportion of DCs in spleen based on expression of CD11c and the ligand for the DC-specific antibody 33D1. Pooled data from 2-3 independent experiments (n=6-10) are shown. All bars depict mean and SEM. No statistical differences were found between control mice and BUS treated mice in any of the measured parameters, using the one-way ANOVA.

Applying our protocol in three independent experiments (**Figure 3.3**, ***A-D***), we induced mixed chimerism in 26 of 27 NOD mice (versus 100% success in B6 mice). In the majority of mice that we monitored chimerism for up to 32 weeks post BMT, mixed chimerism was maintained both in NOD (**Figure 3.3**, ***A-C***) and B6 (**Figure 3.3**, ***D***) mice and never showed a tendency to become full chimerism. Moreover, in those NOD and B6 chimeras that we sacrificed between 37-58 weeks post BMT, all still contained mixed chimerism (**Table 3.1**). Only 1 of 26 NOD chimeras completely lost chimerism eventually (**Figure 3.3**, ***B***). In general, the chimerism level was higher in B6 than NOD mice and it was not increased by injecting twice as many donor cells into the NOD recipients (**Figure 3.3**, ***C*** versus **Figure 3.3**, ***A*** and ***B***).

We examined the lineage composition of donor cells present in NOD and B6 chimeras between 15-17 weeks post BMT and found that the chimerism was multilineage. We detected, in PBMCs, various donor type cells phenotypically consistent with T ($TCR\beta^+$) cells of both sub-lineages ($CD4^+$ or $CD8^+$), B ($CD19^+$) cells, NK ($DX5^+$) cells, macrophages ($CD11b^+$) and dendritic ($CD11c^+$) cells (**Figure 3.3**, ***E***). In this period, chimerism in B6 mice was significantly higher than that in NOD mice. We also noted significant differences in the levels of most donor type hematopoietic lineages found in B6 and NOD chimeras, with consistently lower levels in the latter mice (**Figure 3.3**, ***E***). Between 20-40 weeks post BMT, we failed to detect donor $DX5^+$, $CD11b^+$ or $CD11c^+$ cells in the spleen of most of the 10 NOD chimeras that we analyzed, while multilineage chimerism was still detectable even in the blood of B6 chimeras at greater than 32 weeks post BMT (n=8) and confirmed subsequently in the spleen (n=2) (**Figure 3.4**).

Table 3.1. Donor C3H chimerism in blood and spleen of NOD and B6 mice between 37-58 weeks post BMT.

		% Overall donor ($H\text{-}2K^{k+}$) cells (mean and SEM)	
Chimera	**N**	**Blood**	**Spleen**
NOD	4	3.32 ± 0.99	2.16 ± 0.89
B6	5	7.17 ± 2.48	8.78 ± 4.23

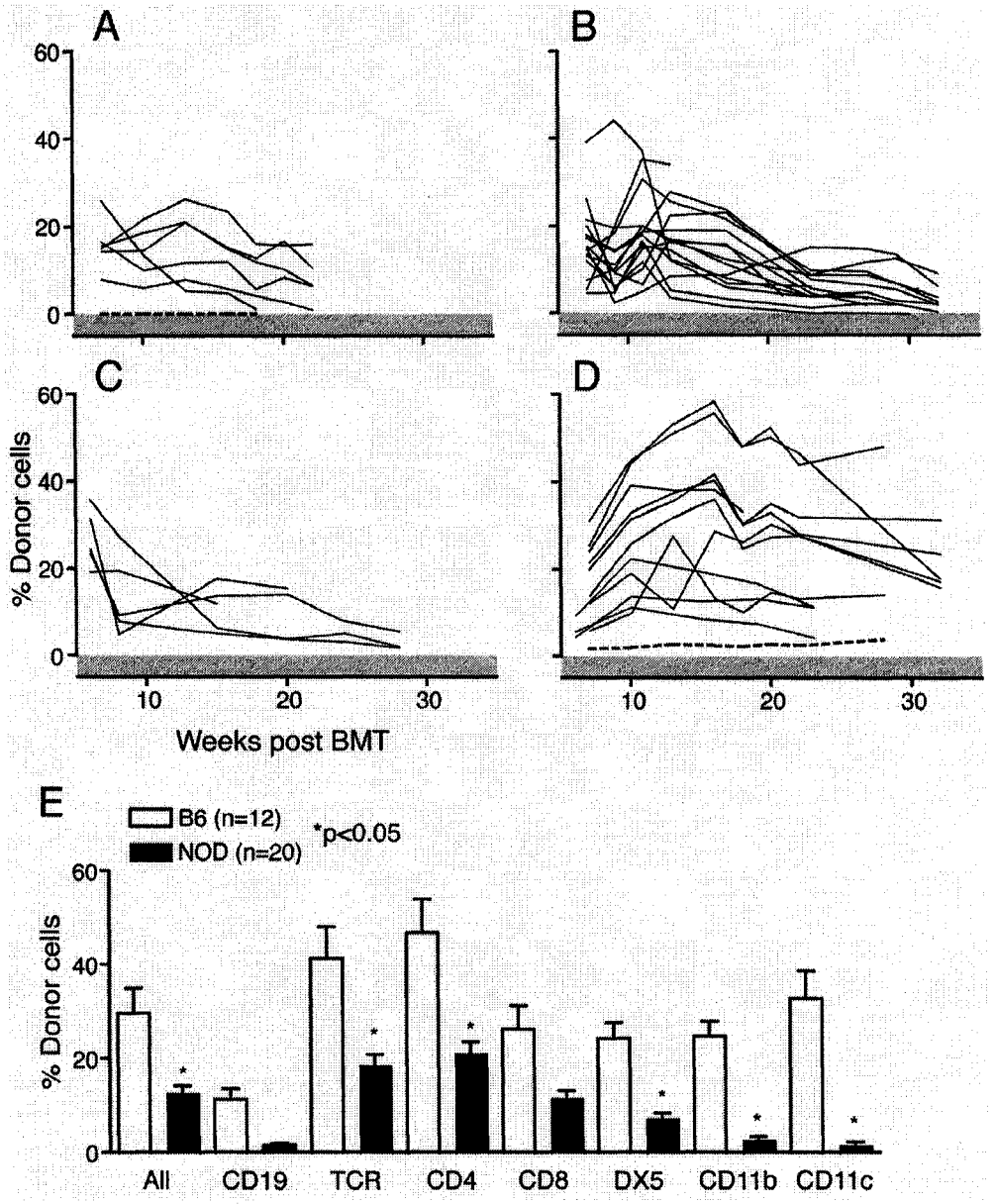

Figure 3.3. Characterization of long-term mixed chimerism in NOD and B6 mice given fully mismatched donor cells. NOD (*A-C*) and B6 (*D*) mice were made chimeric with C3H BM cells (20×10^6 cells injected into all mice except those in *C*, which were given 40×10^6 cells) by an irradiation-free protocol. Chimerism was monitored by flow cytometry of PBMCs beginning as early as 6 weeks post BMT and depicted up to 32 weeks. The percentage of donor cells was determined from among the lymphoid-gated cells, those that stained with antibody against donor versus recipient MHC. Each line represents chimerism monitoring of an individual mouse for all graphs (n=7 in *A*, n=15 in *B*, n=5 in *C* and n=12 in *D*). In *A*, the dotted line represents a mouse that failed to develop chimerism. In *D*, the dotted line represents a mouse that developed and maintained a low-level (<4%) mixed chimerism. Independent repeats of the same experiment are depicted in *A* and *B*. Pooled data from two independent experiments are shown in *D*. In *E*, between 15-17 weeks post BMT, PBMCs of NOD (n=20) and B6 (n=12) mice were analyzed by flow cytometry for the presence of multilineage mixed chimerism. Each bar depicts mean and SEM for the donor hematopoietic lineage stated. NOD and B6 chimeras significantly differed in overall ($p<0.05$), TCR^+ cell ($p<0.001$), $CD4^+$ cell ($p<0.001$), $DX5^+$ cell ($p<0.05$), $CD11b^+$ cell ($p<0.001$) and $CD11c^+$ cell ($p<0.001$) chimerism.

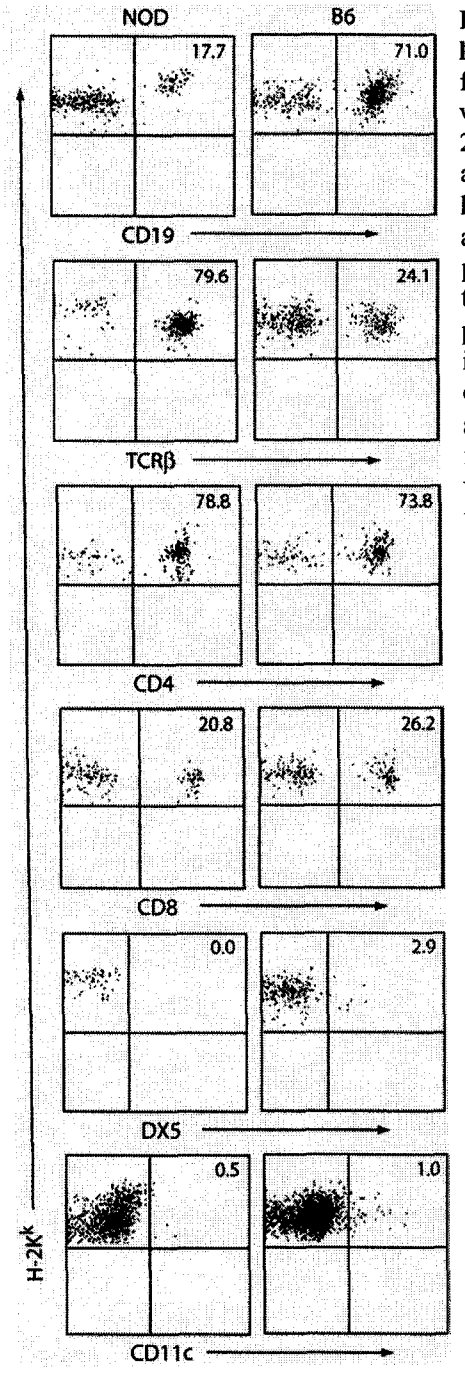

Figure 3.4. Detection of various donor hematopoietic lineages in NOD and B6 chimeras. Shown are FACS plots from one NOD chimera (top) and one B6 chimera (bottom) at 20 weeks and 40 weeks post BMT, respectively, gated on donor (H-$2K^{k+}$) cells within the lymphoid gate. Spleen cells were stained and analyzed by flow cytometry for different donor hematopoietic lineages (not shown is CD11b). For CD19, TCRβ and CD11c, the number in the upper right quadrant reflects the percentage of all H-$2K^k$-expressing cells that stained positive for the indicated marker. For CD4 and CD8, the number reflects the percentage of all H-$2K^{k+}TCR\beta^+$ cells that stained positive for the indicated marker. For the NK cell related marker CD49b (DX5), cells within the lymphoid gate were gated on H-$2K^k$ and analyzed for TCRβ expression. The $TCR\beta^-$ cells were then further gated to examine DX5 expression. The number in the upper right quadrant reflects the percentage of $DX5^+$ cells within this population. Note the relative absence of donor $DX5^+$ cells and $CD11c^+$ cells in the NOD chimera at a much earlier time compared to the persistence of those cells in the B6 chimera at a later time.

When we tracked the level of donor lymphocytes over time, we saw a steady persistence of donor T cells but a precipitous decline and an eventual complete loss of B cell chimerism in the peripheral blood of NOD but not B6 chimeras (**Figure 3.5**, *A*), suggesting that NOD chimeras were split tolerant to the different donor hematopoietic lineages. Subsequent analysis of chimerism in the spleen and BM of 8 NOD chimeras, between 23-40 weeks post BMT, revealed a low to undetectable level of donor B cells in all mice

(**Table 3.2**). Furthermore, in two such chimeras we also failed to detect donor B cells in the peritoneum and the peripheral lymph nodes (**Figure 3.6**). The absence of donor B cells was not simply due to downregulation of the donor MHC class I molecule (H-2K^k) or the B cell marker (CD19) that we stained for, as even when using a different set of markers for donor versus recipient cells (CD45.2 versus CD45.1, respectively) and additional B cell markers (B220, CD21, CD22, sIgM and the donor MHC class II molecule I-A^k) we failed to detect donor B cells in all tissues examined (**Figure 3.6**). However, despite the absence of multilineage mixed chimerism, we were able to detect donor T cells in the thymus and spleen of long-term chimeric NOD mice, confirming the systemic nature of donor chimerism (**Figure 3.5, *B***). To investigate whether this chimerism could lead to deletion of donor-specific T cells, we generated another cohort of NOD chimeras using FVB mice as the source of donor BM. In this combination, the frequency of recipient Vβ10$^+$ T cells expressing the relevant Vβ for binding to FVB-derived superantigen (39) can be monitored, and their decrease in our chimeras would suggest deletion of donor reactive T cells. **Table 3.3** shows that deletion of donor superantigen reactive T cells was not evident at 11 or 19 weeks post BMT in the NOD mixed chimeras, even in the presence of donor B cells that express donor type MHC class II molecules. NOD chimeras were, however, able to delete in a normal fashion Vβ17$^+$ T cells that recognize the corresponding endogenous superantigen, while preserving Vβ6$^+$ T cells that are normally present in NOD mice.

Table 3.2. Relative absence of donor B cell chimerism in spleen and BM of long-term C3H to NOD mixed BM chimeras.

	% Donor B (CD19$^+$) cells[1]	
Chimera	**Spleen**	**BM**
1	<0.15[2]	<0.15
2	<0.15	<0.15
3	<0.15	<0.15
4	<0.15	<0.15
5	<0.15	0.16
6	<0.15	<0.15
7	<0.15	<0.15
8	<0.15	<0.15

[1] Expressed as a percentage of all B cells.
[2] A chimerism level of ≤0.15% was considered to be below the accurate detection limit of flow cytometry in the context of the number of events collected.

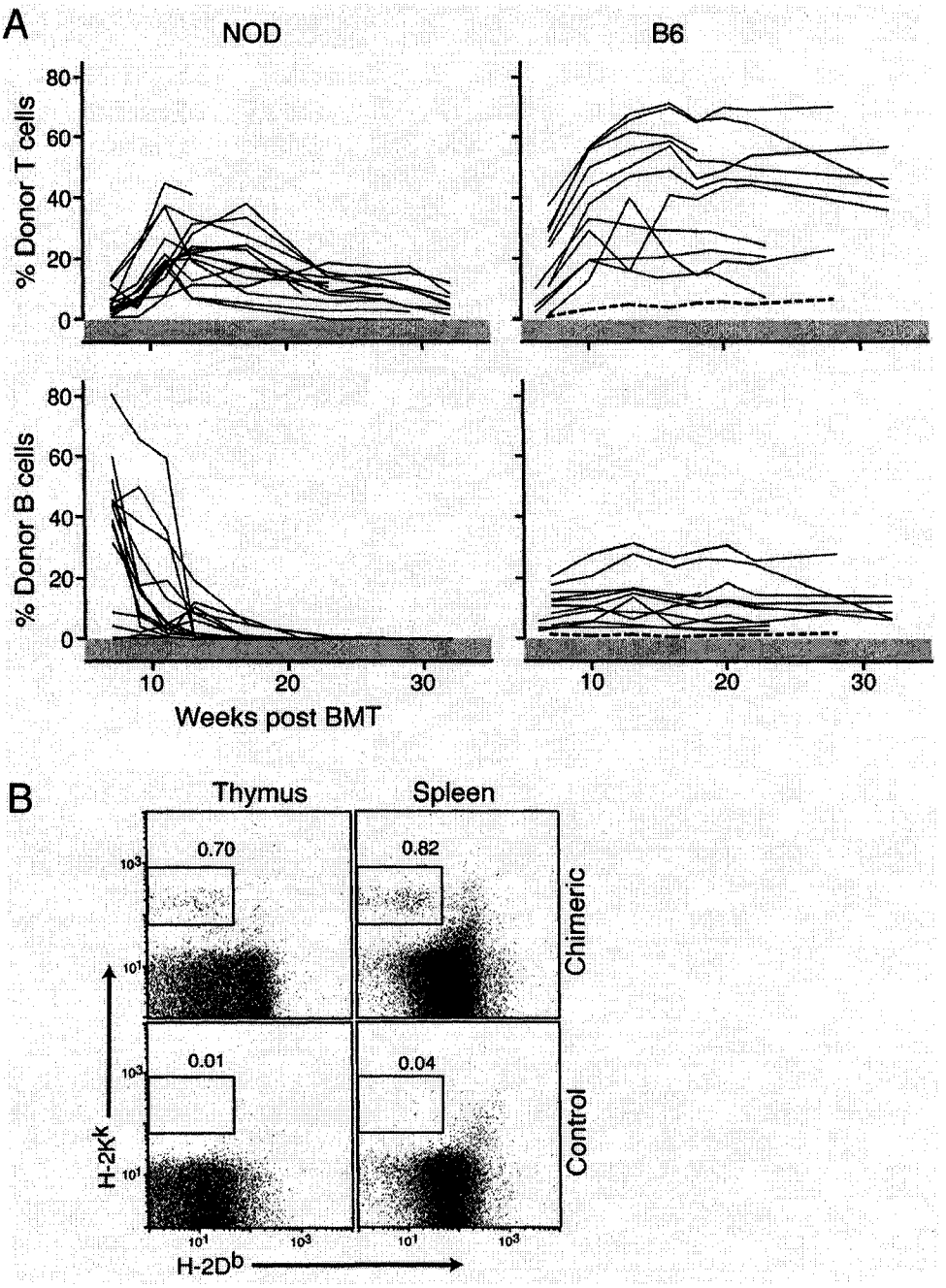

Figure 3.5. Long-term mixed chimerism: distribution and differential survival of donor lymphocytes. (*A*) Long-term monitoring of donor T cells and B cells in PBMCs. Left, top and bottom: Survival of donor T cells and B cells, respectively, in NOD chimeras given 20×10^6 BM cells (n=15) from one experiment. Similar results were obtained in the repeat as well as in the experiment using 40×10^6 BM cells. Right, top and bottom: Survival of donor T cells and B cells, respectively, in B6 chimeras (n=12). The dotted lines represent one mouse that maintained a low-level mixed chimerism consisting of low levels of T cell (0.65-6.42%) and B cell (0.36-1.56%) chimerism. (*B*) Flow cytometric analysis of thymocytes (left) and splenocytes (right) harvested from one NOD chimera at 38 weeks post BMT (representative of 6 mice analyzed between 32-40 weeks post BMT; top row) showed the presence of donor cells in these lymphoid tissues. Bottom row: Thymocytes and splenocytes of a control nonchimeric NOD mouse were stained for comparison.

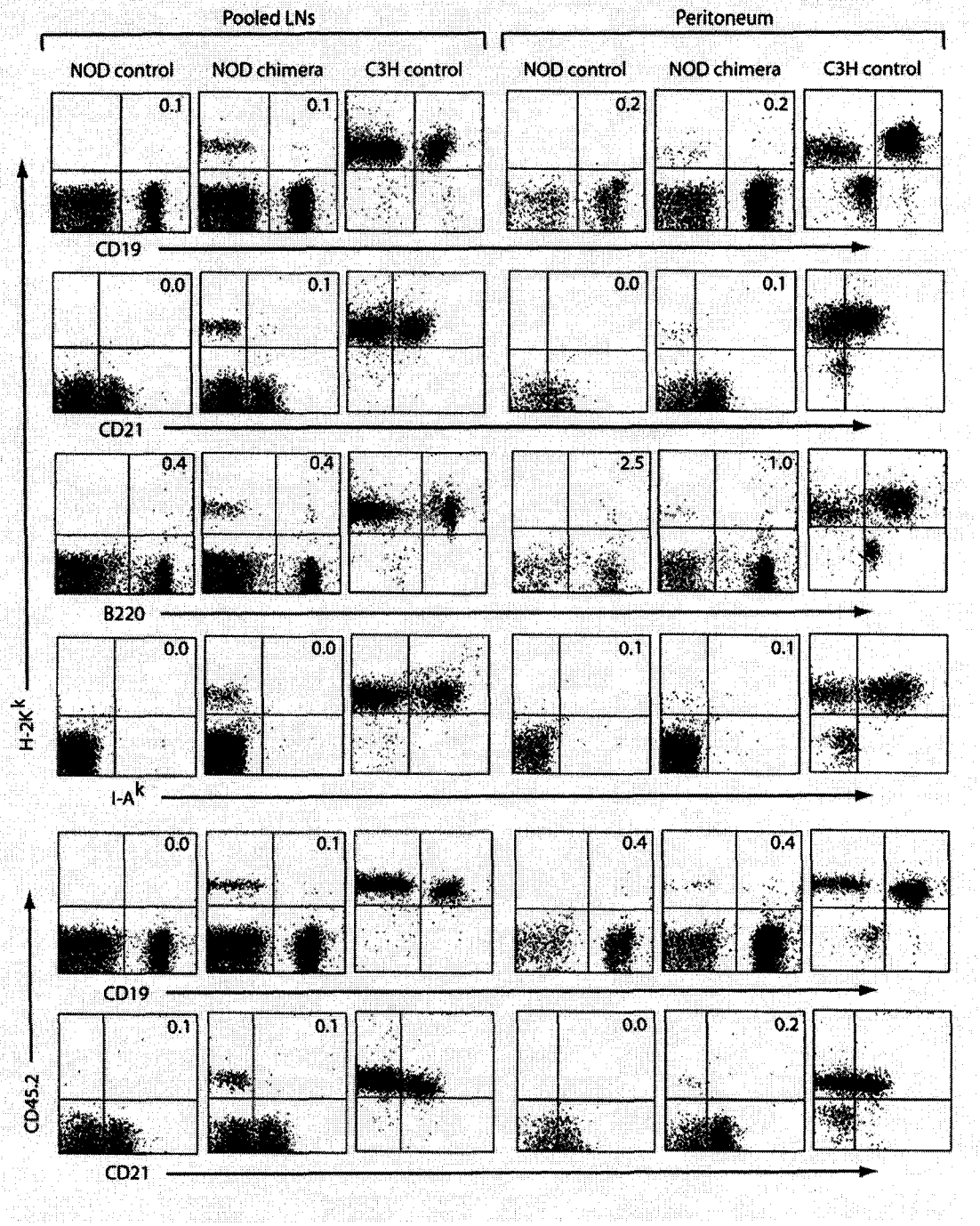

Figure 3.6. Lack of donor B cells in the peritoneum and peripheral lymph nodes of long-term C3H to NOD mixed chimeras. NOD chimeras (n=2) were analyzed for donor B cells at approximately 23 weeks post BMT in their peritoneum and lymph nodes (cells pooled from the axillary, inguinal and cervical lymph nodes). Donor cells were identified based on either H-2K^k or CD45.2 expression. B cell markers used include CD19, CD21, B220 and I-A^k. Similar results were obtained using CD22 and sIgM (not shown). Plots shown are from one chimera. Quadrant percentage for donor B cells detected within the lymphoid gate is shown for the NOD chimera and the negative control NOD. C3H cells harvested from the same two locations were similarly analyzed as a positive control for the stains.

Table 3.3. Vβ expression on peripheral blood T cells from FVB to NOD mixed chimeras.

	Chimera (n=4)		Control (n=2)		Comment
	11 weeks	19 weeks	NOD	FVB	
% Donor cells (all)[1]	4.43±3.75[2]	2.72±2.57	NA	NA	NA[3]
% Donor B cells[1]	1.61±1.41	0.71±0.67	NA	NA	NA
% Donor T cells[1]	5.31±4.30	3.76±3.56	NA	NA	NA
% Vβ6$^+$CD4$^+$	7.89±0.05[4,5]	7.57±0.30[5]	7.94±0.49[5]	6.03±0.13	Control Vβ
% Vβ6$^+$CD4$^-$	9.21±0.34[5]	9.22±1.14[5]	9.83±0.40[5]	15.63±0.05	Control Vβ
% Vβ10$^+$CD4$^+$	2.61±0.17[5]	2.45±0.07[5]	2.75±0.32[5]	0.00±0.00	Absent in FVB
% Vβ10$^+$CD4$^-$	4.45±0.29[5]	4.20±0.41[5]	4.75±0.37[5]	0.05±0.05	Absent in FVB
% Vβ17$^+$CD4$^+$	0.23±0.07	0.15±0.03	0.12±0.07	17.47±0.31	Absent in NOD
% Vβ17$^+$CD4$^-$	0.00±0.00	0.02±0.01	0.05±0.00	3.45±0.15	Absent in NOD

[1] Expressed as a percentage of all cells, all B cells or all T cells respectively.
[2] All data expressed as mean and SEM.
[3] NA: Not applicable.
[4] Vβ percentages reflect H-2D^q/L^{q-}TCRβ$^+$ gated (recipient type) cells.
[5] No statistical significance ($p>0.05$) was found between chimeric and control NOD mice in CD4$^+$ or CD4$^-$ recipient T cells expressing Vβ6 or Vβ10.

Since the donor/recipient combinations (C3H to NOD and C3H to B6) in our chimerism model are fully allogeneic (C3H: D^k K^k I-A^k I-E^k; NOD: D^b K^d I-A^{g7} I-E^{null}; B6: D^b K^b I-A^b I-E^{null}), NK cell killing of donor cells is possible due to their lack of recipient MHC class I expression (40-42). We therefore began to investigate whether NOD or B6 NK cells were relevant effectors in rejecting C3H cells, and what aspect of our chimerism protocol blocked the host NK cell response. First, we tested the ability of NOD or B6 NK cells to reject the few passenger lymphocytes that we have previously shown to be present in islet grafts and readily detectable in recipient systemic circulation post transplant (6). We gave STZ-induced diabetic NOD-RAG-KO or B6-RAG-KO mice (containing NK cells but devoid of T cells and B cells) WT C3H islet transplants and tested for the establishment of chimerism. Unlike NOD-RAG-KO mice that failed to show chimerism, we detected donor cells in B6-RAG-KO mice following islet transplantation, suggesting that allogeneic resistance of C3H cells by NK cells is stronger in NOD mice than B6 mice (**Figure 3.7**) (43). To confirm that NK cells were necessary in the elimination of C3H cells in NOD-RAG-KO hosts, we

directly challenged a naïve cohort of these mice with a mixture of donor and syngeneic FLCs that do not contain mature lymphocytes, and analyzed them for the presence of donor cells when they were either left untreated or previously depleted of NK cells. Recipients of anti-asialo GM1 antibody, compared to untreated mice, contained few NK cells after treatment (**Figure 3.8**, ***A***). Importantly, the absence of NK cells led to chimerism in the antibody-treated mice, while untreated mice were nonchimeric, thus confirming the role of NOD NK cells in rejecting C3H cells (**Figure 3.8**, ***B***).

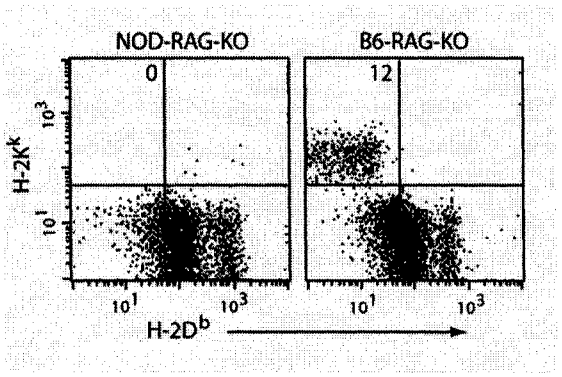

Figure 3.7. C3H passenger cells survived and established chimerism in B6-RAG-KO but not NOD-RAG-KO mice. B6-RAG-KO and NOD-RAG-KO mice (n=2 and 5 respectively) were made diabetic and given C3H islet transplants. More than 40 days later, their peripheral blood was analyzed for C3H (H-2K^{k+}) passenger cells and host (H-2D^{b+}) cells. None of the NOD-RAG-KO but both B6-RAG-KO recipients became chimeric. Representative plots are shown along with the quadrant percentage (lymphoid-gated).

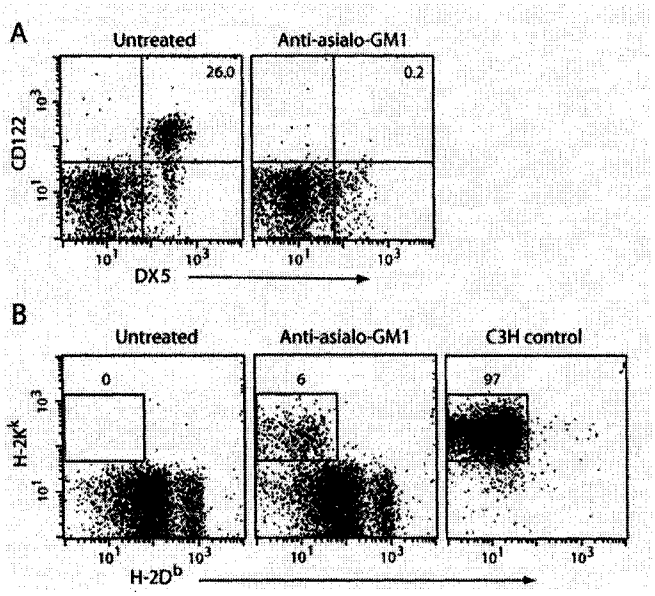

Figure 3.8. Depletion of NK cells promotes engraftment of C3H cells in NOD-RAG-KO mice. NOD-RAG-KO mice were either untreated, or given anti-asialo GM1 to deplete their NK cells (n=4). Two days later, both groups received BUS as conditioning. (*A*) Analysis of peripheral blood NK cells (DX5^{+}CD122^{+}) 3 days after anti-asialo GM1 treatment. Percentage of NK cells detected in the lymphoid gate is shown. (*B*) One day after BUS injection, both groups received a mixture of 15×10^6 C3H and 15×10^6 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-2D^{b+}) cells. A C3H mouse is tested as a positive control. The percentage of donor cells gated in each representative plot is shown.

Based on these studies, it would appear that preventing NK cell killing of donor cells is relatively important in the C3H to NOD combination, but not in B6 mice. Since we showed that our chimerism induction protocol resulted in long-term mixed chimerism in NOD mice (**Figure 3.3**, ***A***), this indicates that one or more components of the protocol (BUS, MR1, CTLA-4-Ig and/or SRL) inhibited host NK cell

function to allow donor BM engraftment. Since the effect of BUS (44) and the role of CD40L (45) in NK cell cytotoxicity is known, while the restricted expression of B7 on hematopoietic cells renders BM stem cells an unlikely target of CTLA-4-Ig (46), we examined the ability of SRL to inhibit NOD NK cells, which has not been tested. NOD-RAG-KO mice were either left untreated or given SRL, transferred with purified C3H B cells (**Figure 3.9**, ***A***) as a source of allogeneic hematopoietic cells and analyzed for donor cells 3 days later. Indeed, significantly more donor cells were detected in SRL-treated mice than untreated mice, but less than mice depleted of NK cells at the time interval examined, indicating that SRL inhibited NK cell cytotoxicity against susceptible target cells *in vivo* (**Figure 3.9**, ***B*** and ***C***). Within this short time frame, inhibition appeared to be of NK cell function rather than their numbers (**Figure 3.9**, ***D*** and ***E***).

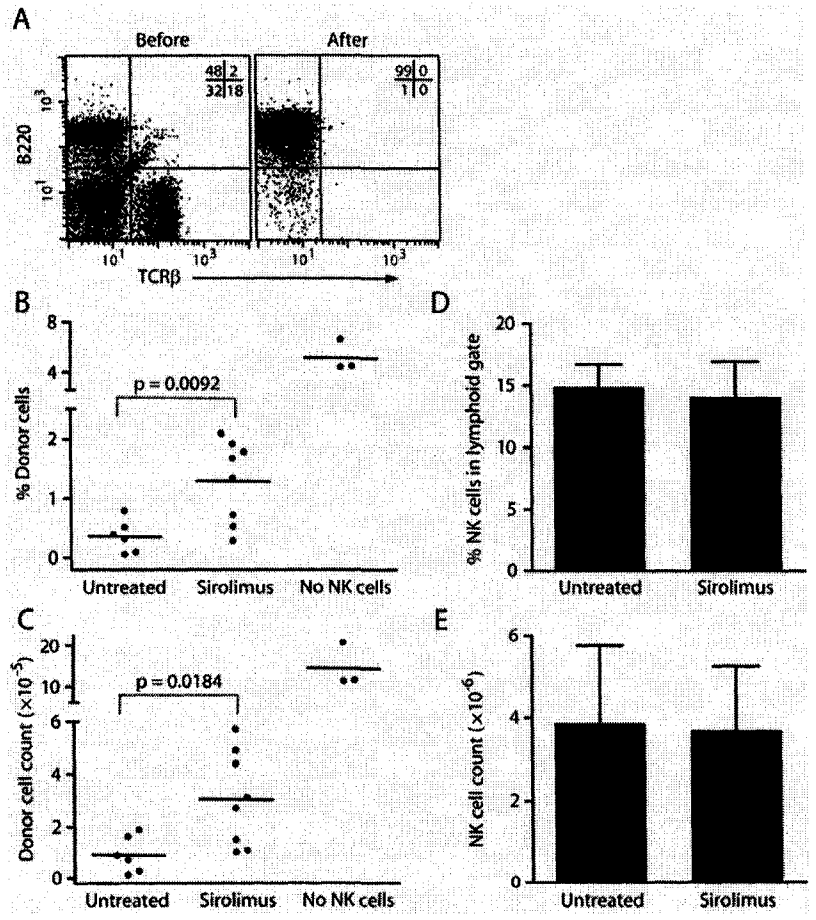

Figure 3.9. SRL inhibits NK cell killing of allogeneic cells. NOD-RAG-KO mice adoptively transferred with 5 × 10^6 magnetically sorted C3H splenic B cells on day 0, were either left untreated (n=6), given SRL 3 mg/kg i.p. on days 0-2 (n=8), or depleted of NK cells by anti-asialo GM1 on day -3 (No NK cells; n=3). On day 0, NK cell depletion was assessed by flow cytometry of PBMCs (0.5 ± 0.2% for antibody-treated mice versus 25.8 ± 7.8% for untreated mice). On day 3, spleens were harvested for analysis. (*A*) After magnetic sorting, C3H spleen cells were analyzed by flow cytometry for its composition, prior to adoptive transfer. The expression of B220 versus TCRβ on lymphoid-gated cells from one experiment is shown and compared to that before sorting. Quadrant percentages are indicated in the legend at the upper right quadrant of each plot. (*B*) Scatter plot of the percentage of donor cells detected on day 3 in each group. (*C*) Absolute number of donor cells. (*D*) Percentage of NK cells (cells coexpressing DX5 and CD122; mean and SD) in the spleen of untreated (n=2) versus SRL-treated (n=3) mice on day 3, determined in one experiment. (*E*) Absolute number of NK cells (mean and SD), determined from mice in *D*, by multiplying the total cell count with the percentage of $DX5^+CD122^+$ NK cells in all lymphoid-gated cells.

3.3.2. Split tolerance extends to donor skin and islet transplants in NOD mixed chimeras lacking overt islet autoimmunity

While B6 mice appeared fully tolerant of donor hematopoietic cells, NOD recipients appeared tolerant of donor T cells but not donor B cells and other non-T cells. To further test the extent of split tolerance, we examined whether the NOD mixed chimeras could reject donor skin and islets. Based on our previous work (13) and that of other investigators (12, 47), showing that split tolerance occurs in MR1-treated nonautoimmune mixed chimeras given donor skin grafts late but not early after BM infusion, we determined whether this would also be the case in our NOD chimeras. At 1-2 days or 14 weeks post BMT, we challenged NOD and B6 chimeras with donor and third party skin grafts. While NOD chimeras acutely rejected third party skin transplants as expected, they also rapidly rejected donor type skin given late, indicating split tolerance (**Figure 3.10**, ***A***). B6 mice made chimeric by the same protocol also rejected donor and third party skin transplants given at 14 weeks. Similar to our previous data with a different MR1-based protocol (13), B6 chimeras demonstrated long-term acceptance of donor type skin grafts given early after BMT. Surprisingly, NOD chimeras transplanted with donor skin at this early time instead rejected the grafts (rejection was delayed compared to late skin grafting, $p<0.01$), demonstrating split tolerance to skin could be reduced in B6 but not NOD mice by giving the graft early (**Figure 3.10**, ***A***).

Having shown that NOD mice appear to have a greater propensity for split tolerance to both donor skin, and certain lineages of donor hematopoietic cells, we next investigated whether split tolerance extended to donor islets. Even at moderate levels, mixed chimerism in nonautoimmune mice led to robust tolerance to strongly mismatched donor islet transplants (48), suggesting that split tolerance may be less of an issue for islets than skin. However, either islet autoimmunity or tolerance resistance of NOD mice could potentially alter the outcome. We found with our protocol, that transplantation of fully mismatched BM did not universally abrogate diabetes (**Figure 3.10**, ***B***). Nevertheless, the general rate of diabetes was significantly reduced in chimeras, reducing the likelihood that any islet rejection we might observe would be due to autoimmunity. Furthermore, we gave a single, high dose of STZ to induce diabetes in those mixed chimeras that remained nondiabetic prior to islet transplantation, a treatment that is known to prevent islet autoimmunity in NOD mice (49). Despite the presence of mixed chimerism, NOD chimeras rejected donor islets as rapidly as control nonchimeric NOD mice (**Figure 3.10**, ***C***), indicating that split

tolerance in relation to tissue transplants was not exclusive to skin. Even donor islets transplanted at the time of BM infusion were rejected by NOD chimeras, despite high levels of chimerism in some of these mice both during and after rejection (**Figure 3.10, *D***). However, syngeneic islets were accepted long-term when transplanted into NOD chimeras that had either rejected donor islets or were not previously challenged, indicating an absence of islet-specific autoimmunity (**Figure 3.10, *C***). While NOD chimeras were split tolerant to donor islets, B6 chimeras showed long-term acceptance of donor islets but not third party islets (**Figure 3.10, *C***). Donor islet acceptance even occurred in the one B6 recipient with long-term low-level (<4%) chimerism (**Figure 3.3, *D***); B6 recipients of the chimerism induction protocol without BMT did not achieve tolerance and acutely rejected donor islets (days of rejection: 12, 14 and 20). Control naïve B6 mice acutely rejected donor and third party islets (**Figure 3.10, *C***). Thus, while we showed that mixed chimerism was achieved in NOD mice, and that they demonstrated an even more pronounced split tolerance to donor skin than nonautoimmune-prone mice, there was additionally split tolerance to donor islets; surprisingly, this was not a result of islet autoimmunity.

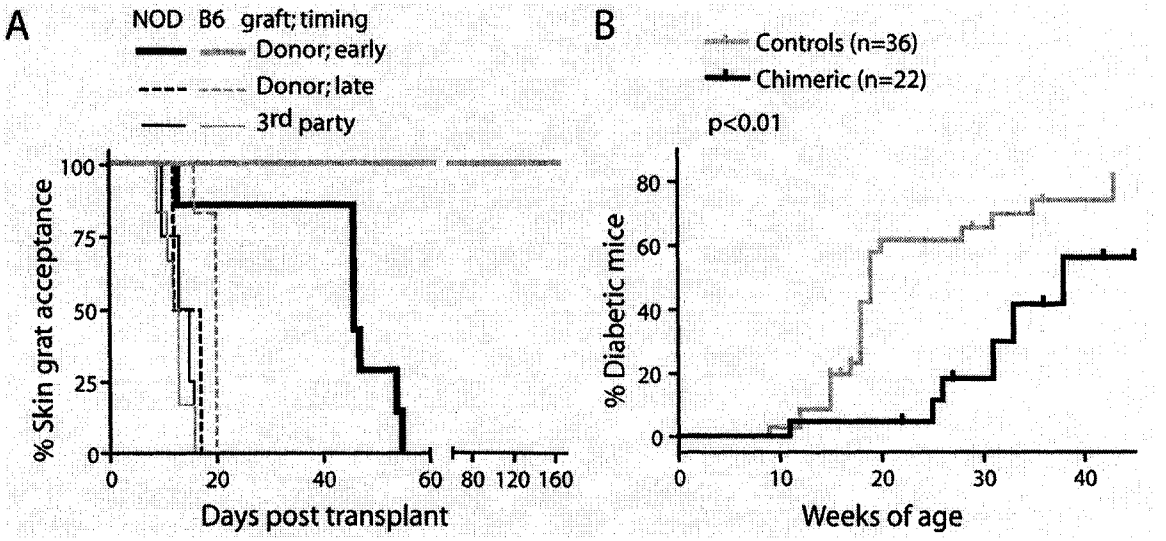

Figure 3.10. Diabetes incidence and full tolerance or split tolerance to skin and islet transplants in chimeras. (*A*) At 1-2 days (early) or 14 weeks (late) post BMT, NOD (early, n=7; late, n=4) and B6 (early, n=2; late, n=6) chimeras in **Figure 3.2, *A*** and ***B*** and **Figure 3.2, *D*** were given donor C3H skin grafts. Chimeras challenged with donor skin grafts late were also given third party (DBA/2) skin grafts (NOD, n=4; B6, n=6). (*B*) Beginning at 7 weeks of age, naïve nondiabetic female NOD mice (controls) and NOD mice treated by our chimerism induction protocol (chimeric) were monitored weekly for diabetes onset.

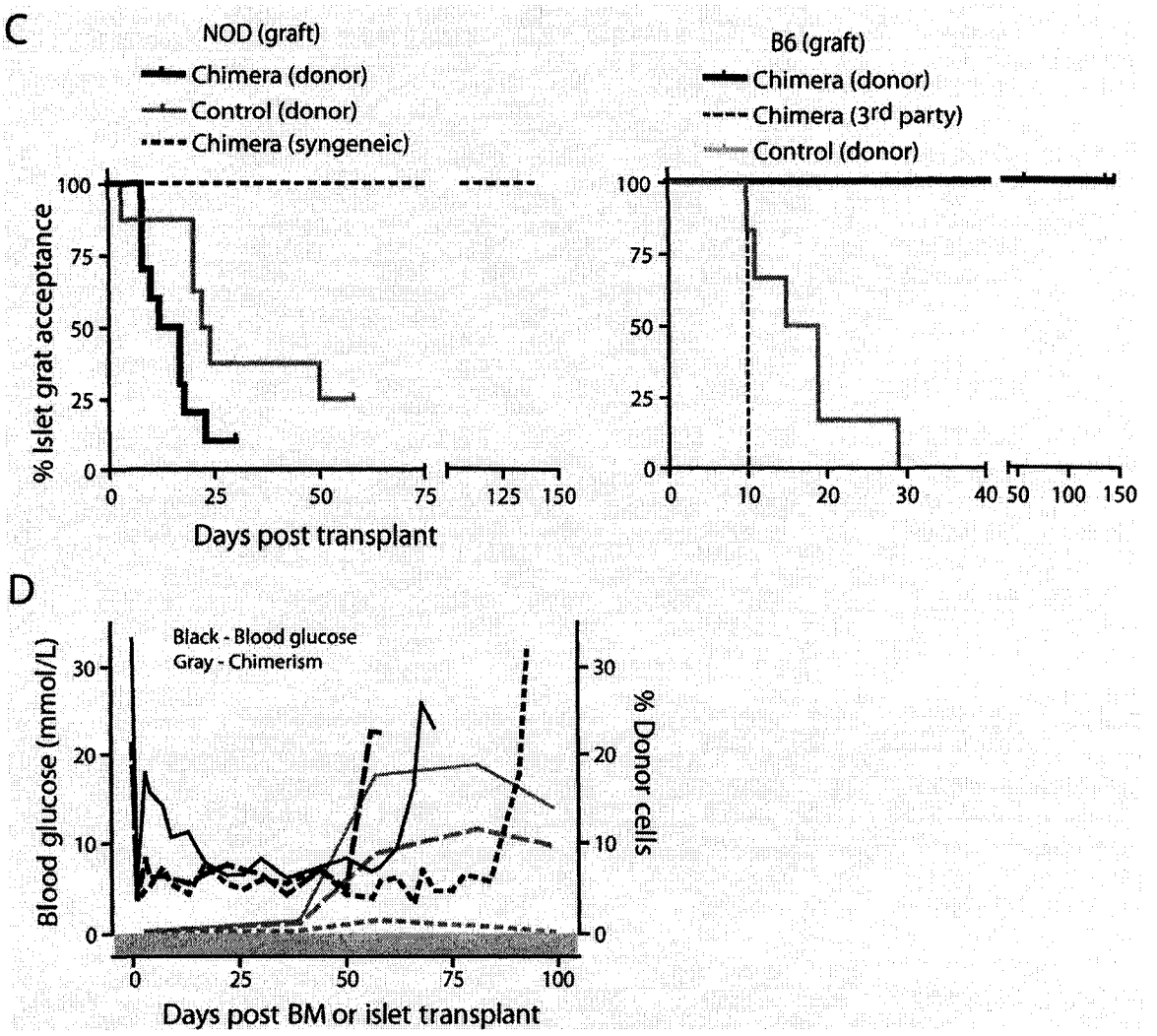

Figure 3.10 (continued). (*C*) Between 13-19 weeks post BMT, nondiabetic NOD chimeras (left; n=10), and B6 chimeras (right; n=6) were made diabetic and given donor C3H islet transplants. As controls, naïve nonchimeric NOD (n=8) and B6 (n=6) mice were also given donor islets. NOD chimeras were also challenged with syngeneic (NOD-RAG-KO) islets (n=7), including a cohort of NOD chimeras (n=4) that rejected donor islets and then challenged with syngeneic islets, and NOD chimeras (n=3) not previously given donor islets that were made diabetic and given syngeneic islets. Islet survival data from NOD chimeras given 20 or 40 $\times$ 10^6 C3H BM cells were pooled. In the chimeric B6 group, on day 138 post C3H islet transplant, third party (NOD or NOD-RAG-KO) islets were given to a cohort (n=2) to test for immune competence. Survival data are shown. (*D*) NOD chimeras were transplanted with donor C3H islets at the time of BMT (day 0; n=3). Blood glucose data are shown for each individual animal (black lines) along with its corresponding chimerism level (gray lines) up to day 99. The last rejection occurred on day 91, and this recipient that had the lowest chimerism level remained chimeric post islet rejection (0.22%). Islet rejection occurred on days 56 and 66 for the other two recipients.

3.3.3. NOD mixed chimeras show partial donor alloreactivity and humoral tolerance extends to third party cells

Since islet-specific autoimmunity was not the cause of split tolerance in NOD chimeras, we tested whether they may demonstrate residual alloreactivity to the donor (*i.e.* tolerance resistance). Between 20-32 weeks post BMT, we performed *in vivo* proliferation assays by transferring CFSE-labelled spleen cells from naïve or chimeric NOD mice to NK cell-depleted syngeneic, donor type and third party RAG-KO recipients. A comparable degree of proliferation by chimeric and control cells was observed in the third party recipients, which was at least 10-fold greater than the background homeostatic proliferation of chimeric and control cells when transferred into syngeneic RAG-KO recipients (**Figure 3.11**). In contrast, we observed more undivided chimeric NOD cells than nonchimeric control cells, in the donor type recipient, based on lower T cell recovery and a higher percentage of T cells with undiluted CFSE (undivided cells) (**Figure 3.11**). However, proliferation and recovery of chimeric NOD cells were still significantly higher in the donor type recipient than in the syngeneic recipient, indicating alloreactivity was not completely abrogated in the chimeras (**Figure 3.11**). Hence chimerism induction in NOD mice led to a partial loss of donor-specific alloreactivity.

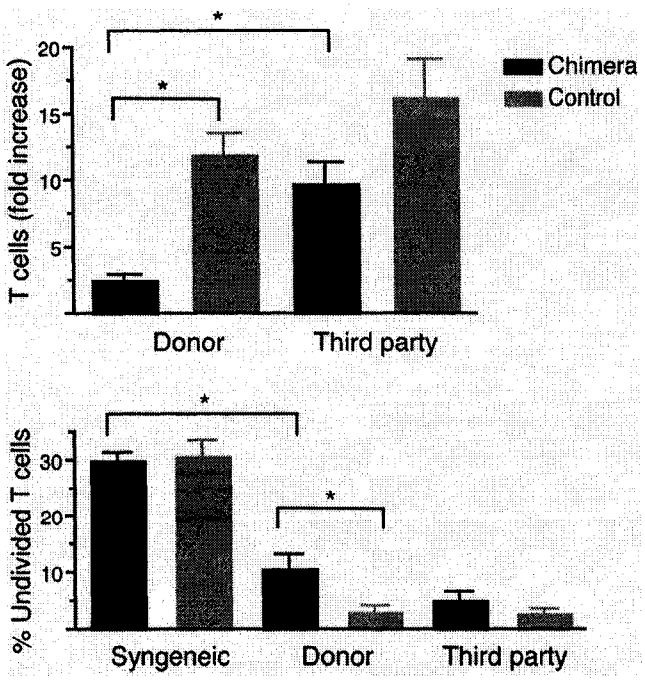

Figure 3.11. NOD chimeras have a partial loss of donor alloreactivity. Between 32-40 weeks post BMT, chimeric NOD mice (n=4) were sacrificed and spleen cells were harvested, CFSE-labelled and adoptively transferred into anti-asialo GM1 treated (to remove endogenous NK cells that could reject the transferred cells) NOD-RAG-KO (syngeneic), C3H-RAG-KO (donor) and B6-RAG-KO (third party) mice. Spleen cells from naïve nonchimeric NOD mice (control; n=6) were similarly transferred into a separate cohort of recipients for comparison. Analysis was performed 4 days later. Top: The number of T cells recovered in each spleen. Data were normalized and depicted as fold increases, determined by dividing the number of T cells recovered in a donor type or third party recipient by that recovered in the syngeneic recipient. Bottom: Recipient spleen cells were analyzed by flow cytometry for CFSE dilution. The percentage of T cells that remained undivided is depicted. All bars represent mean and SEM. An asterisk denotes a comparison made between two groups where the difference was statistically significant.

Since our NOD mixed chimeras were eventually devoid of donor B cells and other donor MHC class II-expressing hematopoietic cells (**Figure 3.4**, **Figure 3.5**, ***A***, **Figure 3.6** and **Table 3.2**), we tested for their production of donor alloantibodies and compared their response with that of B6 chimeras that maintained donor class II-expressing cells. When we assessed serum antibody binding to syngeneic versus donor hematopoietic cells, immunized chimeric B6 sera did not bind donor cells to any greater extent than syngeneic cells, unlike immunized nonchimeric controls, suggesting an absence of alloantibodies and complete B cell tolerance (**Figure 3.12**, ***A***). In contrast, the majority of the sera that we tested from NOD chimeras bound donor cells better than syngeneic cells (although much more weakly than nonchimeric control sera), suggesting that only partial B cell tolerance was achieved in NOD chimeras (**Figure 3.12**, ***A***). These anti-donor antibodies in NOD chimeras predominately bound non-T cells rather than T cells of donor origin (**Figure 3.12**, ***B***), consistent with the apparent tolerance to donor T cells. Interestingly, immunization of nonchimeric NOD and B6 mice with C3H spleen cells resulted in production of alloantibodies that strongly crossreacted with cells from multiple third party strains (**Figure 3.12**, ***C***). We therefore examined whether the alloantibody tolerance generated in C3H to B6 or NOD chimeras resulted in B cell tolerance to a third party strain. **Figure 3.12**, ***D*** shows that B cell complete tolerance or partial tolerance, in B6 and NOD mixed chimeras respectively, extended to third party alloantigens.

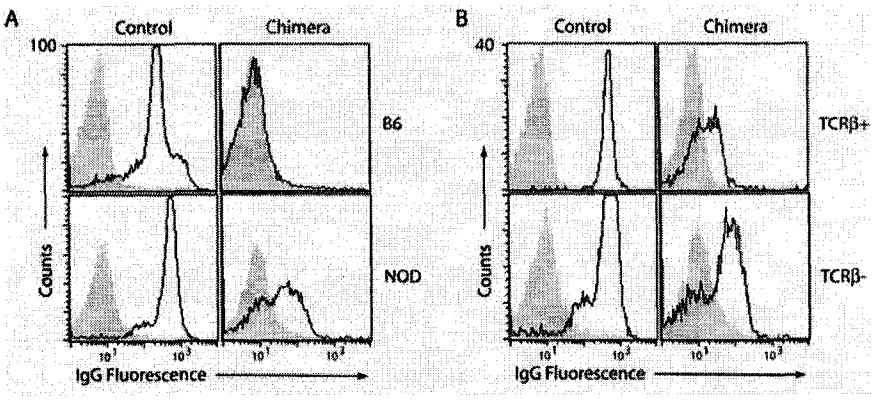

Figure 3.12. Detection of serum alloantibodies in mixed chimeras. (*A*) The reactivity of sera, from immunized C3H to NOD and C3H to B6 chimeras (n=6 and 3, respectively; right) and immunized control nonchimeric NOD and B6 mice (n=8 and 3, respectively; left), to syngeneic (light gray filled) and C3H (black line) donor spleen cells is shown as IgG fluorescence on lymphoid-gated cells, as detected in the FITC channel. Sera of NOD chimeras were tested at a time when most donor B cells were eliminated. Five of 6 NOD chimeras but none of the B6 chimeras had detectable serum alloantibodies after immunization with C3H cells. (*B*) Analysis of antibody binding to $TCR\beta^+$ (top) versus $TCR\beta^-$ (bottom) syngeneic (light gray filled) or donor (black line) spleen cells following incubation with serum from C3H immunized nonchimeric NOD controls (n=8) or NOD chimeras (n=5; chimeras with detectable alloantibodies).

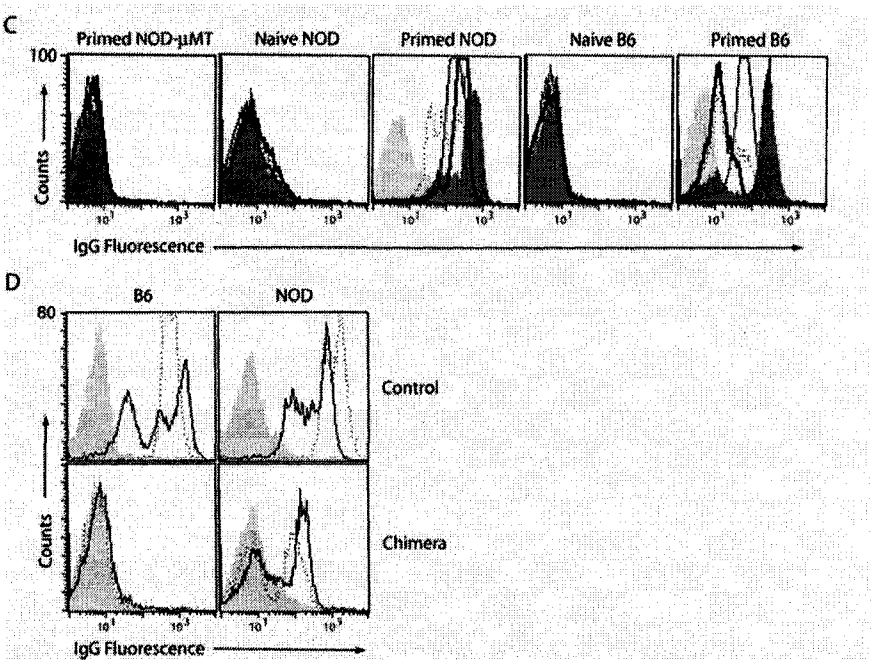

Figure 3.12 (continued). (*C*) Nonchimeric NOD and B6 mice (n=3) were immunized with C3H spleen cells. Their sera were then tested for reactivity to C3H cells (dark gray filled) and different third party cells (thin black line, FVB; dotted black line, BALB/c; thick black line, NOD cells for B6 sera or B6 cells for NOD sera) and compared to syngeneic cells (light gray filled). Sera were obtained from these mice prior to immunization and tested similarly (naïve NOD and naïve B6). As a negative control, B cell-deficient NOD mice (NOD-μMT; n=3) were immunized and their sera tested. (*D*) C3H-immunized NOD (n=4) and B6 (n=3) chimeras from *A* as well as nonchimeric NOD and B6 control mice (n=2) were further immunized with FVB spleen cells. Sera were harvested and tested for antibody reactivity to C3H (solid black line) and FVB (dotted black line) cells as compared to syngeneic cells (light gray filled). In *A-D*, representative plots are shown.

3.3.4. Non-MHC genes control split tolerance development in NOD mixed chimeras

To begin investigating the mechanism responsible for the tolerance resistance of NOD chimeras that was associated with multiple levels of split tolerance, we took a genetic approach and generated three different types of mixed chimeras using our induction protocol: 1) NOD.B10 chimeras, carrying the NOD background genes but expressing H-2^b; 2) B6.g7 chimeras, carrying the B6 background genes but expressing H-2^{g7}; and 3) chimeras in which the recipient background was the F1 generation of crosses between NOD and B6 or BALB/c mice (**Figure 3.13**, ***A***). As shown in **Figure 3.13**, ***B*** and ***C***, split tolerance to hematopoietic cells previously demonstrated by NOD chimeras (**Figure 3.5**) was reproduced only in NOD.B10 chimeras, indicating that the non-MHC genes of the NOD background but not the H-2^{g7} MHC haplotype contributed to this outcome. Moreover, crossing NOD mice with tolerance susceptible B6

or BALB/c mice abrogated split tolerance when chimerism was induced, indicating that this phenotype was recessive to that associated with full tolerance. To begin to determine how genetics may influence split tolerance, at the time when NOD.B10 chimeras lost donor B cells completely, we analyzed the frequency of potential Treg cells in their peripheral blood and compared that to the frequency observed in B6.g7 chimeras as well as in F1 chimeras. We found no significant differences, suggesting that split tolerance to hematopoietic cells could not be attributed to an altered frequency of cells with a Treg phenotype (**Figure 3.13, *D***).

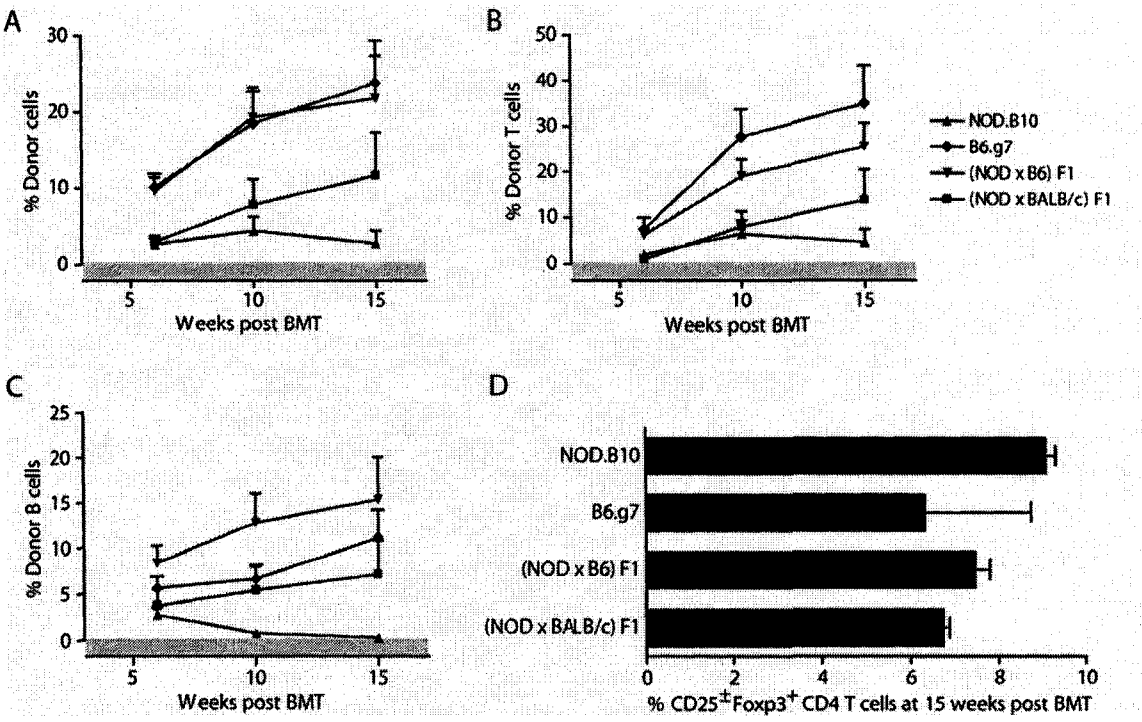

Figure 3.13. Non-MHC genes of the NOD background control split tolerance to donor hematopoietic cells without altering Treg cell frequency. NOD.B10, B6.g7, (NODxB6)F1 and (NODxBALB/c)F1 mice (n=3-4) were made chimeric with C3H BM cells by our chimerism induction protocol. PBMCs were analyzed up to 15 weeks post BMT for chimerism. (*A*) Overall donor cells. (*B*) Donor T cells. (*C*) Donor B cells. Mean and SEM are shown at each time point. Only NOD.B10 chimeras lost donor B cells. (*D*) All groups of chimeras were analyzed for the frequency of potential Treg cells in their peripheral blood at the time when NOD.B10 chimeras lost donor B cells. The bar graph depicts mean and SEM for each group. There was no significant difference in Treg cell frequency between chimeras with or without donor B cells.

3.4. DISCUSSION

Mixed chimerism is a promising approach to induce transplantation tolerance in the clinic because the tolerance induced is the most robust tolerance so far achieved experimentally and because less toxic

conditioning regimens have now been developed. Moreover, the ability of natural tolerance to be applied to allogeneic tolerance depends on the establishment of chimerism when dealing with strong mismatches (**Chapter 2**). While the strength of chimerism-induced tolerance is well appreciated (1-4), a serious potential pitfall of chimerism-induced tolerance, split tolerance (9-13, 15, 16, 18), has received little attention in recent years despite the potential problems it may pose for this approach. We considered that split tolerance may be even more likely to occur in current chimerism protocols; there is a push for milder (clinically feasible) conditioning regimens that leave more of the recipient T cell compartment intact, and hence success becomes more dependent on peripheral tolerance, in both direct and indirect donor reactive T cells. We have shown that stable mixed chimeras induced by nonmyeloablative approaches with few exceptions generate split tolerance, where donor hematopoietic cells persist and donor skin grafts are rejected (13). This split tolerance was attributed to a response against tissue-specific antigens as skin rejection could be prevented by giving the skin graft early, providing a source of donor skin antigens during the "tolerance promoting" treatment with MR1. However, unlike B6 chimeras, the NOD chimeras rejected donor skin grafts even when the grafts were given early (**Figure 3.10, *A***). It therefore seemed likely to us that autoimmunity, or genetic traits associated with autoimmunity or tolerance resistance, may heighten the potential for split tolerance via additional mechanisms.

Indeed, our NOD recipients could be more resistant to chimerism-induced tolerance of islets due to islet autoimmunity (50) and their known resistance to tolerance induction strategies (22-24). However, while islet-specific autoimmunity could lead to donor islet rejection by chimeras, as suggested in a previous study (51), the lack of syngeneic islet rejection in our study indicated that autoimmunity could not explain the split tolerance. The remaining low-level alloreactivity to the donor, detected in NOD chimeras, could instead explain their increased split tolerance as compared to B6 chimeras. As B6 chimeras maintained donor B cells and other donor MHC class II-expressing cells while lacking alloantibodies, this suggests that NOD chimeras failed to become tolerant to donor class II but not donor class I (donor T cells persisted); studies on the specificity of the alloantibodies produced should help clarify this issue. We therefore propose that the "tolerance resistant" phenotype of the NOD mouse (22-24) prevented the MR1-based chimerism induction protocol from generating complete tolerance of antigens in donor hematopoietic cells; a sufficient number of anti-donor CD4 T cells likely remained responsive and induced split tolerance. This

seems to be a likely possibility, as CD4 T cells appear to play a prominent role in the immune reactivity of NOD mice (52-54).

As donor T cells were the only cell type that the NOD chimeras maintained at a stable, low level while other hematopoietic cells and non-hematopoietic tissues were rejected, this also potentially suggests a difference in susceptibility among various cells and tissues to recipient effector mechanisms. In this regard, the indirect pathway of rejection by CD4 T cells is unusually potent in NOD mice (53, 54). Hence, we have recently investigated the issue of differential susceptibility of allogeneic grafts to CD4-mediated indirect rejection in a TCR Tg model. These studies showed that CD4 T cells are able to indirectly reject donor B cells, islets and skin, while in the same recipients the donor T cells are resistant to this pathway of rejection (submitted for publication; see also **Chapter 4**). These data parallel those found here in the NOD chimeras, suggesting a possible mechanism for the multiple levels of split tolerance we have observed.

Notwithstanding, we took a genetic approach and determined that the non-MHC genes of the NOD genetic background contributed to the split tolerance towards donor hematopoietic cells in NOD chimeras. NOD.B10 mixed chimeras but not B6.g7 chimeras showed loss of donor B cells while maintaining donor T cells (**Figure 3.13**, ***A-C***), an observation that clearly attributes split tolerance to genetic elements of the NOD background outside of the MHC. Interestingly, our studies in chimeric F1 hosts suggest that not only the split tolerance phenotype, but also the general tolerance resistance, may be demonstrated to be a recessive trait. This contrasts with existing data showing the resistance of diabetes-free (NOD×B6)F1 mice to tolerance induction by a DST and MR1 treatment (22). The difference in outcomes could be due to the greater potency of our tolerance induction protocol, in combination with chimerism induction, in overriding any inherent tolerance resistance of F1 hosts.

Treg cells are increasingly thought to play a critical role in both self-tolerance and acquired tolerance (55). Therefore, the increased split tolerance in NOD chimeras could alternatively be due to deficient or defective regulatory activity that operates through the tolerance resistance conferred by non-MHC NOD genes. Interestingly, a recent study showed that chimeras generated in an nonautoimmune recipient background that were split tolerant to donor skin or heart transplants, could become fully tolerant if chimerism induction occurred in the presence of donor alloantigen stimulated Treg cells (*in vitro* culture of recipient Treg cells with (donor × recipient)F1 APCs) (56). Thus, Treg cells could potentially play a

role in overcoming split tolerance. Our analysis of the frequency of potential Treg cells in chimeras with or without split tolerance (**Figure 3.13, *D***) suggests that any association between altered Treg cell activity and split tolerance would more likely be related to function rather than frequency, given the lack of differences in the frequency of Foxp3-expressing CD4 T cells. This would be consistent with the idea that the loss of self-tolerance leading to autoimmune diabetes induction is related to a temporal, functional defect in Treg cells rather than their reduced frequency (57). However, at present we cannot rule out a difference in the frequency of donor-specific Treg cells. Furthermore, the possibility that the known thymic defect in NOD mice (58-62) could have contributed to split tolerance in chimeras also warrants future investigation.

Various protocols have been tested to generate long-term mixed chimerism (defined here as $\geq$0.1% donor cells without full chimerism) in NOD mice, but few attempts (39, 63) were made with fully mismatched combinations and none achieved adequate longevity of mixed chimerism induced by practical means. From past studies, chimerism induction either led to initial mixed chimerism that eventually became full chimerism (64), or had an unknown fate because it was not monitored long-term (63), or was sustained but required an extremely high dose of BM cells and generated by potentially more risky infusion of large numbers of donor CD8 T cells (39). Many studies reported the ability of chimerism induction to promote long-term islet acceptance in NOD mice that already manifested autoimmune diabetes (50, 51, 63, 65-68). The strain combinations used in these studies in most cases were not fully mismatched, having either partial (50, 51, 66, 68) or complete MHC matches (67). Difficulty in sustaining mixed chimerism long-term was encountered even in studies that used partially matched donor BM (51, 68). However, in one study fully mismatched islets were accepted by diabetic NOD mice made chimeric, although full chimerism may have explained the tolerance (65). The lack of success in some instances may reflect a competitive developmental advantage of resident NOD hematopoietic stem cells over the exogenously introduced stem cells from nonautoimmune donors (69). We developed a relatively mild conditioning regimen that generated long-term mixed chimerism in NOD mice and extensively characterized the chimeric state and the effects of chimerism on the host immune system. We found that split tolerance can indeed occur in NOD mixed chimeras, and that the split tolerance extended to many more donor tissues (skin, islets and some hematopoietic cells) in NOD chimeras than in B6 chimeras (only donor skin grafts given late). In contrast to our study, Liang *et al.* (39) generated stable multilineage chimerism in

nondiabetic NOD mice and found a lack of split tolerance with skin grafts (islet transplants were not assessed). The differing outcomes might be explained by the induction protocols employed, the strain combinations, the multilineage nature of the chimerism or simply the level of chimerism achieved.

With regards to the type of protocol used and its association with split tolerance induction in chimeras, we and others (12, 13, 47) have shown that the timing of tissue transplantation in relation to the initiation of a chimerism induction protocol that is based on costimulation blockade could influence whether split tolerance is observed. This, however, did not apply to NOD chimeras. Moreover, we tried a number of induction protocols to generate chimerism in NOD mice (**Appendices**, **A.5**, p.189), but with the exception of the protocol reported in this study, we were unable to achieve stable mixed chimerism, thus preventing us from assessing split tolerance. This is clearly consistent with the known tolerance resistance of NOD mice (22-24) and indicates that greater requirements must be met for chimerism induction in the NOD model. However, it does not appear that the level of chimerism by itself explains the rejection of donor islets in NOD but not B6 chimeras, as even a very low-level chimerism (1-3%) was sufficient to prevent rejection of fully mismatched donor islets in B6 mice (**Figure 3.3**, ***D*** and **Figure 3.10**, ***C***). Furthermore, a high level of chimerism in NOD mice was associated with more rapid donor islet rejection than a low level of chimerism (**Figure 3.10**, ***D***). Given these and other data (51), it seems more likely that the threshold level of chimerism required to prevent islet rejection is increased in the autoimmune-prone, tolerance resistant NOD background.

In our chimerism induction protocol, we chose the widely used BUS for host conditioning. BUS is a chemotherapeutic agent used to treat chronic myeloid leukemia (31). Its mechanism of action does not appear to depend on binding and crosslinking of DNA directly, but through insertion of alkyl groups into nucleic acids, it causes linkage of nucleic acids with adjacent proteins and/or other nucleic acids, thereby inhibiting cellular function and triggering cell death (31). BUS targets myeloid cells (30) by an unknown mechanism, as well as early non-dividing hematopoietic stem cells that have the greatest self-renewal capacity (31-35). Loss of BM stem cells occurs as early as 24 hours after BUS administration into murine recipients (70). Use of BUS to create hematopoietic "space" spares the host from irradiation. The level of donor chimerism achieved is titratable to the dose of BUS given; hence variable degrees of myelosuppression can be induced (29). Multiple exposures to this compound lead to lymphopenia (due to

chronic BM failure that is manifested months after BUS injection), resultant quantitative impairment of cellular and humoral immunity (71), and also reduced NK cell cytotoxicity (44, 72). Repeated exposure (71) or single exposure at a high dose (90 mg/kg) (73) to BUS may alter the function of T cells and B cells. However, we determined that a single dose of BUS at 20 mg/kg had little effect on the cellularity or composition of the NOD thymus and spleen (**Figure 3.2**), consistent with the minimal immunosuppressive effects observed in other rodent studies (30, 38). Moreover, BUS given as a single dose has been shown to have no inhibitory effect on NK cell function (44), thus it is unlikely that allogeneic BM engraftment in our model was mediated by this agent. In support of this, infusion of allogeneic FLCs into BUS-treated NOD-RAG-KO recipients that are deficient in T cells and B cells but not NK cells did not lead to chimerism; however, similarly conditioned hosts that were additionally depleted of NK cells by anti-asialo GM1 became chimeric (**Figure 3.8**).

Since NK cells play an important role in the rejection of allogeneic BM cells (74), successful chimerism induction requires overcoming the NK cell barrier, either by infusing a sufficiently large dose of donor cells to overwhelm the cytotoxic capacity of NK cells (75-77), eliminating NK cells (78-80), or blocking NK cell function (77). NK cells have originally been thought to participate in the rejection of allogeneic hematopoietic cells by antibody-mediated cellular cytotoxicity (ADCC), due to the coating of allogeneic cells, upon transfer, by pre-existing alloreactive antibodies (81). However, the demonstration that SCID mice, which contains NK cells but lack T cells and antibody producing B cells, could reject allogeneic BM cells indicated that ADCC was not the sole mechanism and suggested that NK cells could alone be involved in cytotoxicity (82), either through a cell contact-dependent mechanism or the release of cytokines (83). Interestingly, cell contact-dependent killing potentially occurs without perforin or Fas ligand (84), but this is controversial (75).

Our chimerism model employed fully allogeneic donor/recipient combinations, thus allogeneic resistance by host NK cells is a relevant issue. Resistance to C3H cells appeared to be stronger in NOD mice than B6 mice (**Figure 3.7**). Mechanistically, poor rejection of C3H cells expressing H-2^k by B6 NK cells is likely due to the expression of inhibitory Ly-49 receptors on B6 NK cells specific for the MHC class I molecules H-$2K^k$ or H-$2D^k$ (*e.g.* Ly-49A or Ly-49C) of the C3H background, and the absence of an H-2^k-specific activating Ly-49 receptor (*e.g.* Ly-49L) (85, 86). In contrast, in the C3H to NOD

combination, the expression of Ly-49W, an activating receptor that can bind H-2D^k, by NOD NK cells likely explains their robust resistance against H-2^k-expressing cells (87). However, the ability for C3H BM cells to engraft in the conditioned NOD recipients of our study suggests reduced host NK cell activity due to the chimerism protocol, with or without contribution from injecting a large dose of allogeneic BM cells that could break the recipient NK cell barrier. The latter mechanism could be more relevant to B6 recipients than NOD recipients due to their differential expression of activating versus inhibitory Ly-49 receptors.

To investigate how allogeneic resistance by NK cells could be blocked in NOD mice to facilitate BM engraftment, we tested whether SRL could inhibit NOD NK cell function. Using NOD-RAG-KO mice as recipients of allogeneic cells, we observed better survival of donor cells in recipients that were given a short course of SRL than untreated recipients (**Figure 3.9**), indicating that SRL can inhibit NK cell cytotoxicity. Besides our data here (that were not included in the "in press" manuscript related to this chapter), there have not yet been published reports in the mouse model on the effect of SRL on NK cells. However, previous studies in rats have shown that SRL blocked NK cell proliferation and cytotoxicity and reduced the level of peripheral NK cells following treatment, while IFN-γ production was unaffected (88). Moreover, SRL also appears to reduce the cytotoxicity of human NK cells (89). Altogether, overcoming the NK cell barrier to BM engraftment in our model was likely assisted by SRL.

SRL is a potent anti-fungal macrocyclic antibiotic produced by the filamentous bacterium, *Streptomyces hygroscopicus*; however, it is also a nonspecific anti-proliferative compound that can act on T cells, B cells, mast cells, lymphoid and non-lymphoid tumour cells, smooth muscle cells, hepatocytes and fibroblasts (90, 91). Importantly, the ability to depress the function of immune cells makes SRL an immunosuppressant. Proliferation of T cells or B cells following stimulation by mitogens, phorbol esters and calcium ionophore, or antibodies is reduced in the presence of SRL (92, 93). SRL binds an intracellular protein known as the mammalian target of rapamycin, and blocks its function (90). In T cells, this protein is required for cell cycle progression from G_1 to S phase (91, 94). In the setting of BMT to induce chimerism, besides its effect on NK cells as discussed, a known key function of SRL is the prevention of GVHD (95, 96) by inhibiting the expansion of donor T cells that are cotransplanted with whole BM cells, while preserving the engraftment potential of donor BM cells required to establish

chimerism (95). BM engraftment is also enhanced due to SRL-mediated inhibition of anti-donor immunity induced by recipient T cells (97).

Our induction protocol also consisted of two other potent immunomodulatory agents: MR1 and CTLA-4-Ig. MR1 is an antibody that binds CD40L, a costimulatory molecule expressed by B cells, platelets and activated T cells (98). In the mouse, NK cells can also express CD40L upon stimulation by IL-21 (99). NK cells can induce cytotoxicity via CD40L (45). The corresponding expression of CD40 by hematopoietic progenitors (100) suggests that inhibition of NK cell killing of allogeneic BM cells in our chimeras could also potentially be mediated by MR1 blockade of CD40L. However, CD40-expressing cells could also trigger NK cells that do not express CD40L (101), thus the role of MR1 in inhibiting NK cell function could be less relevant. Indeed, in another chimerism model that targeted costimulatory signals, the role of costimulation blockade by itself in inhibiting BM rejection by NK cells appears to be minor; elimination of NK cells by depleting antibodies had a significantly better effect (77). Nevertheless, MR1 is important in blocking the T cell response, as it functions to prevent GVHD, and blocks BM rejection by a host versus graft T cell response (102). A potential mechanism by which these effects are induced is the induction of IL-2-reversible hyporesponsiveness in T cells following MR1 treatment (103), associated with impaired cytokine production (104).

MR1 is generally thought to block CD40/CD40L signalling in T cells due to its specificity for CD40L expressed on activated T cells. Studies performed in mice deficient in complement or Fc receptors, however, have suggested that MR1 may alternatively deplete T cells as a mechanism to block T cell immunity, due to the inability of MR1 to prolong transplant survival in recipients lacking C3 or Fc receptor gamma-chain (105). MR1 was also ineffective when used as $F(ab')_2$ fragments in WT recipients of allogeneic transplants, consistent with the possibility that its mechanism of action is Fc-mediated (105). This ineffectiveness may be dose-dependent, however, as $F(ab')_2$ fragments used at a high lose but not a low dose inhibited T cell proliferation (106). Together, it appears that MR1 acts on T cells by either one of these mechanisms depending on the dose used and the type of immune response studied. The fate of T cells following blockade of CD40/CD40L signalling by MR1 may or may not depend on Treg cell activity. MR1 induces T cell anergy (103) and inhibits T cell function independent of Treg cell activity (107).

Nevertheless, MR1 potentially has a second mechanism of action via an enhancement of the suppressive capacity of Treg cells (107), although how this occurs has not yet been clarified.

Similarly, CTLA-4-Ig is an agent that binds costimulatory molecules. It is a fusion protein containing the extracellular domain of CTLA-4 that can bind either B7-1 or B7-2, therefore competing with CD28 for its ligand (108, 109). CTLA-4-Ig potently suppresses T cell and B cell responses *in vitro* and *in vivo* (108-111). Moreover, it synergizes with MR1 to prolong allograft survival (112). Tumour cells transfected with B7 showed increased sensitivity to NK cell killing that could be blocked by CTLA-4-Ig (101, 113). This raises the possibility that infusion of allogeneic BM cells in the presence of CTLA-4-Ig may show improved engraftment due to blockade of a potential NK cell ligand. However, B7 expression on hematopoietic cells is highly restricted and has not been observed on stem cells (46), thus the role of CTLA-4-Ig in blocking NK cell activity in our BM recipients to promote allogeneic engraftment and chimerism is unlikely. Indeed, rejection of allogeneic BM cells was not prevented by costimulatory blockade involving CTLA-4-Ig (77). In contrast, due to its competition with CD28 expressed on T cells for B7 binding, CTLA-4-Ig inhibits T cell activation (109). In addition, CTLA-4-Ig appears to enhance the catabolism of tryptophan that is required for T cell function, thus blocking T cell immunity (114). Thus, the combination of MR1 and CTLA-4-Ig in our chimerism induction protocol offers significant benefits that facilitate chimerism and tolerance induction, however, these effects were evident in the B6 but not NOD recipients, which we observed to be highly tolerance resistant.

To definitely show that donor islet rejection by NOD chimeras was due to alloimmunity and not islet-specific autoimmunity, we administered a high dose of STZ to the islet recipients, which not only induced diabetes but has been shown to abrogate the autoimmune response (49). The absence of syngeneic islet rejection confirmed this and clearly indicated that the NOD chimeras rejected donor islets by alloimmunity. However, independent of the effects of STZ, chimerism induction in NOD mice has also been shown to inhibit the development of autoimmune diabetes. Infusion of non-NOD BM into NOD mice could alter the developmental pathway of disease, such that following an allogeneic BMT, the recipients failed to show metabolic and histological hallmarks of autoimmune diabetes over time (115). Replacement of all (63, 64, 116, 117) and even some (39, 50, 118, 119) recipient hematopoietic cells by those derived from donor precursors was sufficient to abrogate diabetes development in prediabetic NOD mice.

Interestingly, not only did long-term mixed chimerism fail to prevent donor islet rejection in our study, it also failed to fully prevent diabetes; in some cases diabetes was initiated but at a delayed rate (**Figure 3.10, *B***). This suggests that the specific characteristics of the chimeric state are important, as long-term systemic donor chimerism *per se* does not necessarily abrogate diabetes development.

The mechanism by which the establishment of allogeneic chimerism prevents islet-specific autoimmunity is not fully understood. One possibility is that allogeneic stem cells can generate non-H-2^{g7}-expressing APCs that present self-antigens to T cells developing in the NOD thymus, contributing to the selection of a T cell repertoire lacking islet-specific autoreactive T cells. Alternatively, since a NOD-specific defect at the level of the T cell precursor could be responsible for the generation of autoreactive T cells without contribution by the thymic epithelium (60), allogeneic chimerism may be able to prevent diabetes potentially by displacing T cell precursors of NOD origin, reducing or eliminating the development of autoreactive clones. However, it is more likely that allogeneic chimerism is able to reverse autoimmunity by correcting multiple levels of defects relating to T cell development that require further investigation (118, 120).

We were able to detect alloantibodies in NOD chimeras but not B6 chimeras, suggesting more complete B cell tolerance in the latter hosts. It was surprising to find that this partial or full humoral tolerance extended to a third party strain fully mismatched with the recipient. In most other studies of humoral immunity in mixed chimeras, alloantibody production to third party cells was not investigated (121-123). The lack of alloantibody responses to third party cells is unlikely to be due to the previously described general immunodeficiencies in mixed chimeras (124), attributed to MHC mismatches between the host thymus and peripheral APCs; the alloantibody producing B cells in NOD chimeras are of the host type, expressing the appropriate MHC that is also present in the host thymus. In addition, Serreze et al. (123) examined antibody responses after generation of mixed chimerism and observed an intact response to nominal antigen in adjuvant. Further supporting the conclusion that the absence of alloantibodies was due to tolerance rather than immunodeficiency, NOD mixed chimeras were often able to make alloantibodies to donor (and third party) non-T cells, while maintaining specific tolerance in terms of the reduced alloantibodies specific to antigens on donor T cells. Instead, the extension of alloantibody tolerance to third party cells is explained by the crossreactive nature of the alloantibody response. Such crossreactivity

has been observed previously (125-130). However, humoral tolerance to third party cells was surprising, in that an alloantibody response to private specificities might have been expected. This suggests that humoral tolerance may involve a dominant mechanism (*e.g.* antibody feedback through Fc receptors (131), or dominant tolerance due to Treg cell activity that inhibits the ability of donor-specific helper T cells to help activate alloantibody producing recipient B cells) to public specificities on MHC or additional antigens (130, 132), and not simply deletion of donor-specific B cells (133). While crossreactive alloimmunity has been well demonstrated, we are not aware of any published data showing that humoral tolerance to one donor can extend to additional donors. One previous study did find that, in xenogeneic chimeras, xenoreactive antibodies crossreactive to third party cells were absent; however, it was not determined whether these recipients were humorally tolerant or simply ignorant of third party cells (recipients were not immunized with third party cells prior to assessment of humoral immunity) (130). An important implication of our finding is in the setting of transplantation from multiple consecutive tissue donors, as occurs in clinical islet transplantation (134), where tolerance to many donors could be of substantial benefit (135). In addition, the relatively donor-nonspecific humoral tolerance could play a role in third party heart graft acceptance found in a neonatal tolerance model (136).

Collectively, our data indicate that in addition to the recognized role of tissue-specific antigens, an inherent tolerance resistant genotype may contribute to the development of split tolerance. This suggests that split tolerance is likely to be a more important obstacle to the success of chimerism approaches than previously considered. In contrast, the potential for multiple donor humoral tolerance appears to be a distinct advantage of the mixed chimerism approach.

3.5. AUTHOR CONTRIBUTIONS

WFC designed and performed all experiments described, analyzed data and wrote the paper. Data in Figure 4.9, *A* were obtained in collaboration with HR, who also analyzed data and revised the paper. Data in Figure 4.2, *C* were obtained in collaboration with BL, who also designed the chimerism induction protocol to be applied in NOD mice, analyzed data and revised the paper. AMJS advised on the design of research and revised the paper. CCA conceived the study of split tolerance in NOD chimeras, advised on the design of research, analyzed data and wrote the paper.

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Chapter 4: Differential susceptibility of allogeneic grafts to indirect CD4 immunity

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4.1. INTRODUCTION

CD4 T cells frequently help to activate CD8 T cells and B cells. However, CD4 T cells have also been demonstrated to be alone sufficient to induce transplant rejection (1-7) or tumour clearance (8). Consistent with the passenger leukocyte hypothesis (9, 10), graft rejection can occur following T cell activation by direct allorecognition, which is generally agreed to lead to acute graft loss (11). However, indirect allorecognition of donor MHC (12) or non-MHC (13-15) peptides is now viewed to be relevant to the overall immunity towards donor grafts. Certain alloantigens may even be preferentially presented to recipient T cells indirectly (16). CD4 T cells with known antigenic specificities have been shown to reject transplants following direct (6, 17, 18) or indirect (6, 17) activation, in the absence of immunity by CD8 T cells or B cells, and in the absence of any demonstrable cross-reactivity (6). Moreover, indirect CD4 responses appear to be particularly important in xenograft rejection (19), and alloimmunity and/or autoimmunity generated in NOD mice towards islets (7, 20, 21). Tolerance induction in certain transplant situations may also depend on the indirect pathway (22). Our studies in NOD mixed chimeras revealed a relatively intact anti-donor response that was sufficient to reject certain types of hematopoietic cells as well as skin and islet transplants but not T cells (**Chapter 3**). This anti-donor response was alloimmune even in islet rejection, as mixed chimeras accepted syngeneic islets. Given these observations and the importance of indirect CD4 immunity in NOD mice (7, 20, 21), whether indirect CD4 alloimmunity is equally destructive towards different types of allogeneic grafts warrants investigation. Using a TCR Tg mouse model in which indirect CD4 alloimmunity alone to a defined antigen can be studied, we provide evidence that the indirect response can, in fact, be futile in eliminating certain allogeneic grafts but highly effective towards others, thereby generating a split tolerant phenotype that mirrors split tolerance in NOD mixed chimeras. Surprisingly, the deficiency of indirect CD4 alloimmunity could convert immunity to tolerance.

4.2. MATERIALS AND METHODS

4.2.1. Animals. Adult B6 (H-2^b), CD45.1-expressing B6, BALB/c (H-2^d) and BALB/c-SCID mice were purchased from NCI-Frederick (Frederick, MD). MHC class II-deficient B6.129-*H2-Ab1*^{tm1Gru} mice were purchased from Taconic Farms (Germantown, NY). (C57BL/6J × C57BL/10SgSnAi)-[KO]γc-[KO]*Rag2*

mice (23) that lack T, B and NK cells (RAG/γc-KO) were obtained through the NIAID Exchange Program. B6.C-*H2-Ab1*^{bm12} (bm12; H-2^{bm12}) and FVB (H-2^{q}) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6.129S7-*Rag1*^{tm1Mom} (RAG-KO), NOD.129S7(B6)-*Rag1*^{tm1Mom} (NOD-RAG-KO) and TCR Tg Marilyn (24) mice on the B6-RAG-KO background were bred on-site. Fetuses were used at days 14-15 of gestation. All care and handling of animals was carried out in accordance with CCAC guidelines.

4.2.2. FLC transplantation. FLs were harvested and homogenized into single cell suspensions in PBS. Male FLCs were distinguished from females by PCR (see **Chapter 2**, p.51). FLCs were pooled according to sex, and 10×10^6 male or female CD45.1-expressing B6, or bm12 FLCs, were injected i.v.

4.2.3. Skin transplantation. Full thickness tail skin grafts were transplanted onto the lateral thoracic wall of anaesthetized recipients. Grafts were secured with sutures and protected with gauze and bandage for a minimum of 7 days. Health of donor skin was monitored by visual and tactile inspection. The day of skin rejection was defined as graft necrosis of approximately 100%.

4.2.4. Islet isolation and transplantation, nephrectomy and glucose monitoring. Islet isolation was carried out as previously described (25). Mice were made diabetic by a single i.p. injection of STZ (Sigma-Aldrich Canada, Oakville, ON) at 200 mg/kg. Similar to WT mice with a complete T cell repertoire (26) (also **Appendices, A.3**; p.168), STZ-induced acute diabetes in TCR Tg Marilyn mice (**Figure 4.1, *A***) led to a significant reduction in thymic cellularity associated with a decreased proportion of DP thymocytes and an increased proportion of CD4 SP thymocytes (**Figure 4.1, *B***), but had a lesser effect on the spleen (**Figure 4.1, *C***). Diabetes was confirmed by a blood glucose of >20.0 mmol/L. Five hundred islets were transplanted into the renal subcapsular space of diabetic recipients. Recipient blood glucose was monitored to detect rejection (>15.0 mmol/L in two consecutive readings on different days). Some recipients that rejected the islets were given a second, syngeneic transplant to the contralateral kidney, or an insulin pellet (LinShin Canada, Scarborough, ON) subcutaneously (s.c.), to restore normoglycemia. Some recipients that showed long-term acceptance of donor islets (>110 days post transplant) underwent nephrectomy to determine their dependence on donor islets to maintain normoglycemia.

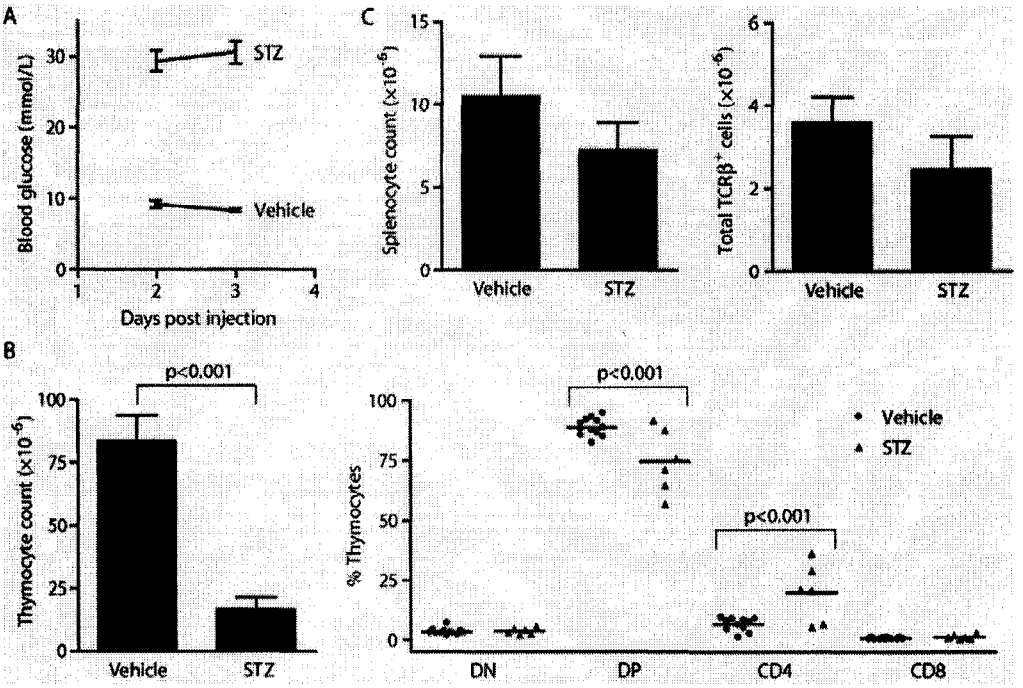

Figure 4.1. Effect of STZ-induced acute diabetes on the Marilyn immune system. Naïve female Marilyn mice (6-11 weeks) were either given acetate buffer (Vehicle; n=12), or STZ at 200 mg/kg i.p. (STZ; n=6). (*A*) Blood glucose on days 2 and 3 post injection (mean and SEM). (*B*) On day 3, vehicle-treated nondiabetic and STZ-induced diabetic Marilyn mice were sacrificed. Their thymi were harvested to obtain total cell count (left) and analyze thymocyte composition by flow cytometry (right). (*C*) Total cell count (left) and absolute T cell count (right; multiplying total cell count by the percentage of T cells in lymphoid-gated spleen cells) of spleen on day 3. No statistically significant differences were found in the spleen. All data shown were pooled from at least two independent experiments.

4.2.5. Flow cytometry. Multi-color flow cytometry was performed following Fc receptor blocking and staining with antibody cocktails. Antibodies used were specific for H-2D^b (KH95), H-2D^d (34-2-12), I-A^b (KH74), MHC class II (M5/114.15.2), B220 (RA3-6B2), CD4 (RM4-5), CD8α (53-6.7), CD19 (6D5), CD45.1 (A20), CD45.2 (104), TCRβ (H57-597) or Vβ6 (RR4-7). Biotinylated antibodies were detected with streptavidin conjugated to Tricolor or allophycocyanin. Reagents were purchased from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA) and Caltag (Burlingame, CA). Data were acquired using a FACSCalibur™ (Becton Dickson, Sunnyvale, CA) and analyzed with CellQuest™. To detect passenger cells in isolated islets, islets were incubated in enzyme-free cell dissociation buffer (Invitrogen, Burlington, ON) at 37°C for minutes, with mechanical dispersion and resuspension of cells by pipetting at the beginning of the incubation and every 10 minutes thereafter. Cells were then stained and analyzed.

4.2.6. Serum cytokine quantitation. Islet recipients that underwent rejection were anaesthetized by i.v. injection of Avertin (2,2,2-tribromoethanol; see **Appendices, A.1.1**; p.158). They were then exsanguinated by cardiac puncture using a 1-mL syringe capped with a 23-gauge needle. Blood was allowed to clot for at least 15 minutes before centrifugation at >2,000 × *g* for 20 minutes to recover serum. Serum was stored at -80°C if analysis was not immediately performed. Serum cytokines were quantitated by a mouse cytokine-specific multiplex antibody bead assay (BioSource, Camarillo, CA). Data were acquired using a Luminex 100™ IS System (Applied Cytometry Systems, Sacramento, CA) and the STarStation software.

4.2.7. *In vitro* proliferation assay. *In vitro* Marilyn responses were tested by a standard MLR assay. Fifty thousand female Marilyn responder splenocytes were cocultured with titrated numbers (starting at 1×10^6) of gamma-irradiated (1500 rads; ^{137}Cs irradiator, Gammacell 40, Atomic Energy of Canada, Ottawa, ON) stimulator splenocytes for 72 hours in supplemented Iscove's Modified Dulbecco's Medium (see **Appendices, A.1.3.1**; p.158). Cultures were then pulsed with ^{3}H-thymidine at 1 μCi/well and incubated for an additional 16 hours before harvesting onto filters, and counting of radioactive incorporation.

4.2.8. Statistical analysis. Means were reported either with SD or SEM as indicated. Two-tailed Student's *t*-test was used for comparison of means between two groups. Log-rank test was used to compare survival curves. All statistical analyses were done using Prism 4 (GraphPad Software, San Diego, CA) with statistical significance defined as $p<0.05$.

4.3. RESULTS

4.3.1. Indirect CD4 alloimmunity alone rejects islet transplants but not their passenger cells; passenger cells play opposing roles in transplantation immunity

Marilyn, a TCR Tg mouse that contains a monoclonal population of CD4 T cells specific for the male histocompatibility antigen, H-Y, presented in I-A^b (24), has been used to demonstrate that indirect CD4 immunity to a defined antigen, alone, can be sufficient to acutely reject skin transplants (6) and tumour cells (8) but not heart (18) or thymus (27) transplants. In these recipients, only male antigen-expressing

tissues evoke T cell immunity, the activation of which is MHC-restricted and does not occur by cross-reactivity (6). Thus, manipulation of the donor/recipient combination permits the indirect pathway of T cell activation alone to be studied. To assess the relative effectiveness of indirect CD4 alloimmunity in rejecting different types of cells and tissues, we began by testing its ability to reject islet transplants and the few passenger lymphocytes (primarily donor T cells) in islets that are able to migrate out of the transplant (28). Specifically, we examined the ability of Marilyn mice to indirectly reject male islet transplants and their passenger lymphocytes, the latter cells being readily detectable *in vivo* in immunodeficient hosts bearing healed-in grafts from immunocompetent donors (28), as well as *ex vivo* in islets harvested from immunocompetent (BALB/c, B6 and FVB) but not immunodeficient (NOD-RAG-KO) mice (**Figure 4.2** and data not shown). We gave Marilyn, male or female fully MHC-mismatched WT BALB/c islets and found that the indirect CD4 response alone was sufficient to induce alloantigen-specific islet rejection (**Figure 4.3**, ***A***). At the time of male islet rejection, however, we did not detect, in blood (**Figure 4.3, *B***) and spleen (data not shown), passenger lymphocytes that migrated out from the donor graft. As Marilyn mice contained not only monoclonal CD4 T cells but also NK cells, the inability to detect passenger lymphocytes could be due to their rejection by recipient NK cells (29). We confirmed this by transplanting BALB/c islets into NK cell-replete RAG-KO or NK cell-deficient RAG/γc-KO mice and detected passenger lymphocytes, consisting of T cells but not B cells, only in RAG/γc-KO recipients (**Figure 4.3, *C***).

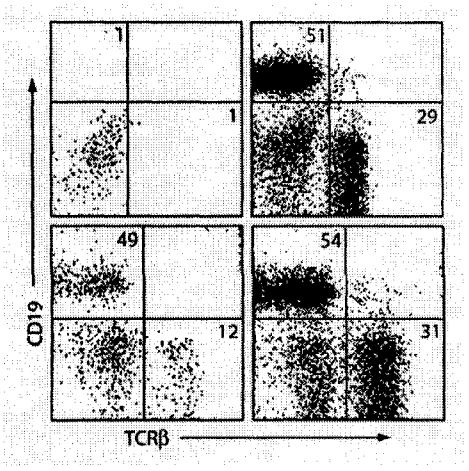

Figure 4.2. Detection of passenger lymphocytes in islet preparations. Shown are FACS plots of immunodeficient NOD-RAG-KO (top left) and immunocompetent WT BALB/c (bottom left) donor islets, stained for passenger lymphocytes by anti-TCRβ and anti-CD19 antibodies. For comparison, WT NOD (top right) and BALB/c (bottom right) spleen cells were analyzed similarly. Two different aliquots of islets were tested for each donor strain with similar results. Results comparable to BALB/c islets were also obtained with B6 and FVB islets (not shown). Quadrant percentages are provided.

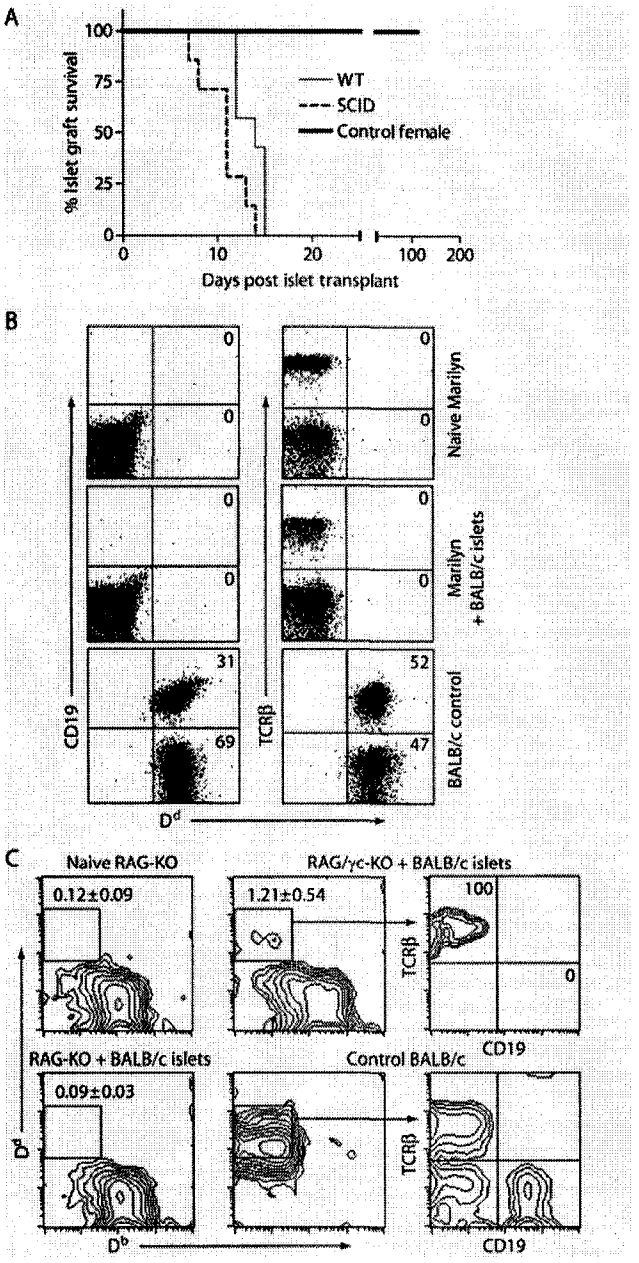

Figure 4.3. Alloantigen-specific islet rejection by indirect CD4 immunity. (*A*) STZ-induced diabetic female Marilyn mice were given male BALB/c-SCID (n=7), or male (n=7) or control female (n=2) BALB/c islets. Survival curves are shown; p=0.0396 between male and female BALB/c islets, p=0.025 between male BALB/c-SCID and female BALB/c islets, and p=0.0151 between male BALB/c-SCID and male BALB/c islets. (*B*) Female Marilyn mice given BALB/c islets (Marilyn + BALB/c islets) were analyzed for donor (D^{d+}) passenger T cells (TCRβ) or B cells (CD19). Shown are FACS plots of one mouse 2 days after rejection. Peripheral blood was compared to a naïve Marilyn and a BALB/c mouse. Data are representative of four mice that rejected their transplants. A fifth mouse that had not yet rejected its islets at the same time period also did not show chimerism. Number in the upper right quadrant indicates the percentage of donor T or B cells detected. Number in the lower right quadrant indicates the percentage of other donor cells detected. (*C*) BALB/c islets were transplanted into diabetic RAG-KO (n=5) or RAG/γc-KO (n=3) recipients (D^{b+}) that were analyzed for passenger lymphocytes (D^{d+}) in the peripheral blood. Shown are representative plots at 9-10 days post transplantation, along with mean and SEM of the percentage of gated cells. Donor MHC-gated cells were tested for their expression of TCRβ or CD19. Naïve RAG-KO and BALB/c cells served as negative and positive controls, respectively.

Because of the confounding host NK cell rejection of fully MHC-mismatched passenger lymphocytes, we repeated the study using male or female bm12 islet transplants (bm12 mice are fully class I-matched with B6 but carry an MHC class II molecule mutated from I-A^b due to gene conversion, in which the beta chain of the I-A heterodimer differs from the WT product by three nucleotides) (30, 31) and again observed antigen-specific indirect islet rejection (**Figure 4.4**, *A*). Rejection was associated with a predominance of Th1 cytokines, including IL-12, IFN-γ and IFN-γ associated chemokines, monokine induced by gamma interferon (MIG) and 10 kDa IFN-γ-induced protein (IP-10) (32, 33), as detected in

recipient serum (**Figure 4.5**). Upon rejection of male islets, we gave the recipients "syngeneic" female RAG-KO islets to maintain their normoglycemia (**Figure 4.4, *B***) and then analyzed their peripheral blood for the presence of passenger lymphocytes (*i.e.* non-Vβ6-expressing CD4 and/or CD8 T cells; Tg TCR uses Vβ6). Importantly, Marilyn mice that indirectly rejected male bm12 islets were found to be chimeric for passenger T cells (**Figure 4.4, *C***) that we were also able to detect directly *ex vivo* in islet preparations (**Figure 4.4, *D***). This suggested that the establishment of chimerism was due to the migration of passenger cells from donor islets into host systemic circulation. Moreover, there was clearly an inability of the host immune response to eliminate donor T cells but not the islet grafts in which they resided (**Figure 4.4, *A*** and ***C***).

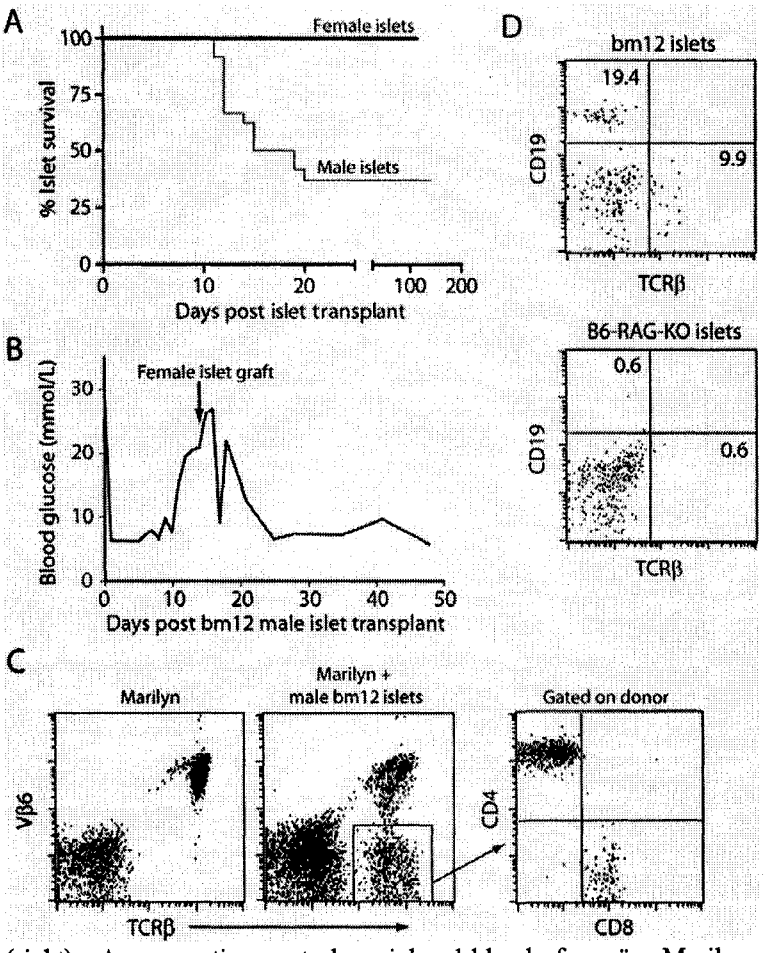

Figure 4.4. Differential susceptibility of islets and passenger lymphocytes to indirect CD4 alloimmunity. (*A*) STZ-induced diabetic female Marilyn mice were given male (n=24) or female (n=8) bm12 islet transplants. Survival curves are shown; p=0.0051 between male and female islets. (*B*) A cohort of recipients (n=6) that rejected male islets 11-14 days post transplantation was given "syngeneic" female RAG-KO islets in the opposite kidney (or an insulin pellet s.c. in one recipient) to maintain normoglycemia. Shown is a representative blood glucose profile of a Marilyn post transplantation of male bm12 and syngeneic islets. (*C*) Five weeks later, the presence of passenger lymphocytes in the peripheral blood was assessed. Shown are representative dot plots of the presence of passenger lymphocytes in one Marilyn that previously rejected male bm12 islets. Donor cells ($TCR\beta^+V\beta6^-$) were gated (middle) and assessed for CD4 and CD8 expression (right). As a negative control, peripheral blood of a naïve Marilyn was analyzed (left). (*D*) Top: Islets were isolated from two male bm12 donors, divided into two equal aliquots (approximately 200 islets each) and analyzed for passenger $TCR\beta^+$ cells and $CD19^+$ B cells by flow cytometry. Shown is a dot plot for one aliquot along with quadrant percentages. Similar results were obtained from the second aliquot, and when a second repeat experiment was done. Bottom: Islets isolated from B6-RAG-KO donors genetically deficient in passenger T cells and B cells were stained and analyzed for comparison.

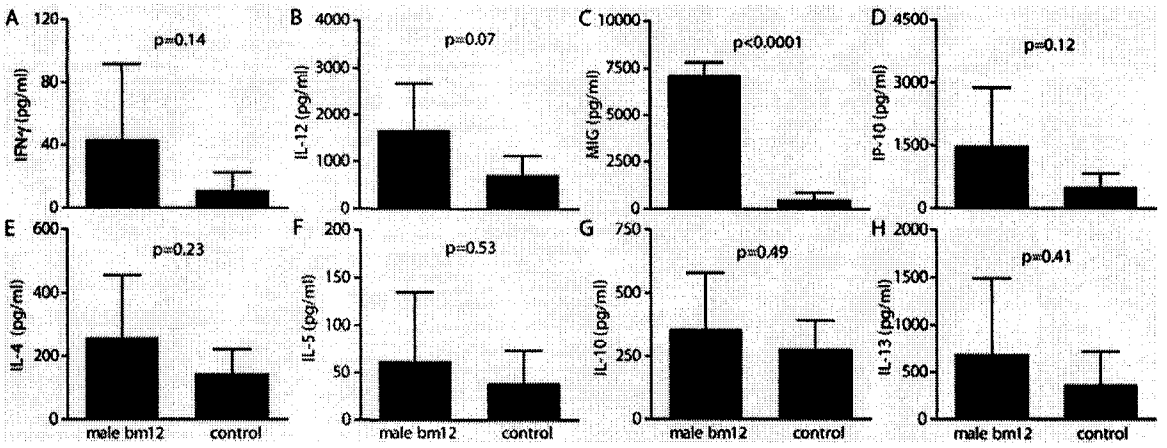

Figure 4.5. Serum cytokine quantitation in female Marilyn that underwent indirect rejection. STZ-induced diabetic female Marilyn mice were given male bm12 islets (n=3). Their sera were collected 2-6 days following islet rejection (approximately 3 weeks post transplant), and tested for the cytokines indicated by a multiplex assay. Control sera (n=6) were obtained from female Marilyn mice that were either not given a transplant, or given female bm12 islets for approximately 3 weeks (sera were harvested after these recipients underwent nephrectomy of the islet-bearing kidney to recapitulate the hyperglycemic condition in the male bm12 group, and results from the two types of controls were pooled as they were similar). All bars depict mean and SD. Values of statistical significance are provided.

Consistent with the passenger leukocyte hypothesis, removal of passenger cells from transplants usually results in improved graft survival (34-40), which is reversible when donor leukocytes are re-introduced (41, 42). To determine what effect the absence of passenger lymphocytes has on the indirect CD4 response towards islet grafts, we challenged Marilyn with male islets provided by donor mice genetically deficient in T cells and B cells and compared the survival of these transplants to those from WT, immunocompetent donors. Marilyn showed significantly faster rejection of fully MHC-mismatched BALB/c-SCID islets that lack passenger lymphocytes than WT BALB/c islets (**Figure 4.3**, ***A***). This difference was not simply due to a peculiarity of the BALB/c background or the *scid* mutation. Marilyn rejected male B6-RAG-KO islets that also lack passenger lymphocytes, significantly faster than WT B6 islets (**Figure 4.6**, ***A***), thus confirming the ability of passenger lymphocytes to delay islet rejection in this model. Together, these data indicate that the vigour of indirect islet rejection by CD4 T cells could be diminished by the presence of passenger lymphocytes. Initially, this seemed contradictory to the concept that passenger cell depletion enhances allograft acceptance, as described above. This difference could potentially be due to the removal of passenger DCs in previous studies, while our study instead involved removal of passenger lymphocytes. However, we had previously shown that passenger T cells could be immunogenic within the context of a full recipient T cell repertoire containing both CD4 and CD8 T cells

(29), although this might have been dependent on the lymphopenic hosts employed. We therefore tested whether genetic elimination of passenger lymphocytes in islet transplants would enhance or delay islet rejection in non-lymphopenic hosts containing polyclonal CD4 and CD8 T cells. **Figure 4.6, *B*** shows that passenger lymphocyte-replete WT islet transplants were rejected significantly faster than islets from lymphocyte deficient donors. Collectively, these data indicate that passenger T cells (but not islets) are able to withstand indirect CD4 effector mechanisms, and that they contribute to more rapid graft rejection. Where the response is restricted to indirect CD4 immunity, however, passenger T cells inhibit rejection.

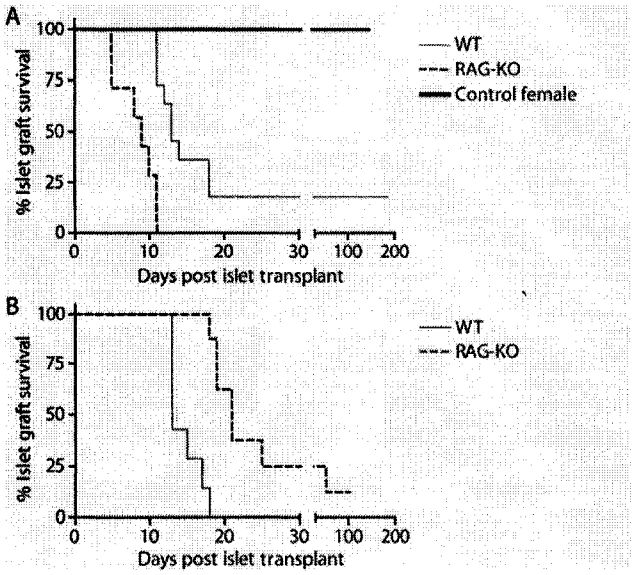

Figure 4.6. Passenger lymphocytes delay indirect islet rejection by CD4 T cells, but enhance rejection in the presence of a full repertoire. (*A*) STZ-induced diabetic female Marilyn mice were given male B6-RAG-KO (n=7), or male (n=11) or control female (n=7) WT B6 islet transplants and monitored by blood glucose for rejection. Survival curves are shown; p=0.0015 between WT male and female B6 islets, p=0.0002 between male RAG-KO and control female islets, and p=0.0001 between male WT versus RAG-KO islets. (*B*) STZ-induced diabetic BALB/c mice given WT B6 (n=7) versus RAG-KO (n=8) islet transplants were monitored for rejection; p=0.0001.

4.3.2. Indirect CD4 immunity is highly destructive for donor skin and B cells but the inability to eliminate donor T cells switches immunity to tolerance

To further address whether indirect CD4 immunity would be equally destructive towards different types of donor grafts, we determined the sensitivity of male hematopoietic cells versus male skin grafts to rejection by Marilyn T cells. We challenged Marilyn with male or control female congenic (CD45.1) B6 FLCs (a source of hematopoietic stem cells that can generate T cells and B cells *de novo*, and lack MHC class II expression) (**Figure 4.7**, ***A***) (43-45) and examined the survival and differentiation of the donor cells (*i.e.* establishment of chimerism). Marilyn demonstrated a significant but transient rejection of male FLCs (**Figure 4.7, *B***). The inability to completely eliminate the FLCs was not due to a general inability of CD4 T cells to kill hematopoietic cells. Marilyn fully rejected the male B cells that developed from FL precursors (**Figure 4.7**, ***C***). In contrast, following a transient rejection, the level of donor male T cells

steadily increased. Thus, the indirect CD4 response may not be equally destructive towards donor T cells and B cells (or their precursors). However, rejection of male B6 B cells did not prove that B cells were cleared efficiently by an indirect CD4 response. B cells, but not T cells, express MHC class II in mice and this might have made the B6 B cells targets of direct rejection by Marilyn T cells. To stringently test whether the indirect CD4 response can efficiently eliminate donor B cells, we challenged Marilyn mice with male or control female bm12 FLCs. We confirmed that male cells expressing I-A^{bm12} were unable to directly trigger proliferation of Marilyn T cells, unlike I-A^{b}-expressing male cells (**Figure 4.7, *D***). Strikingly, in Marilyn given male or female bm12 FLCs, male bm12 B cells were efficiently rejected (female bm12 B cells persisted) (**Figure 4.7, *C***), indicating that indirect CD4 alloimmunity is effective in B cell but not T cell rejection.

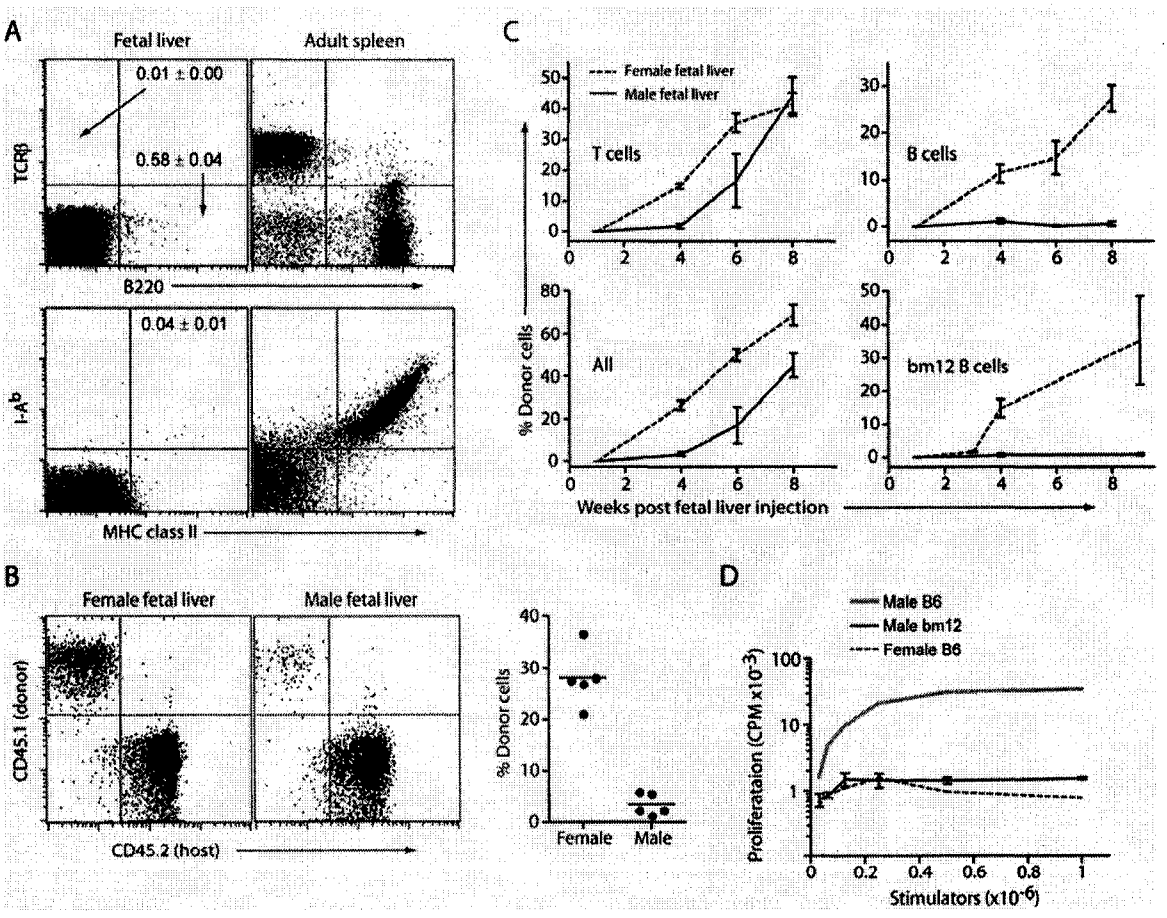

Figure 4.7. Differential susceptibility of allogeneic T cells and B cells to indirect CD4 immunity. (*A*) Left: FLCs from CD45.1-expressing B6 fetuses (n=6) were stained and analyzed for the presence of pre-existing T cells and B cells (top) and cells expressing MHC class II molecules (bottom), within the lymphocyte gate. Right: Adult B6 spleen cells shown as a positive control. Quadrant percentages are provided for B6 FLCs (mean and SEM). The low percentage of $B220^{+}$ cells detected are most likely B cell

precursors (lacking expression of surface IgM) that have already arisen in FL at the gestational age examined (46). Data shown are from one of two independent experiments. (*B*) Female or male CD45.1-expressing B6 FLCs were injected into female Marilyn recipients. Peripheral blood was analyzed for donor cells at 4 weeks post injection. Left: Representative dot plots. Right: Individual chimerism levels, $p<0.001$. (*C*) Marilyn mice receiving female or male CD45.1-expressing B6 FLCs were monitored for the frequency of donor cells over time. The percentage of cells in: the lymphocyte gate that were donor cells (All; bottom left), the T cell gate that were donor T cells (top left), or the B cell gate that were donor B cells (top right), is depicted. Also shown are Marilyn recipients of male or female bm12 FLCs, with donor B cells depicted (bottom right). Data were obtained from 3-5 animals per group. (*D*) Marilyn cells failed to proliferate when stimulated by male bm12 cells. Female Marilyn spleen cells were stimulated with titrated numbers of male B6 (positive control) or female B6 (negative control), or male bm12 spleen cells *in vitro*. Data for male bm12 stimulation represent mean and SEM of 5 animals.

Having established that CD4-mediated indirect rejection was effective for elimination of donor B cells but not T cells, we next asked if this "split tolerance" extended to donor skin grafts. We gave Marilyn mice that received male or control female B6 FLCs three days previously, a male and a female MHC class II-deficient B6 skin graft that could only be rejected indirectly. We observed that Marilyn recipients of male FLCs were able to reject donor male (but not female) skin grafts like the control recipients (**Figure 4.8**, ***A***), despite the long lasting presence of donor male hematopoietic cells that consisted of T cells but not B cells (**Figure 4.7**, ***C***). Thus, Marilyn T cells mounted an effective response against male B cells and skin transplants, but the same response ongoing within the same animal was relatively futile in eliminating male T cells.-

To assess whether the split tolerant state could persist long-term, we tested whether Marilyn mice that received male or female B6 FLCs approximately 12 weeks previously (and that subsequently became mixed hematopoietic chimeras) (**Figure 4.8**, ***B***), would accept male and female class II-deficient B6 skin grafts. Surprisingly, we found that the previous outcome of split tolerance towards donor skin transplants (**Figure 4.8**, ***A***) was no longer present in Marilyn recipients of male FLCs, as specific tolerance towards male skin transplants given late was achieved (**Figure 4.8**, ***C***). At the time of skin transplantation, however, the frequency of Marilyn T cells was similar between recipients of male versus female FLCs (**Figure 4.8**, ***D***), suggesting that a nondeletional mechanism of tolerance was responsible for skin graft acceptance in Marilyn given male FLCs. Downregulation of the CD4 coreceptor on Marilyn T cells but not donor T cells appeared to contribute to the tolerant state (**Figure 4.9**).

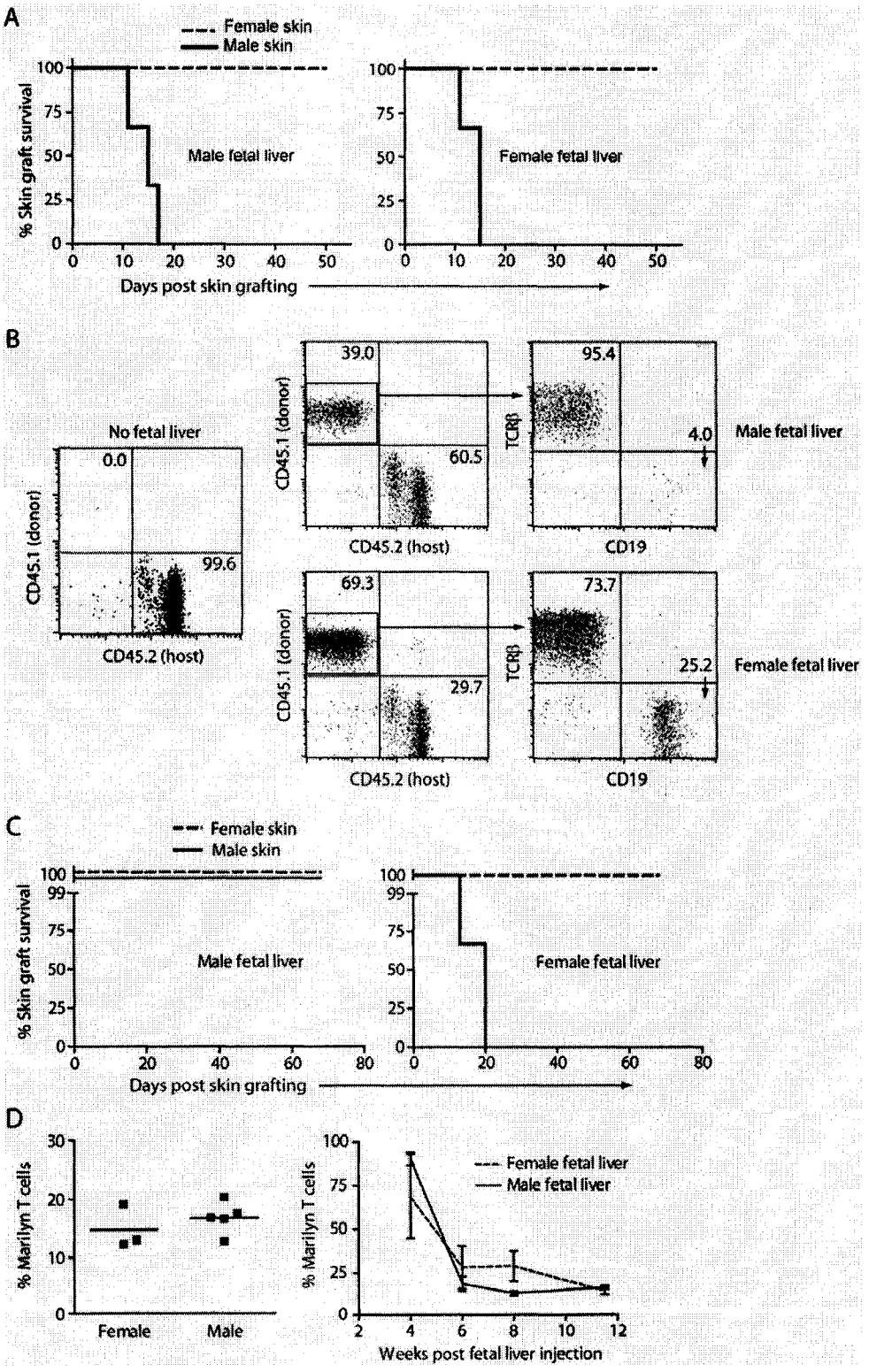

Figure 4.8. Differential susceptibility of allogeneic skin grafts and hematopoietic cells to indirect CD4 immunity generates split tolerance; failure to eliminate chimerism results in robust tolerance towards skin transplants. (*A*) Three days post male versus female CD45.1-expressing B6 FLC injection, recipients from **Figure 4.7**, ***C*** were challenged with MHC class II-deficient B6 male and female skin grafts, and monitored for rejection (p=0.0242). (*B*) A cohort of Marilyn given male (n=5) versus female (n=3) B6

FLCs approximately 12 weeks earlier was analyzed for peripheral blood chimerism. Donor cells were gated and analyzed for the presence of T cells and B cells. Representative dot plots from one recipient in each group are shown, along with staining of a Marilyn not given FLCs as a negative control. (*C*) Twelve weeks post FLC injection, recipients in *B* were challenged with MHC class II-deficient B6 male and female skin grafts. Survival curves are shown; p=0.0295 in the female FLC group. (*D*) Left: Frequency of Marilyn T cells, as a percentage of all T cells, in individual mice given female or male B6 FLCs as detected at the time of skin grafting. Horizontal bar denotes the mean in each group. There was no statistical significance between the groups. Right: Time course of the frequency of Marilyn T cells from 4 weeks post FLC injection up to the time of skin grafting (approximately 12 weeks as described above).

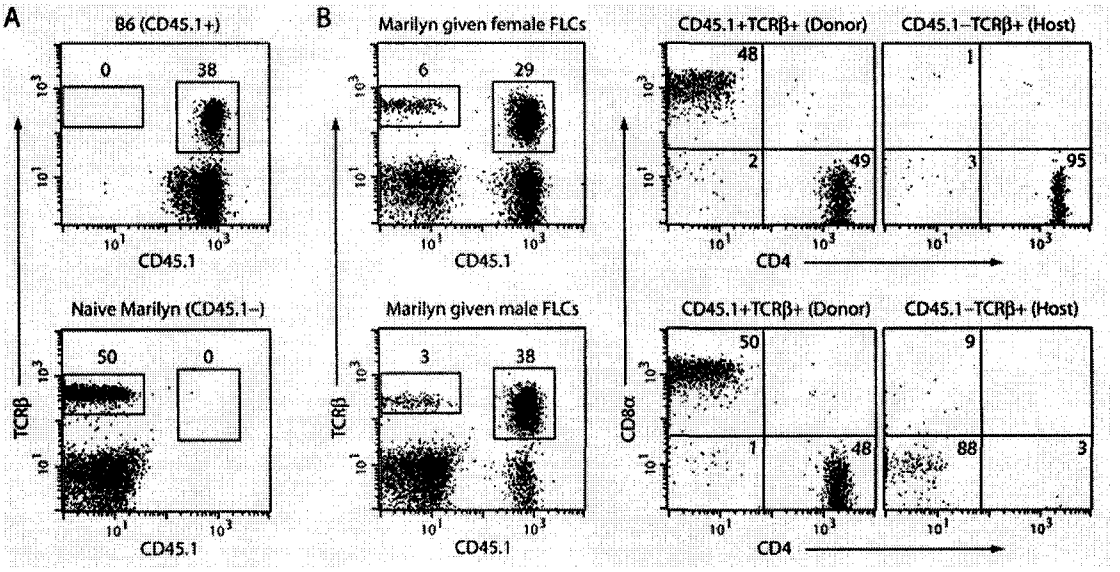

Figure 4.9. Downregulation of CD4 on Marilyn T cells in chimeric Marilyn mice. Female Marilyn mice (CD45.1$^-$) that were given male or female CD45.1-expressing B6 FLCs (n=3-6 from two independent experiments) and became long-term mixed chimeras were assessed for the expression of CD4 versus CD8α on Marilyn TCRβ$^+$ cells at >32 weeks post FLC injection. (*A*) Control staining done on a CD45.1-expressing B6 mouse (top) and a naïve Marilyn (bottom), showing the expression of CD45.1 versus TCRβ. (*B*) Top row: A representative Marilyn given female FLCs. Donor and host T cells were examined for CD4 versus CD8α expression. Bottom row: A representative Marilyn given male FLCs and analyzed similarly. Number above the rectangular box reflects the percentage of lymphoid-gated cells that are T cells of either donor (CD45.1$^+$TCRβ$^+$) or host (CD45.1$^-$TCRβ$^+$) origin. Relevant quadrant percentages are provided in the plots showing CD4 versus CD8α expression. The loss of CD4 expression on host T cells of Marilyn mice given male FLCs was also observed at >49 weeks post injection (not shown).

Collectively, these data indicate that the indirect CD4 response is not equally destructive towards different types of donor cells and tissues. It is especially ineffective in eliminating donor T cells, the futility of which could lead to tolerance induction.

4.4. DISCUSSION

CD4 T cells are important in adaptive immunity, either acting as helpers or effectors. The latter function is not traditionally associated with CD4 T cells but is gradually becoming better appreciated especially in

transplant immunity. CD4 T cells alone can be sufficient in skin rejection (2, 5) and can be necessary and sufficient in allogeneic (1) or xenogeneic (4) islet rejection. Moreover, CD4 T cells may preferentially respond by the indirect pathway (7, 19-22). However, whether indirect CD4 alloimmunity is equally destructive towards different types of allogeneic grafts has not been extensively assessed. In this study, we used Marilyn (24), a TCR Tg mouse model specific for the male antigen, H-Y, to demonstrate that indirect CD4 responses are highly destructive towards allogeneic B cells, skin and islet transplants but are ineffective at eliminating allogeneic T cells within the same animal.

We found that Marilyn mice undergoing indirect rejection of islet transplants simultaneously developed mixed T cell chimerism due to passenger lymphocyte migration. This suggested that the same indirect CD4 response that was sufficient for allogeneic islet rejection was ineffective in eliminating donor T cells. We also observed that Marilyn given male FLCs became mixed chimeras of T cells but efficiently rejected donor B cells (or their precursors) by the indirect pathway (**Figure 4.7, *C***). The reason for the reduced effectiveness of the indirect Marilyn response in male T cell but not B cell elimination is currently unknown. Potentially, T cells are intrinsically more refractory than B cells to indirect CD4 immunity.

In support of this hypothesis, Marilyn mice contain CD4 T cells of a single specificity and lack CD8 T cells and B cells, rendering them immunodeficient both qualitatively and quantitatively. Given the ability of T cells to homeostatically proliferate due to a deficient in T cell number (47), the few donor T cells that were initially generated from FLCs could have expanded upon entering the periphery and acquired a phenotype that conferred further resistance to indirect CD4 immunity. In contrast, B cells, while also capable of homeostatic proliferation, appear to do so at a slower rate than T cells and retain a quiescent phenotype (48, 49) (*i.e.* do not acquire memory-like properties as detected in T cells (50)). Therefore, these phenotypic differences may account for the resistance of T cells but not B cells to indirect rejection, but future studies are required to clarify this issue. However, susceptibility to a delayed-type hypersensitivity (DTH) effector mechanism may also explain our data. Indirect CD4 alloimmunity is likely to involve a DTH response involving Th1 cytokines (6, 16, 18, 51). Consistent with this possibility, Marilyn mice that underwent islet rejection indirectly, produced Th1 cytokines as well as chemokines known to depend on IFN-γ for their release (33) (**Figure 4.5**). Further on the mechanism of rejection, Marilyn T cells do not appear to upregulate Fas ligand or tumour necrosis factor-related apoptosis inducing ligand, while the

detectable presence of granzyme B is not associated with the induced expression of CD107, a marker for degranulation, suggesting that these alternative effector pathways may play a minor role in Marilyn T cell responses (52). In contrast, neutralization of IFN-γ leads to prolonged graft survival (52) suggesting the dominance of this mechanism.

Alternatively, the opposite fates of donor T cells and B cells in face of indirect CD4 alloimmunity may relate to the ability of T cells but not B cells to function as veto cells (53, 54). The veto concept was originally proposed as one of the mechanisms by which self-tolerance could be achieved (53). In this phenomenon, a host cell expressing self-antigen (known as the veto cell, and conventionally viewed to be a T cell) interacts with an autoreactive T cell in a unidirectional manner; upon recognition of self-antigen on the veto cell, the autoreactive T cell is rendered non-functional. Importantly, the veto effect has also been reported to occur in host response towards alloantigens (55). In this context, a donor T cell bearing alloantigens and capable of the veto effect is recognized by an anti-donor recipient T cell that consequently becomes inactivated. Furthermore, it has been suggested that CD4 cells, lacking MHC class II expression in mice, are able to regulate their own function by acquiring peptide/MHC complexes from APCs and presenting them to other CD4 cells in an inhibitory manner (56, 57). Thus, passenger CD4 cells from male islet transplants or CD4 cells developed from male FLCs could potentially inhibit Marilyn T cells thereby preventing donor T cell rejection.

Previously, the susceptibility of different types of allografts to T cell-mediated rejection has been examined in relation to CD8 T cells responding to either an intact allogeneic class I molecule (58) or a minor-H antigen presented on recipient MHC class I (59). In terms of CD8-mediated rejection, graft size may be an important factor that dictates the susceptibility of different tissue transplants to rejection. However, based on our study, if different types of donor grafts demonstrate a hierarchy of susceptibility towards indirect CD4 alloimmunity, the susceptibility of B cells, skin and islet transplants and the resistance of T cells to the Marilyn response would unlikely be attributed to differences in graft size. Instead, a clear difference between tissues and hematopoietic cells as targets of elimination is the distribution of their antigens. By their nature, target antigens of tissue transplants are concentrated in a single physical location in the recipient while those of hematopoietic cells can be broadly and systemically distributed, thus making hematopoietic cells a more difficult target to completely eliminate (60). Certain

mechanisms of rejection like immune-mediated damage to the vasculature supplying a transplant, which could lead to graft loss without the direct contact of immune cells with the parenchymal cells of the graft, could be irrelevant to rejection of hematopoietic cells (60). Such differences may explain the futility of the indirect CD4 response in eliminating allogeneic T cells, especially if they already demonstrate some intrinsic resistance. However, these latter considerations would not explain the differential susceptibility to indirect rejection of allogeneic B cells versus T cells.

When we challenged Marilyn recipients of male FLCs with male skin transplants that could only be rejected indirectly, we found that host immunity was sufficient to induce skin rejection. Interestingly, the inability to clear donor hematopoietic cells caused split tolerance towards donor skin grafts to turn into full tolerance, as male skin transplants given late were accepted long-term, as compared to acute rejection when given early (**Figure 4.8**). Mechanistically, this was associated with downregulated CD4 expression on Marilyn T cells (**Figure 4.9**), which, to our knowledge, has not been reported in allogeneic tolerance induction. However, CD4 downregulation was previously reported in a rat model of experimental allergic encephalomyelitis (61). Similarly, downregulation of CD8 has been observed as a mechanism for self-tolerance (62, 63). Furthermore, we expect that tolerance induction was also achieved in two Marilyn mice that showed long-term acceptance of male B6 islets (**Figure 4.6**, ***A***), as they were chimeric for donor T cells that were not eliminated (data not shown). The deficiency of indirect CD4 alloimmunity may, therefore, be exploited in developing new tolerance induction protocols.

It is also interesting to note that the failure of the indirect CD4 response to eliminate donor T cells may have a second consequence. As shown in **Figure 4.4**, ***A***, Marilyn mice did not always reject male bm12 islets. Only 15 of 24 (63%) recipients showed rejection. Of the remaining nine recipients, eight developed ascites typically occurring by 7 weeks post transplant, a time at which mixed T cell chimerism would have been established. As bm12 and Marilyn mice differ only at MHC class II, negating the ability of recipient NK cells to reject donor T cells, we surmise that the inability of Marilyn T cells to eliminate male bm12 T cells indirectly, and in combination with the absence of an alloreactive CD8 T cell response, led to a graft versus host reaction associated with ascites development. In addition to this potential effect, we observed that passenger T cells delayed indirect CD4-mediated rejection. However, within the context of a complete host T cell repertoire capable of both direct and indirect immunity, passenger T cells

enhanced rejection. In our previous studies suggesting that passenger T cells could provide an immunogenic source of antigen (29), we could not rule out the possibility that the immunity observed was dependent on a lymphopenic environment and consequent homeostatic effects. The current data (**Figure 4.6, *B***), in non-lymphopenic recipients, clearly substantiate the immunogenic nature of passenger lymphocyte-derived antigens when they are encountered by a complete repertoire. Thus, in terms of immunogenic passenger cells within a graft, our data indicate that it is not just donor DCs that are important, but also donor lymphocytes.

As indicated previously, Marilyn recipients of male FLCs and male skin transplants showed donor skin and B cell rejection but not T cell rejection, reflecting a form of split tolerance. Split tolerance can be generally defined as the simultaneous presence of immunity towards one type of donor cell/tissue but tolerance towards a second type of cell/tissue of the same donor origin (60, 64-67). It is most frequently manifested as skin rejection by hematopoietic chimeras due to immunity towards skin-specific antigens (60, 64-73). However, since we studied immunity towards the well-defined male antigen that is not tissue-specific, our data would suggest that a form of split tolerance that occurs independently of tissue-specific antigens may be possible and mediated through the variable effectiveness of indirect CD4 responses in eliminating different types of allogeneic cells/tissues. In this regard, diabetes-prone NOD mice demonstrate potent indirect CD4 responses (7, 21), and we have recently found that generation of mixed chimerism in NOD recipients can result in a split tolerance characterized by rejection of donor B cells, skin and islets and survival of donor T cells (see **Chapter 3**), paralleling the split tolerance data shown here in a TCR Tg recipient. It is also worth noting that the susceptibility of B cells to indirect rejection that we have shown here has implications for understanding B cell deletion induced by T cell killing of B cells (74, 75). Since CD4 T cells rejected bm12 B cells that were unable to present the relevant antigen, our data bring into question the previous conclusion that CD4 T cell killing of naïve B cells occurs via cognate interaction between the two cell types (75).

Collectively, our data provide evidence that indirect CD4 alloimmunity can either be highly destructive or relatively futile depending on its target of elimination. Differential susceptibility of allogeneic cells and tissues to indirect rejection potentially explains the mechanism driving split tolerance

in NOD mixed chimeras. Serendipitously, the "natural" deficiency of the indirect CD4 response may benefit our attempts to induce transplantation tolerance.

4.5. AUTHOR CONTRIBUTIONS

WFC designed and performed all experiments described, analyzed data and wrote the paper. Data in Figure 5.7, *B* and *C*, Figure 5.8 and Figure 5.9 were obtained in collaboration with HR, who also analyzed data and revised the paper. CCA conceived the study, advised on the design of research, analyzed data and wrote the paper.

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Chapter 5: Discussion

5.1. FUTURE DIRECTIONS

In this thesis, I performed studies that indicate the importance of chimerism in generating robust tolerance, by natural tolerance mechanisms (i.e. invoking mechanisms that have been naturally evolved to deal with self-tolerance, and not mechanisms induced through modulation of the immune system using pharmacologic agents or agonists/antagonists of specific receptors/ligands), towards an allogeneic islet transplant containing multiple minor-H or greater mismatches. However, I found that induction of allogeneic chimerism in the NOD mouse, a relevant model to test islet transplantation tolerance due to its predisposition to autoimmune diabetes, was associated with multiple levels of split tolerance, including the rejection of donor islets. Based on these data, I examined at the genetic level the origin of defect that results in this split tolerance phenotype. My initial studies in congenic NOD mice have indicated that one or more of the NOD background genes confer split tolerance in NOD mixed chimeras. To help to define the role of these genes, it would be important in future studies to first identify the cell(s), whether hematopoietic or non-hematopoietic (*e.g.* recipient thymic epithelium) in origin, that are required to generate split tolerance. The subsequent use of microarrays on these cells to detect significant transcriptional changes compared to cells from chimeras showing robust tolerance would be useful in screening for the relevant genes that mediate split tolerance.

As indicated previously, mixed chimeras generated through mild host conditioning could be more prone to split tolerance particularly because the recipient T cell compartment is left considerably intact. Resistance of these cells to the tolerizing therapeutic agents that are used to induce peripheral tolerance would allow them to maintain their ability for allogeneic resistance. Thus, the relative importance of any pre-existing peripheral host T cells (naïve and/or memory), especially those capable of anti-donor immunity, in driving split tolerance also requires further investigation. One way to test this would be to generate chimerism in a host devoid of pre-existing mature NOD T cells (*i.e.* use of a NOD-RAG-KO recipient). Through such an approach all NOD T cells would develop in the presence of donor cells; should split tolerance be detected this would argue against the need for pre-existing cells.

Even though split tolerance was likely the result of T cell immunity, it would still be important to determine whether a response by other host immune cells, such as B cells or NK cells, could influence the T cell response. This could be achieved by depleting a specific cell type from chimeric hosts with

antibodies, or by generating chimerism in a host genetically devoid of a specific type, followed by an assessment for split tolerance. Regarding NK cells, the use of a fully allogeneic donor/recipient combination means that host NK cells could participate in rejection, but whether their role is limited to the conventional rejection of allogeneic hematopoietic cells, or whether they could contribute to the rejection of solid tissue grafts in our model, is currently unclear. Moreover, the importance of achieving recipient NK cell tolerance to donor alloantigens needs to be addressed. Namely, would a lack of NK cell tolerance contribute to split tolerance? In the B6 model, establishing NK cell tolerance may not be as relevant due to the specific donor/recipient combination that we employed, in which the presence of donor alloantigen-specific inhibitory Ly-49 receptors on recipient NK cells (hence a genetically determined inability to reject allogeneic cells of a specific mouse strain) potentially obviates such a requirement. In contrast, NK cell tolerance towards donor alloantigens could be critical in NOD hosts, as NOD NK cells were capable of rejecting hematopoietic cells of the donor mouse strain tested.

There is also a need to provide further evidence to support our hypothesis that differential susceptibility of allogeneic grafts to indirect CD4 immunity was either partially or wholly responsible for split tolerance in NOD mixed chimeras, beyond what was observed in the TCR Tg model. In this regard, an investigation into why T cells are potentially more resistant to indirect rejection than other cells and tissues is warranted. Importantly, whether NOD CD4 T cells responding only indirectly are necessary and sufficient in generating split tolerance will need to be determined. Furthermore, given that our NOD mixed chimeras were chimeric for donor T cells only, the requirement for sustainable, long-term multilineage chimerism in preventing split tolerance would also need to be evaluated. This could be done by testing additional chimerism induction protocols in NOD recipients and correlating the presence or absence of split tolerance with the quality and longevity of chimerism achieved. Lastly, whether the robust tolerance induced in B6 mixed chimeras was due to a dominant mechanism warrants clarification. Should the removal of Treg cells in chimeric B6 mice generated by the standard protocol be associated with split tolerance rather than robust tolerance, either the absence of tolerance inducing Treg cells or the presence of defective Treg cells in NOD mixed chimeras could explain the multiple levels of split tolerance observed.

5.2. RELEVANCE OF SPLIT TOLERANCE IN CLINICAL TRANSPLANTATION

The original (1-3) and many subsequent studies (4-7) in which split tolerance was observed were focussed on the rejection of donor skin grafts by hematopoietic murine chimeras. Immunity to donor skin-specific antigens was the likely culprit of split tolerance, but is not a response restricted to the mouse immune system since chimeric dogs have also been shown to reject skin transplants from hematopoietic cell donors (8, 9). Furthermore, dizygotic cattle twins that became chimeric via sharing of their placental circulation rejected skin grafts from each other (4). Since allogeneic skin is not transplanted clinically for treatment, the issue of split tolerance may be easily dismissed and considered simply by many as an academic curiosity (7). However, it can be said that many studies on tolerance induced by chimerism have, by design, avoided the potential for split tolerance, as the donor/recipient combinations used were matched for minor-H antigens (that would code for polymorphic tissue-specific antigens) and therefore were clinically irrelevant (7). Surprisingly, the acknowledgement by some that testing for tolerance experimentally in the presence of complete mismatches would be more stringent and more relevant clinically has not been linked with the dissociation between chimerism and tolerance that can evidently occur in these systems (10). The reason for this is unclear.

However, the work in a small animal model presented in this thesis suggests for the first time that split tolerance is a potential complication if chimerism is induced for generating islet transplantation tolerance in humans. Immunity to donor islet-specific antigens needs to be considered, and early studies in rabbits provided evidence for the existence of pancreatic isoantigens (11), thus supporting this possibility. Moreover, early studies in dogs made chimeric and given heart transplants that were later rejected (12) suggested yet another non-skin tissue that could be implicated in split tolerance in humans. Consistent with a previous proposal (13), a recent study has also shown in a mouse model that split tolerance could indeed develop in fully allogeneic chimeras given donor heart transplants (14). Clearly, a clarification on whether split tolerance involving these tissues would be a likely occurrence in humans should be undertaken in larger animal models.

Aside from allogeneic skin grafts, and possibly with allogeneic islet or heart grafts, certain transplants like the kidney may not be associated with split tolerance even when transplanted into humans, but this appears to be controversial. A recent case report on the combined transplantation of kidney and

hematopoietic cells from a donor matched with the recipient for human leukocyte antigens (HLA; the designation for human MHC molecules) described relatively stable mixed chimerism associated with donor-specific tolerance to hematopoietic cell antigens as demonstrated *in vitro*, and survival of the kidney transplant for close to 2 years after the discontinuation of immunosuppression (15). This is in contrast with the results from another group also employing HLA-matched kidneys, where kidney survival occurred in spite of the loss of hematopoietic chimerism (16, 17). The same observation was made when transplantation of HLA-mismatched kidney and BM cells was performed (18). Interestingly, the possibility that immunity towards antigens expressed on donor hematopoietic cells but not on the kidney (*i.e.* split tolerance in the reverse manner) was raised (17). Alternatively, the dissociation between hematopoietic chimerism and allograft survival could relate to the use of cyclosporine in the treatment protocol, which was found experimentally to impede tolerance induction by chimerism (19). However, cyclosporine was also used in the study in which there was maintenance of both chimerism and kidney survival (15), thus the disparate outcomes remain to be explained. Importantly, the long-term fate of kidney allografts transplanted in all these studies will be of great interest. Unlike small animal models, transplant outcomes in humans are conceivably more difficult to predict due to a greater biological complexity of humans and a greater likelihood for disturbances in their immune status to arise (a reflection of the relatively uncontrolled environmental conditions faced by humans compared to laboratory animals).

Collectively, split tolerance may not necessarily be a barrier whenever chimerism is induced to generate transplantation tolerance even in humans. This should be a consolation to those who favour such an approach (20). Based on the available experimental and clinical evidence, however, the continued pursuit of hematopoietic chimerism warrants consideration of the potential for split tolerance, and most importantly, protocol refinement and implementation of strategies to reduce or eliminate this potential.

5.3. OVERCOMING SPLIT TOLERANCE

To induce in humans the most robust form of immunological tolerance towards islet alloantigens via hematopoietic chimerism, while avoiding split tolerance, it would be necessary to fully eliminate the recipient's adaptive immune system during the conditioning phase in which hematopoietic "space" is

created (21) (**Figure 5.1**). NK cells that can reject allogeneic hematopoietic cells (22) also need to be physically eliminated or functionally inactivated to facilitate engraftment. Since transplantation of donor BM cells alone that lead to full chimerism (5) is potentially detrimental to the host due to the generation of a non-functional immune system (10, 23), rebuilding the immune system to contain both donor and recipient hematopoietic cells may be required. Thus, a mixed human chimera can be generated by simultaneously transplanting donor and recipient stem cells, the latter obtained by, for example, mobilizing stem cells with granulocyte/macrophage colony stimulating factor prior to conditioning (10).

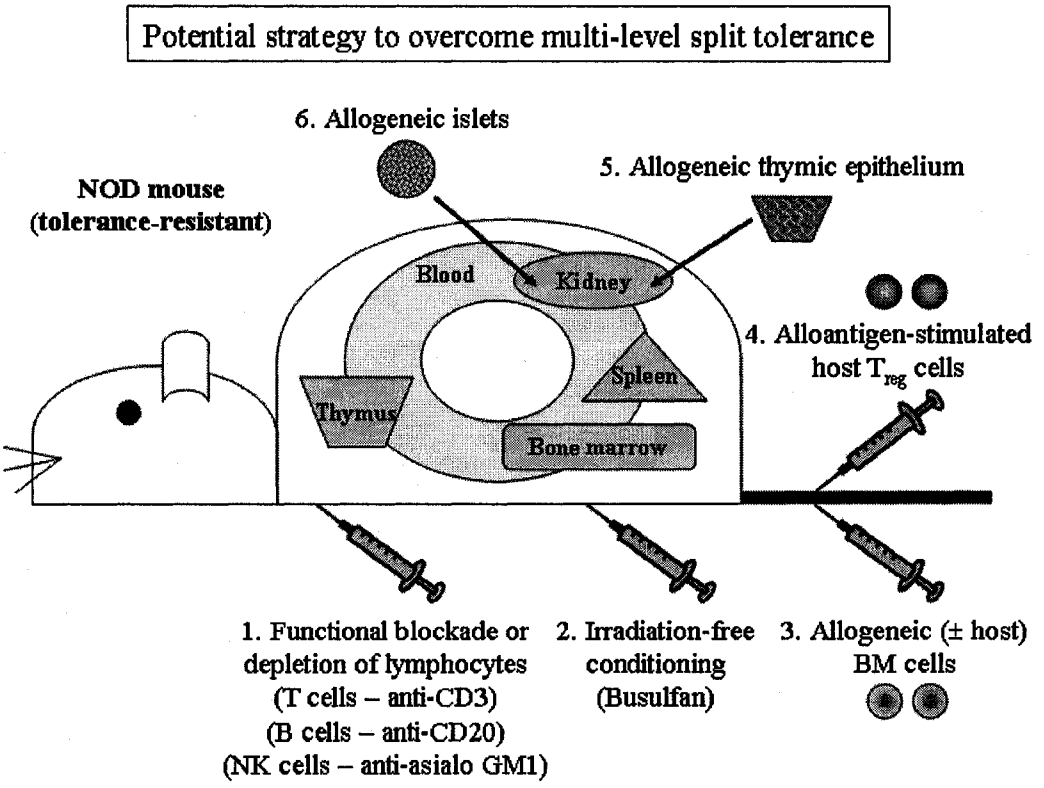

Figure 5.1. Overcoming split tolerance in mixed hematopoietic chimeras. Chimerism induction has been associated with robust tolerance in various experimental models of transplantation. However, due to a high degree of tolerance resistance, NOD mice that were made into mixed hematopoietic chimeras using fully allogeneic BM cells under nonmyeloablative conditioning showed multiple levels of split tolerance that involved donor islet rejection. To overcome this, thereby enabling the tolerogenic effects of chimerism to generate robust allogeneic tolerance, I propose that rebuilding of the NOD immune system in the presence of allogeneic cells would be necessary. A potential strategy is illustrated in the above schematic. Key features of this strategy include physical and/or functional elimination of host lymphocytes (1), creating hematopoietic "space" with an agent that effectively does so with minimal toxicity (2), transplantation of allogeneic (with or without host) BM stem cells (3) along with infusion of host type Treg cells previously stimulated *in vitro* by donor alloantigens (4), transplantation of donor thymic epithelium (5), and transplantation of allogeneic islets early during tolerance induction (6).

Unlike the success achieved when allogeneic chimerism is induced in nonautoimmune hosts that are also given donor islet transplants (24), the strong tolerance resistance in autoimmune hosts as demonstrated previously (25-27) and in this thesis is a critical reason for eliminating the recipient's existing immune system and rebuilding a new one. Even without an intrinsic resistance to tolerance induction, immunity towards an islet transplant given clinically (if in the absence of immunosuppression) would be particularly potent because transplantation is performed into a recipient whose immune system can induce rejection by either autoimmunity or alloimmunity or the combination thereof (28). This by itself already presents a challenging barrier to tolerance, which is heightened when genetically determined tolerance resistance is functional. One of the ways that tolerance resistance is potentially manifested is through the anti-donor reactivity of recipient T cells (notably memory T cells) left behind after nonmyeloablative chimerism induction. Even in small animals, memory T cells capable of heterologous immunity (29) are difficult to tolerize (30). Moreover, in the presence of Treg cells, memory T cells but not naïve T cells retain their ability to reject allografts (31). Depending on how chimerism is induced, the generation of a peripheral environment that promotes homeostatic proliferation of pre-existing recipient T cells that subsequently resist tolerance induction (32) is an additional, major complication. The combination of these problems means that the tolerogenic effects of chimerism can only be obtained by resetting the immunological "clock".

Construction of the immune system *de novo* in this manner allows the recognition of donor alloantigens by developing cognate T cells that leads to their deletion or inactivation. There is also reciprocal induction of tolerance to recipient alloantigens by cognate T cells developed from donor precursors (33). These events occur simultaneously with the establishment of self-tolerance (34). Furthermore, those T cells developing from recipient precursors that have an islet-specific autoimmune potential and are able to escape central tolerance in the native environment (35-37) can either be selected against in the thymus or functionally inhibited in the presence of chimeric nonautoimmune cells thereby abrogating the recurrence of autoimmune diabetes (38).

As proposed by others (10), tolerizing T cells, B cells and NK cells that are the relevant effector cells in resisting allogeneic cells and tissues is likely necessary to generate robust tolerance. The concept that NK cells must be rendered tolerant is relatively new compared to the long history of appreciation for

T cell and B cell tolerance in relation to long-term transplant survival without immunosuppression. This stems from the observation that NK cells, that are not conventionally associated with adaptive immunity and do not express somatically rearranged antigen-specific receptors, undergo a form of self-tolerance (NK cells generally kill cells lacking self MHC class I molecules, but class I-deficient mice that contain NK cells do not exhibit autoimmunity) (39, 40). Moreover, NK cells have become increasingly important participants in transplant immunity (41). Hence, establishing NK cell tolerance in mixed chimeras is likely required and has been demonstrated with either minimal or full mismatches (42-44). The mechanism by which NK cells are tolerized remains unclear; altered expression of Ly-49 receptors has been observed (43, 44) but is not a consistent finding (42). Nevertheless, the idea that NK cell reactivity against donor alloantigens can "make or break" tolerance (44) is one that cannot be taken lightly.

Compounds like BUS that preferentially target hematopoietic stem cells over mature immune cells could be used to generate hematopoietic space. This would be done along with depletion of lymphocytes by antibodies (possible candidates include anti-CD52 for T cells and anti-CD20 for B cells in humans). Memory T cells that are more resistant to depletion by conventional depleting antibodies (45-47) would require targeting by novel antibody clones that are capable of depleting memory cells (48) (remain to be developed in humans). NK cells can be targeted either by depleting them or blocking their function using agents like SRL.

To avoid split tolerance to islets due to recipient immunity towards donor tissue-specific antigens, simultaneous transplantation of donor BM cells and islets will likely give the greatest chance of success in generating allogeneic tolerance. This is based on the idea that during reconstitution of the recipient's immune system, donor tissue-specific antigens that are potentially expressed only on the islets but not the BM cells would be available for direct or indirect presentation for tolerance if the islet transplant has been placed around the time of BMT (7). Transplantation of donor thymic epithelial cells may further expand the scope of tolerance that could possibly be achieved (21). The expression of Aire that promotes promiscuous thymic expression of antigens found in peripheral tissues (49) augments the repertoire of antigens expressed by donor hematopoietic cells that are available for presentation. Moreover, thymic medullary epithelial cells can either directly present their antigens to delete cognate T cells, or they can be a source of antigens that are presented by APCs circulating in the thymus to induce

central deletion (50). An intriguing idea is whether the control of antigen expression by Aire includes those peripheral antigens that could be polymorphic and differ between individuals.

Since the revival of suppressor T cells (51, 52) as Treg cells (53-55), this unique subset of T cells, with their ability to block the function of other T cells not endowed with a regulatory phenotype, has been highly valued for their potential in inducing transplantation tolerance. Although a convincing demonstration of their suppressive capacity has been troublesome *in vitro* (56), some *in vivo* models of tolerance have been found to contain Treg cells that are highly potent in controlling T cell immunity towards alloantigens (57, 58). In certain chimerism models where tolerance towards allogeneic skin grafts was achieved, elimination of putative Treg cells by antibody depletion led to a state of split tolerance characterized by donor skin rejection in spite of the maintenance of hematopoietic chimerism, suggesting that Treg cells could be involved in overcoming immunity towards donor tissue-specific antigens (59). On the other hand, murine chimeras that were adoptively transferred with Treg cells stimulated *in vitro* with donor alloantigens were able to convert their split tolerance to donor solid tissue grafts to full tolerance (14). Although this study involved recipient strains that are generally susceptible to tolerance induction, the use of a similar strategy in the tolerance resistant NOD model as described in this thesis is potentially beneficial in correcting its propensity for split tolerance, and may be applicable to future endeavours in generating chimerism in humans. The synergism between dominant tolerance induced by Treg cells and recessive tolerance resulting from chimerism should provide the necessary ammunition to generate islet transplantation tolerance clinically.

5.4. FINAL THOUGHTS

In retrospect, I think that our search for a solution to host resistance towards allogeneic transplants (the "Holy Grail" as many refer to it) is an act of rebellion against nature. By nature, I mean the combination of the environment in which all living and nonliving things exist, and the selective pressures of evolution that define those genes and their coded functions that provide the greatest survival advantage and therefore persist with time. The purpose of immunity towards alloantigens is consistent with that of the naturally evolved ability of the immune system to protect the host against the myriad of potential

pathogens existing outside the host (60). Hence, preventing such a response from being generated is an apparent sabotage.

However, I believe that viewing our efforts to find the Holy Grail from such a perspective is interesting but counterproductive. Transplantation is an effective way to treating a disease that causes the loss of function of an organ or tissue, in spite of its being a target of immunological insult when it is not syngeneic in origin. A successfully performed, well functioning transplant prolongs the life of the ill and improves quality of life. Therefore, transplantation has a key place in medicine now and in future, as supported statistically (61). How have we dealt with transplant immunity? Although effective in prolonging graft survival, the use of various immunosuppressive agents (62) that are lifelong and associated with numerous side effects cannot be considered the ideal solution; withdrawal usually restores immunity against the transplant. Instead, tolerance induction is the preference of many and therefore has been aggressively pursued. Sharing the consensus view, I believe that transplantation tolerance is the Holy Grail.

Spontaneous blood chimerism observed in dizygotic cattle twins (63) or chickens (64) that shared blood circulation during early development, as well as allogeneic tolerance observed in neonatal mice injected with donor spleen cells (65), were not only serendipitous biological discoveries of a purely scientific interest, but discoveries that were crucial in raising hope for transplantation tolerance to be achieved ultimately. Hematopoietic chimerism appears to be the driving force behind acquired tolerance. The observation of spontaneous chimerism made by Starzl and others in human recipients of organ transplants (66-70) is worth mentioning here since cases of putative immunological tolerance could be identified (67). Starzl and Zinkernagel proposed that in a chimera, striking a balance between host versus graft and graft versus host effects that involve clonal exhaustion and deletion reciprocally, in combination with some level of immunological ignorance, is responsible for tolerance induction (71). Interestingly, chimerism (at a low level that can only be detected by sensitive techniques such as PCR) seems to be a natural effect of pregnancy in which exchange of cells occurs via the fetomaternal circulation (72). As demonstrated in this thesis, the establishment of chimerism spontaneously and its impact on tolerance induction (see **Chapter 2** and **Chapter 4**) resonate with both experimental and clinical observations that have been described. Importantly, if split tolerance in allogeneic chimeras can be averted and tolerance in

all the relevant effector cell types is induced, the possibilities for the future are endless. Selection of donor/recipient combinations would clearly be less restricted. Moreover, the potential for split tolerance to occur in xenogeneic chimeras (73-75) would also be eliminated and replaced by robust tolerance, thus xenogeneic transplants can be broadly used to solve donor shortage.

For now, we continue our quest to achieve tolerance, as biology is never so simple, and the best solution has not yet been derived. I think our persistence in this endeavour is the correct attitude. Would we ever be able to tame our immune system that is primed to strike in self-defence? What we have working for us is our innovation, our fearlessness and our willingness to take on great challenges. Regarding chimerism induction, sound and reproducible experimental data demonstrating its ability to generate a state of tolerance that does not involve split tolerance will be the backbone that leads to success. I believe our continued efforts will yield great dividends in the future.

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Appendices

A.1. REAGENTS AND ASSAY PROTOCOLS

A.1.1. Avertin (2,2,2-Tribromoethanol)

1. Heat 1 L of double-distilled water (ddH_2O) on a hot plate in a 1 L Erlenmeyer flask.
2. In a 50 mL conical tube, add 20 g of 2,2,2-tribromoethanol to 20 mL of tert-amyl alcohol.
3. Place tube above the heating water to help contents dissolve.
4. Transfer contents into a 1 L volumetric flask, and bring up to volume with ddH_2O.
5. Cover flask with parafilm and invert to mix. Aliquot solution into tubes and store at 4°C.

A.1.2. βTC-tet cell culture medium

Per one 500 mL bottle of Dulbecco's Modified Eagle's Medium, add 50 mL of fetal bovine serum (FBS), 5 mL of penicillin/streptomycin (10,000 units/mL of penicillin G sodium and 10,000 μg/mL of streptomycin sulphate), and 5 mL of 200 mM L-glutamine. Mix and sterilize by vacuum filtration. Store at 4°C.

A.1.3.1. CTL/MLR culture medium

Per one 500 mL bottle of Iscove's Modified Dulbecco's Medium (IMDM) with sodium bicarbonate, add 50 mL of FBS, 5 mL of penicillin/streptomycin (10,000 units/mL of penicillin G sodium and 10,000 μg/mL of streptomycin sulphate), 5 mL of 200 mM L-glutamine, 500 μL of 10 mg/mL gentamicin and 500 μL of 5×10^{-3} M 2-mercaptoethanol. Mix and sterilize by vacuum filtration. Store at 4°C.

A.1.3.2. CTL killing assay medium

Per one 500 mL bottle of IMDM with sodium bicarbonate, add 13 mL of FBS. Mix and sterilize by vacuum filtration. Store at 4°C.

A.1.3.3. CTL killing assay protocol

1. Culture responder and stimulator spleen cells in 24-well flat bottom polystyrene tissue culture-treated plates using CTL/MLR culture medium (2 wells for each responder/stimulator combination, each well containing a total volume of 2 mL). Two million gamma-irradiated (1500 rads) stimulators are added

to each well. For fully allogeneic stimulation, use 5×10^6 responders and culture for 5 days at 37°C with 5% carbon dioxide. For single or multiple minor-H stimulation, use 6×10^6 responders and culture for 6 days (responders must come from a previously immunized host).

2. Approximately 40 hours before assaying the effectors from the responder/stimulator cultures for target killing, set up Con A-stimulated blast targets. Con A (type IV lyophilized powder) is dissolved in Roswell Park Memorial Institute 1640 medium, aliquotted and stored at -20°C. Culture responder (syngeneic control) and stimulator (test) strain spleen cells (37°C with 5% carbon dioxide) separately at 1.5×10^6 cells/well in 24-well plates (8-10 wells for each strain) using CTL/MLR culture medium, adding Con A to each well at a final concentration of 1.25 μg/mL, for a total volume of 2 mL/well. Pulse Con A blast targets with 10 μCi ^{3}H-thymidine per well (5 μCi/mL) for 3 hours before setting up CTL killing assay (*i.e.* at about 39 hours after setting up Con A blast cultures).
3. CTL killing assay: Harvest responder/stimulator cultures. Pool duplicate wells into one 15 mL conical tube. Spin tubes at 1,200 rpm for 10 minutes. Discard supernatant and resuspend cell pellet in 600 μL of warm CTL killing assay medium. Aliquot 150 μL of cells into 4 wells of a 96-well U-bottom polystyrene tissue culture-treated plate (these are the "neat" wells). Serially dilute the cells 3-fold from the neat wells by adding 100 μL of CTL assay medium into adjacent wells and transferring 50 μL of cells from the neat wells to the next set of wells, mixing thoroughly, then transferring 50 μL to the next set and so forth. Discard the 50 μL from the last set of wells after mixing.
4. Plate setup (R1 and R2 refer to two different types of responders to be assayed):

1:81 R1	1:81 R1	1:81 R2	1:81 R2		Targets alone	Target alone
1:27 R1	1:27 R1	1:27 R2	1:27 R2	Test	Targets alone	Target alone
1:9 R1	1:9 R1	1:9 R2	1:9 R2	targets	Targets alone	Target alone
1:3 R1	1:3 R1	1:3 R2	1:3 R2	↔	Targets alone	Target alone
Neat R1	Neat R1	Neat R2	Neat R2		Targets alone	Target alone
Neat R1	Neat R1	Neat R2	Neat R2	Control	Targets alone	Target alone
1:3 R1	1:3 R1	1:3 R2	1:3 R2	targets	Targets alone	Target alone
1:9 R1	1:9 R1	1:9 R2	1:9 R2	↔	Targets alone	Target alone

5. Harvest targets while responder/stimulator cultures are being spun in step 3. Due to their fragility, harvest targets with a large bore pipette (10 mL) by gently resuspending cells and transferring them to a 15 mL conical tube. Spin pooled targets at 800 rpm for 10 minutes. Discard supernatant and resuspend targets in 10 mL of warm CTL killing assay medium. Perform a manual count of the targets

using a hemacytometer, counting only large blasts. Adjust concentration to 1×10^5 blasts/mL. Add 100 µL of the appropriate targets to each well according to plate setup. Total volume in each well is 200 µL. For wells containing targets alone, add 100 µL of medium to make up final volume.

6. Set up a second 96-well plate that contains each of the target types alone. Harvest cells immediately after setup, using the harvester (time 0 targets).
7. Incubate plates with responders and targets for 3.5 hours 37°C with 5% carbon dioxide. Harvest cells.
8. Obtain radioactivity counts from all the harvested plates using the beta counter. The difference in counts between time 0 targets and targets alone that were incubated for 3.5 hours gives an indication of the relative health of the targets (*i.e.* spontaneous death of targets during the incubation period). A difference of 5-20% is acceptable.
9. Plot CTL assay data as the percentage of killing at each responder-to-target ratio.

A.1.4.1. Acetate buffer (solvent for streptozotocin)

1. To 0.7775 mL of glacial acetic acid, add ddH_2O to a final volume of 50 mL (Solution A).
2. To 1.36 g of sodium acetate trihydrate, add ddH_2O to a final volume of 50 mL (Solution B).
3. Combine 15.25 mL of Solution A and 9.75 mL of Solution B together and add ddH_2O to a final volume of 50 mL.
4. Add 0.45 g of sodium chloride to the combined solution and mix.
5. Adjust pH to 4.5, sterilize by vacuum filtration and store at 4°C protected in aluminum foil.

A.1.4.2. Preparation of streptozotocin for injection

1. Only prepare streptozotocin (STZ) just before use. Weigh STZ powder in milligrams (*e.g.* 20 mg) and dissolve in 1 mL of acetate buffer.
2. Determine the dose of STZ (*e.g.* 200 mg/kg). Weigh recipients in grams (*e.g.* 20 g).
3. Divide the STZ dose by the concentration of STZ prepared (*i.e.* 200 mg/kg ÷ 20 mg/mL = 10 mL/kg = 10 µL/g).
4. Multiply the quotient by the body weight to determine the volume of STZ to be injected (*i.e.* 10 µL/g × 20 g = 200 µL). Inject STZ i.p.

A.1.5.1. Phosphate-buffered saline (10×)

Per 1 L, add 2 g of potassium chloride, 2 g of potassium dihydrogen phosphate, 80 g of sodium chloride and 9.2 g of disodium hydrogen phosphate to ddH_2O. Stir to dissolve. Adjust pH to 7.2-7.4, sterilize by vacuum filtration and store at room temperature.

A.1.5.2. Diluent for antibodies used in flow cytometry

Per 50 mL, add 5 mL of dialyzed 10% (w/v) bovine serum albumin (BSA) to 45 mL of 1× phosphate-buffered saline (PBS). Sterilize by vacuum filtration. Store at 4°C.

A.1.5.3. Fc receptor blocking solution

Per 10 mL, mix together 3.333 mL each of mouse, rat and hamster serum. Add 300 μg of anti-CD16/32 antibody (2.4G2). Mix, sterilize by vacuum filtration and store at 4°C in small aliquots for use.

A.1.5.4. Cell labelling by carboxyfluorescein diacetate succinimidyl ester

10 mM stock solution:

To one stock vial (500 μg), add 90 μL of dimethyl sulphoxide. Aliquot and store at -20°C.

Labelling/washing buffer:

To 5 mL of 10% BSA, add 1× PBS to a final volume of 500 mL. Mix and sterilize by vacuum filtration. Store at room temperature.

Labelling protocol:

1. Prepare a single cell suspension of the cells to be labelled at a concentration of 10-20 × 10^6 cells/mL in labelling/washing buffer.
2. Add 10 mM stock solution to cells to a final concentration of 5 μM. Mix and incubate cells at 37°C for 10 minutes.
3. Quench staining by adding FBS to cells to a final concentration of 5% and incubating on ice for 5 minutes.

4. Wash cells with labelling/washing buffer. Centrifuge at 1,200 rpm for 10 minutes and discard supernatant. Perform a second wash.
5. Wash cells for a third time with 1× PBS.
6. Resuspend cells in 1× PBS at the desired concentration for injection.

A.1.5.5. Red blood cell lysis buffer (1×)

Per 1 L, add 8.29 g of ammonium chloride, 1 g of potassium bicarbonate and 37.2 mg of disodium ethylenediaminetetraacetic acid to ddH_2O. Stir to dissolve. Adjust pH to 7.2-7.4. Store at 4°C.

A.1.5.6. Antibody staining protocol

Staining with fluorescently labelled primary antibodies only:

1. Typically, stain 25 µL of cells (heparinised blood, spleen, lymph nodes, thymus, BM, peritoneum *etc.*).
2. Add Fc receptor blocking solution at 1:1 ratio. Incubate at room temperature for 5 minutes.
3. Add antibody cocktail (each previously titrated antibody is used at a 1:1 ratio to cells). Maximum of four colors can be used on the two-laser FACSCalibur™, one antibody for each fluorescence channel. Vortex and incubate at 4°C for at least 15 minutes.
4. Wash cells with 3 mL of cold red blood cell lysis buffer if working with blood, or 3 mL of cold 1× PBS for all other types of samples. Centrifuge at 1,200 rpm at 4°C for 10 minutes.
5. Discard supernatant and resuspend cells in 200-300 µL of cold 1× PBS for analysis on flow cytometer.

Staining with a combination of biotinylated and fluorescently labelled primary antibodies:

1. Follow the above protocol to step 3 inclusive. Wash cells with 3 mL of cold 1× PBS. Centrifuge at 1,200 rpm at 4°C for 10 minutes. Discard supernatant.
2. Add streptavidin conjugated to Tricolor or allophycocyanin at a 1:1 ratio to cells. Vortex and incubate at 4°C for at least 15 minutes.
3. Wash cells with 3 mL of cold red blood cell lysis buffer if working with blood, or 3 mL of cold 1× PBS for all other types of samples. Centrifuge at 1,200 rpm at 4°C for 10 minutes.
4. Discard supernatant and resuspend cells in 200-300 µL of cold 1× PBS for analysis on flow cytometer.

A.1.6.1. Hematoxylin and eosin stain for histology

This is an adapted version of the protocol provided by Dr. Greg Korbutt.

1. Dehydrate paraffin slides as follows:
    a. Histoclear, 3 washes (5 minutes per wash);
    b. 100% ethanol, 3 washes (2 minutes per wash);
    c. 95% ethanol, 1 wash (1 minute);
    d. 70% ethanol, 1 wash (1 minute);
    e. Distilled water (5 minutes).
2. Harris Hematoxylin (1 minute 30 seconds). Rinse slides in running tap water until clear.
3. 0.25% acid alcohol (3 seconds). Agitate slides in water (5 seconds).
4. Lithium carbonate (30 seconds). Rinse in water (30 seconds).
5. 95% ethanol (30 seconds).
6. Alcoholic Eosin Y (35 seconds).
7. Dehydrate slides as follows:
    a. 95% ethanol (30 seconds);
    b. 100% ethanol, 4 washes (30 seconds per wash);
    c. Xylene, 3 washes (5 minutes per wash).
8. Apply coverslip using mounting media.

A.1.6.2. Insulin stain by immunohistochemistry

This is an adapted version of the protocol provided by Dr. Greg Korbutt.

1. Islet grafts are first fixed overnight in a buffered zinc formalin fixative (Z-Fix) before processing.
2. Paraffin-embedded tissue is sectioned at 3 μm and placed on histobond slides.
3. Sections are rehydrated to water, followed by quenching of endogenous peroxidases by a solution of 20% hydrogen peroxide in methanol for 6 minutes.
4. Blocking is performed with 20% normal goat serum for 15 minutes at room temperature.
5. Primary antibody incubation (1:1000 dilution of guinea pig anti-insulin antibody, Dako Cytomation, Mississauga, ON) is performed for 30 minutes at room temperature followed by 3 washes in PBS.

6. Secondary antibody incubation (1:200 dilution of biotinylated goat anti-guinea pig IgG, Jackson ImmunoResearch) is performed for 20 minutes at room temperature.
7. After 3 washes in PBS, incubation with the avidin-biotinylated enzyme complex (Vector Laboratories, Burlingame, CA) is performed for 40 minutes at room temperature.
8. After a further 3 washes in PBS, staining is detected using diaminobenzidine as the chromagen (Signet Laboratories Inc., Dedham, MA).
9. Sections can then be counterstained and coverslipped.

A.1.7. Mouse islet isolation

This is an adapted version of the protocol provided by Dr. Ray Rajotte.

Preparation of Ficoll:

1. Prepare, per litre of Hanks' Buffered Salt Solution (HBSS), a solution containing penicillin/streptomycin at 0.5% and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 2.5%. Make up to 5 L.
2. Add 1.5 kg of Ficoll® 400 (Type 400-DL; Sigma-Aldrich) to 4.42 L of supplemented HBSS in a container with a stirring rod. Mix overnight.
3. Check density of solution (between 1.098-1.105 g/mL) and set pH to 7.0-7.2.
4. Place one end of a Silastic® tubing into the container, feeding it through a peristaltic pump and attaching it to a 0.22-μm capsule filter.
5. Pump Ficoll through the filter into sterile bottles for storage. These are 25% Ficoll stocks.
6. Use the following chart to prepare Ficoll gradients for mouse islet isolation:

Density (g/mL)	25% Ficoll stock (mL)	Supplemented HBSS (mL)
25% (1.101)	100	0
23% (1.097)	92	8
21.5% (1.088)	86	14
11.5% (1.045)	44	56

Excision of pancreas and islet isolation:

1. Islet donor mice are anaesthetized by i.p. injection of Avertin.
2. With the donor positioned on its back, a full length incision is made from the groin past the breastbone.

3. The common bile duct is clamped where it joins with the small intestine.
4. After exposing the bifurcation at the common bile duct, the duct is cannulated at the bifurcation by a 27-gauge needle capping a syringe that contains the collagenase solution. Cold collagenase is then injected slowly. Collagenase (Type V; Sigma-Aldrich) is usually used at between 1-2 mg/mL with 5 mL injected per mouse. It is dissolved in supplemented HBSS as described in step 5.
5. The needle is retrieved and the perfused pancreas is excised from the connective tissue and placed in 15 mL of cold HBSS (supplemented with 50% dextrose at 0.5%, penicillin/streptomycin at 0.5% and HEPES at 0.5%) until the next step.
6. The pancreas is transferred from HBSS to a tube containing the collagenase solution, capped and placed in a shaking water bath at 37°C for digestion (time varies between 9-14 minutes).
7. Pancreas is removed from the bath and cold HBSS is added to stop digestion. Centrifuge at 1,500 rpm for 30 seconds and remove supernatant by vacuum.
8. Resuspend pellet in 15 mL of HBSS and vortex.
9. Suspended tissue is poured through a funnel fitted with a 500-μm screen. Rinse digestion tube.
10. The filter is rinsed with 10 mL of HBSS to dislodge any attached islets.
11. Split the filtrate evenly into new tubes. Fill tubes with HBSS and centrifuge at 1,500 rpm for 30 seconds. Remove supernatant.
12. Add 10 mL of 25% Ficoll to each tube and vortex to resuspend.
13. Slowly, in descending density, add 6 mL of each of the remaining Ficoll solutions (*i.e.* 23%, 21.5% and 11.5%). Centrifuge at 2,000 rpm for 10 minutes.
14. Using a 10-mL syringe and a 14-gauge Insyte™ catheter (Becton Dickinson) to remove the islets from the first Ficoll interface and place in a tube containing a second set of gradients.
15. Fill tube with HBSS and centrifuge at 2,000 rpm for 1 minute. Remove the top layer containing the HBSS supernatant. Repeat wash.
16. Remove all supernatant and add 10 mL of 25% Ficoll, followed by the remaining layers. Centrifuge at 2,000 rpm for 2 minutes.
17. Remove the islets from the Ficoll interface and place them into a new tube.
18. Add HBSS and centrifuge at 2,000 rpm for 1 minute. Remove supernatant and repeat wash.

19. Remove all supernatant and resuspend islets in 10 mL of Medium 199 (supplemented with newborn calf serum at 10% and penicillin/streptomycin at 0.5%).
20. Pour islets into a petri dish. Rinse the tube with M199 and add to dish.
21. Using a Pasteur pipette, pick out all the islets and transfer to a new dish.
22. Maintain islets in culture in CMRL or Ham's F-10 medium at 37°C prior to use.

A.1.8.1. Agarose gel (2%)

For a gel containing 50 wells:

1. Add 5 g of agarose to 250 mL of 1× Tris-borate-ethylenediaminetetraacetic-acid buffer.
2. Heat mixture in microwave to dissolve agarose (until boiling). Cool to ~60°C.
3. Add 12.5 µL of 10 mg/mL ethidium bromide and mix.
4. Pour gel and allow it to solidify.

A.1.8.2. Loading buffer (6×)

Per 1 mL, add 2.5 mg of bromophenol blue and 400 mg of sucrose to 1 mL of ddH_2O. Mix to dissolve. Store at 4°C.

A.1.8.3. Tris-borate-ethylenediaminetetraacetic-acid buffer (10×)

Per 1L, add 108 g of Tris base, 55 g of boric acid and 40 mL of 0.5 M ethylenediaminetetraacetic acid (pH 8.0) to ddH_2O. Stir to dissolve. Store at room temperature.

A.2. ESTABLISHMENT OF NATURAL TOLERANCE TO HEART TRANSPLANTS

(All data shown were generated by C. Anderson.)

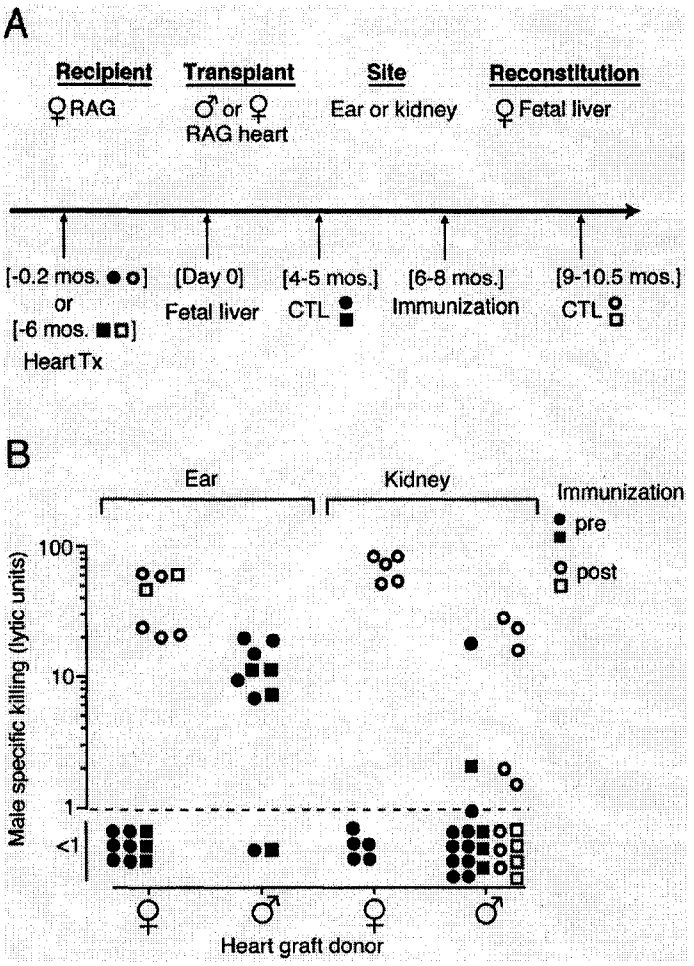

Figure A.2.1. Pre-immunocompetence single minor-H-mismatched heart grafts are not ignored but instead trigger immunity or natural tolerance depending on the graft site. (*A*) Experimental design and timeline. (*B*) Male-specific killing by spleen cell responders from individual mice is depicted. Female RAG mice were given a RAG male or female neonatal heart graft under the kidney capsule or the skin of the ear pinna. Less than 10 days (circles) or 6 months (mos.; squares) later these mice were reconstituted with FLCs from female B6 fetuses. Four to 5 months after reconstitution, recipients were hemi-splenectomized and tested *in vitro* for CTLs specific to the H-Y antigen that had been primed *in vivo* (unprimed mice do not generate a CTL response to minor-H antigens *in vitro*) (pre-immunization; solid symbols). Killing of targets was tested at various responder-to-target ratios. After killing of control syngeneic targets was subtracted, donor-specific killing was expressed as lytic units calculated as the dilution of responders giving 20% killing of targets. Pre-immunization: male versus female heart graft, $p<0.001$ in the ear and $p>0.05$ in the kidney. Some of the mice were later immunized i.p. with 3 $\times 10^6$ B6 male spleen cells and 6 or more weeks after immunization (*i.e.* 9-10.5 months after immune reconstitution) they were tested again *in vitro* for CTL priming to H-Y (post-immunization; open symbols). For grafts in the kidney, $p<0.001$ for female heart (pre- versus post-immunization), $p>0.05$ for male heart (pre- versus post-immunization), and $p<0.01$ for post-immunization male versus female heart.

A.3. IMMUNOSUPPRESSIVE EFFECTS OF ACUTE DIABETES

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A.3.1. INTRODUCTION

Protocols to induce donor-specific tolerance in transplant recipients have commonly been tested using a single type of donor tissue (*e.g.* heart, skin or islets) in rodent models. The recent advances in clinical islet transplantation (1) and the relative inconsequence of islet rejection on patient morbidity have made islet transplantation an important area to test translational tolerance strategies. However, when islet transplants are carried out in rodents, they are generally performed under conditions of severe acute diabetes and marked dehydration, and in the absence of insulin treatment. This is quite different from the chronic, insulin stabilized state of patients receiving islet infusions. Immunomodifying effects of diabetogenic drugs such as STZ or alloxan have been observed, and occur promptly after drug administration, suggesting the possibility that the acute diabetic state may, in addition to its chronic effects, rapidly lead to a degree of immunosuppression. However, the current view is that immunosuppression post diabetogenic drug administration is a result of direct immunosuppressive properties of the drugs rather than a consequence of the diabetes they induce (2-5). This conclusion has appeared sound because it is based on observations from a number of approaches. For example, STZ is toxic to lymphocytes *in vitro* (6); insulin injections only have a partial impact on STZ-induced immunosuppression (7); and islet transplant rejection in NOD mice that are already diabetic can be blocked by injection of STZ (2, 5). However, each of these approaches has considerable limitations, as compounds that are toxic *in vitro* are not necessarily toxic *in vivo*; insulin injections do not fully reverse the diabetic state; and STZ affects presentation of autoantigens targeted by NOD T cells (8). Furthermore, while increased rates of infection occur in diabetic patients and reduced immunity has been noted in animal models of chronic diabetes (9-12), it is not clear whether the

immunosuppressed state requires the cumulated effects of multiple episodes of hyperglycemia or if it is a consequence of much earlier events. Suggestive of the latter possibility, hyperglycemia is a common finding in critically ill patients and intensive insulin therapy for such patients reduces infections and mortality (13). A link between hyperglycemia and suppressed adaptive responses has received little attention; reduced innate rather than adaptive immunity was speculated to be the basis for increased infections in these hyperglycemic patients (13). In view of the above considerations we designed experiments to evaluate the effect of acute diabetes on cellular immunity. Dissecting out the potential immunosuppressive properties of diabetes versus diabetogenic drugs is also critical for the evaluation of potential translational tolerogenic strategies in islet transplantation. Should acute diabetes itself lead to substantial impairment in immunity then the conventional method of testing for tolerance to islets (removal of long-term accepted islets, returning the recipient to a diabetic state, followed by challenge with a second donor islet transplant) could substantially overestimate the potency of a given tolerogenic protocol. We therefore examined the possibility that acute diabetes may rapidly induce an immunosuppressed state.

A.3.2. MATERIALS AND METHODS

A.3.2.1. Animals. Adult CBA/J (H-2^k) and spontaneously diabetic C57BL/6-*Ins2*^{Akita} (Akita; H-2^b) (14, 15) mice were purchased from Jackson Laboratories (Bar Harbor, ME), C3H (H-2^k) and B6 (H-2^b) mice from NCI-Frederick (Frederick, MD), and BALB/c (H-2^d) mice from Health Sciences Laboratory Animal Services (University of Alberta, Edmonton, AB). B6.129S7-*Rag1*^{tm1Mom} mice (B6-RAG-KO) from Jackson Laboratories were bred on-site at the University of Alberta. These were housed under viral antigen free conditions. All other mice were housed under standard conditions. Animal care was in accordance with the guidelines established by the CCAC.

A.3.2.2. Induction of diabetes, islet transplantation, thymectomy and adrenalectomy. A single optimal dose of STZ (Sigma-Aldrich Canada, Oakville, ON) in acetate buffer was injected i.p. or i.v. to 8-12 weeks old B6 (200 mg/kg) and CBA/J mice (210-375 mg/kg, depending on the lot of STZ) to induce stable hyperglycemia; diabetes was confirmed 48 hours later if the blood glucose level was >18 mmol/L. Donor

islets were isolated as previously described (16). Approximately 450 islets were transplanted in the renal subcapsular space of recipients. Successful engraftment was defined by correction of blood glucose level to <10 mmol/L by the second day post transplant; rejection was defined as a rise to >20 mmol/L for 2 consecutive days. Sham transplantation was performed by surgical exposure and dissection of the kidney. To evaluate islet graft survival in nondiabetic recipients, STZ-induced diabetic mice were first transplanted with syngeneic islets to correct hyperglycemia. After 2-3 weeks these mice received donor islets under the right, contralateral renal capsule. The syngeneic islets were then removed by left nephrectomy within 10 days. To evaluate tolerance induced by antibodies targeting putative costimulatory molecules, islet allograft recipients were treated, as described (16), with monoclonal anti-CD40L antibody (MR1; 0.25 mg/day on days 0, 2, 4 and 6) and anti-inducible costimulator (ICOS) antibody (12A8; 0.1 mg/day for 14 days) beginning on the day of transplantation. In some experiments we used adult thymectomized mice (thymus exposed by making a small incision above the sternum). In the indicated experiments some mice received bilateral adrenalectomy or a sham surgery. Adrenalectomized mice received drinking water containing 0.9% sodium chloride.

A.3.2.3. βTC-tet cell implantation. Five million cultured (see **Appendices, A.1.2**, for the formulation of culture medium; p.158) insulin secreting βTC-tet cells (generation and functional analysis of this cell line was previously described) (17, 18) were injected s.c. in PBS. Blood glucose was monitored twice a week, for 8 weeks. Tumour engraftment was defined by hypoglycemia, a decrease in blood glucose to <4 mmol/L beyond 5 days post tumour injection (with or without syngeneic islet transplantation). The engraftment criteria eliminate false positives (hypoglycemia) due to release of insulin by cells dying shortly after islet transplantation and tumour injection.

A.3.2.4. Tissue analysis, CTL responses, *in vivo* proliferation, flow cytometry and serum corticosterone measurement. Cellularity of spleen and thymus was assessed by viable cell counts under phase contrast microscopy. CTL responses were assayed by the JAM Test as described (19). Briefly, recipient splenocytes were stimulated for 6 days with gamma-irradiated (1500 rads; ^{137}Cs irradiator, Gammacell 40, Atomic Energy of Canada, Ottawa, ON) donor or third party splenocytes. Targets were ^{3}H-

thymidine labelled Con A (Sigma-Aldrich Canada, Oakville, ON) stimulated splenocyte blasts. Killing of targets was tested at various responder-to-target ratios and killing at a ratio of 67:1 is depicted. Killing of control syngeneic targets was subtracted. *In vivo* proliferation of T cells and B cells was assessed by detecting incorporation of BrdU (Sigma-Aldrich) following the manufacturer's instructions (BrdU Flow Kit, BD Pharmingen, San Diego, CA). Briefly, BrdU was given at 0.8 mg/mL in the drinking water from days 5-9 post STZ administration, then spleens were harvested and B cells and T cell subsets were assessed by four-color flow cytometry. Control animals that received acetate buffer (STZ vehicle) and remained nondiabetic were similarly examined. Flow cytometry included staining with antibodies to CD4 (RM4-5), CD62L (MEL-14), B220 (RA3-6B2) (Caltag, Burlingame, CA), BrdU (3D4), CD8α (53-6.7), TCRβ (H57-597) (BD Pharmingen), and CD44 (IM7; eBioscience, San Diego, CA), and was done with a FACSCalibur™ and CellQuest™ software (Becton Dickinson, Sunnyvale, CA). Insulin staining of islet grafts was done as described previously (20). An enzyme immunoassay kit for the determination of corticosterone in mouse and rat sera (OCTEIA Corticosterone, IDS Inc, Fountain Hills, AZ) was used according to the manufacturer's recommendations.

A.3.2.5. Statistical analysis. Graft survival was analyzed using the Kaplan-Meier method and the log rank test (GraphPad Prism Software, San Diego, CA). Other data, either expressed as individual values or mean and SEM, were analyzed using one-way ANOVA and Tukey's multiple comparison test.

A.3.3. RESULTS

A.3.3.1. The acute diabetic state induces a rapid decrease in thymocytes and splenic T cells

Diabetogenic drugs are known to cause reduced thymic and splenic cellularity (7), although it has not been established whether this includes reduced peripheral T cell numbers. Furthermore, it is not clear whether the drugs directly affect cellularity or instead do so via the acute diabetes they induce. To examine this we assessed cellularity in drug-treated mice cured of their diabetes. To provide a more complete and physiological control of the diabetic state post STZ administration than can be achieved with insulin injections (7), we transplanted syngeneic islets soon after the onset of diabetes (2 days after STZ). Sham

operation controls included untreated mice and STZ-treated mice without a syngeneic islet transplant. We performed an extensive time course analysis of the effect of STZ versus diabetes on cellularity. As early as 5 days after STZ (3 days after islet transplant or sham operation), the total number of thymocytes from diabetic mice decreased dramatically to 4.5% of control mice and remained around this level thereafter. In mice made diabetic with STZ and then cured of diabetes with syngeneic islets, the number of thymocytes decreased to 10.6% of controls at day 5, almost as low as the diabetic animals. Thus, the initial drop in thymus cellularity could have been due either to the diabetic state, present initially for both the STZ alone and the STZ plus syngeneic islet groups, or a direct toxic effect of STZ. However, in mice that had their diabetes cured with an islet transplant, thymus cellularity recovered significantly at day 7 and completely by day 14 (**Figure A.3.1, *A***). Syngeneic islets similarly reversed the hypocellularity in mice made diabetic with alloxan. These data suggested that the hypocellularity was induced by diabetes. However, it did not rule out the more complex possibility that the initial hypocellularity was due to a direct toxic effect of STZ, and that recovery required normoglycemic conditions.

Total splenocyte numbers, including $B220^{+}$ B cells, from diabetic B6 mice also dropped significantly although much less than thymocyte numbers; the B cell reduction appeared to be strain-dependent as B cell numbers were reduced in B6 mice (approximately 40%) but instead were increased in CBA/J mice post STZ administration (data not shown). **Figure A.3.1, *B*** shows that STZ triggered a reduction (approximately 50%) in the absolute number of splenic T cells, predominately affecting T cells with a naïve phenotype ($CD44^{lo}CD62L^{hi}$) at day 5. T cell numbers were partially recovered either by the transplantation of syngeneic islets or even spontaneously by day 9 post STZ administration. The latter suggested the possibility that homeostatic proliferation was triggered by STZ or the lymphopenia it induces. Consistent with this possibility, we found that STZ treatment resulted in an early loss of T cells with a memory phenotype ($CD44^{hi}CD62L^{lo}$) followed by a recovery in the number of these cells even in the absence of thymic T cell output (thymectomized mice) (**Figure A.3.1, *C***). Together these results indicated that developing and mature T cells were decreased in STZ-induced diabetic mice and that correction of hyperglycemia resulted in substantial or complete (in the case of the thymus) recovery of cellularity.

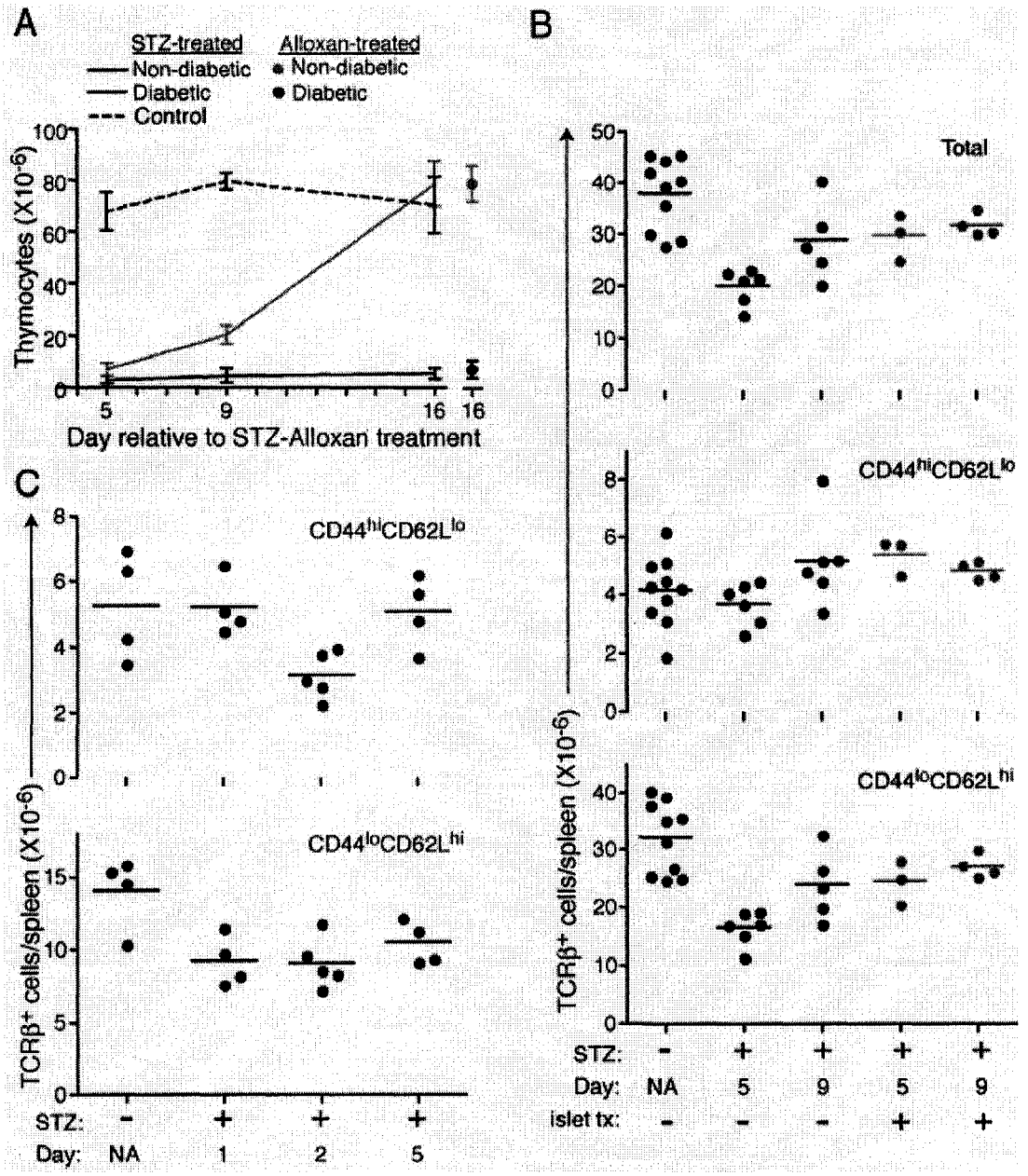

Figure A.3.1. Diabetes rapidly affects thymocyte and T cell numbers. In *A* and *B*, two days after STZ or alloxan treatment, B6 mice were either cured of diabetes by a syngeneic islet transplant (non-diabetic) or had a sham operation and remained hyperglycemic (diabetic). Untreated control mice were also given a sham operation. (*A*) Various days after islet transplantation or sham operation, thymocytes were counted (n=3-6). (*B*) Top: Splenic T cell numbers 5 or 9 days post STZ administration were determined by staining for TCRβ and multiplying the percentage of $TCR\beta^+$ cells within the lymphoid gate by the total cell count. Memory (middle) versus naïve (bottom) phenotype T cell numbers were determined by staining for TCRβ, CD44 and CD62L, and multiplying the percentage of relevant cells within the T cell gate by the absolute T cell count. (*C*) Splenic T cell numbers of the memory (top) and naïve (bottom) phenotype in adult thymectomized B6 mice were determined 1, 2, and 5 days post STZ or control acetate buffer treatment (NA = not applicable).

Although there was a reduction in splenic T cells and thymocytes 5 days after STZ administration, it was still unclear whether diabetes alone was responsible for this early loss. To address this we tested the effect of diabetes alone by removing an islet transplant from mice treated with STZ weeks earlier. Two days after STZ induction of diabetes, we gave them syngeneic islets and allowed them to maintain normoglycemia for 3 weeks (sufficient time for recovery of cellularity) (**Figure A.3.1**, ***A***). We then separated the mice into 3 groups: some we nephrectomized the islet-bearing kidney and these mice became diabetic (diabetes not caused by STZ); some we treated again with STZ to induce diabetes; and the rest we left normoglycemic but performed a mock nephrectomy. Five days later (**Figure A.3.2**, ***A***) the thymocyte and splenic T cell numbers dropped approximately 90% ($p<0.001$) and 35% ($p<0.01$) respectively compared to controls, in both the nephrectomy and re-STZ groups. Cellularity was not significantly different between the nephrectomy and re-STZ groups ($p>0.05$). Nephrectomy of normal mice did not lead to thymus hypocellularity (data not shown), indicating that it was the loss of the islet graft, rather than the loss of the kidney that affected cellularity. Thymocyte loss preferentially affected the immature CD4 and CD8 DP cells (**Figure A.3.2**, ***B***), consistent with the stronger effect on the thymus compared to the spleen. These results demonstrated that diabetes alone could cause a rapid loss of lymphocytes. To test this idea further in a different diabetes model that does not involve STZ, we used chronically diabetic nonobese Akita ($H\text{-}2^b$) mice that have early onset, spontaneous, nonautoimmune diabetes (14, 15) due to a mutated insulin 2 gene that disrupts insulin processing from proinsulin and thereby proper insulin secretion (21). Hyperglycemia in these mice is treatable by islet transplantation (22). **Figure A.3.2**, ***C*** shows that diabetic Akita mice had greatly reduced thymocytes ($p<0.01$) and splenocytes ($p<0.001$) compared to nondiabetic controls that received syngeneic B6 islets. Together these data indicated that, in addition to any direct effects of STZ, diabetes itself causes lymphoid hypocellularity, including a loss of T cells.

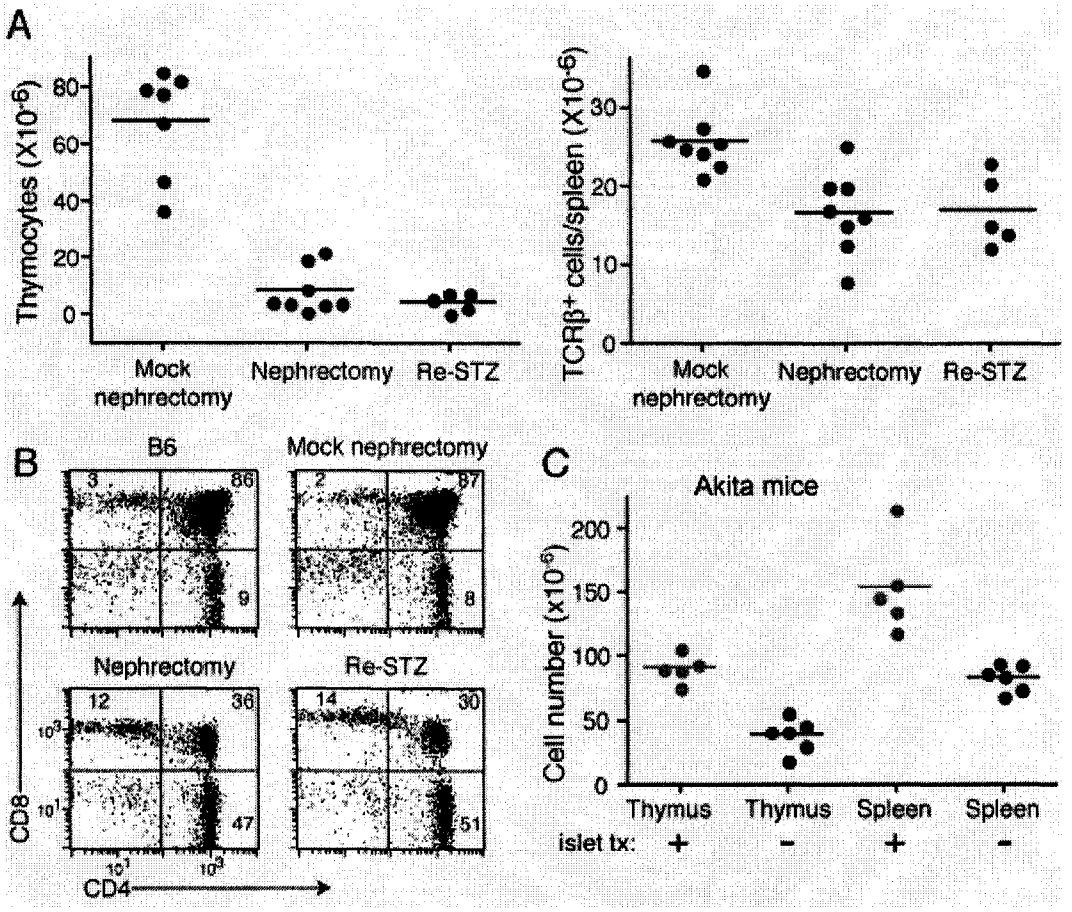

Figure A.3.2. Lymphopenia is a result of diabetes rather than a direct immune system toxicity of STZ. In *A* and *B*, two days after STZ treatment diabetic B6 mice were transplanted with syngeneic islets and then made diabetic again 3 weeks later either by removing the islet-bearing kidney (diabetes not caused by STZ; Nephrectomy) or by a second STZ treatment (Re-STZ). Control (Mock nephrectomy) mice were left normoglycemic. (*A*) Thymocytes and splenic T cell numbers from individual mice were counted 5 days later, as in **Figure A.3.1**. (*B*) Representative (three experiments) flow cytometry plots of thymocyte subpopulations. Percentage of cells in each quadrant is indicated. (*C*) Diabetic Akita mice were either given a B6 islet transplant (nondiabetic) or a sham islet transplant (diabetic). Two months later, thymocytes and splenocytes of both groups were counted.

To begin to assess the mechanism of diabetes-induced lymphoid hypocellularity we tested whether glucocorticoids may be involved. Diabetes elevates plasma corticosterone (23, 24) and corticosterone increases (increased by means other than diabetes) can cause lymphocyte/thymus hypocellularity (25, 26). However, multiple factors induced by diabetes could potentially reduce cellularity. We therefore tested if removal of the adrenal glands could fully prevent the diabetes-induced lymphoid hypocellularity. **Figure A.3.3** shows that removal of the adrenals eliminated circulating corticosterone and completely prevented the thymus hypocellularity induced by diabetes.

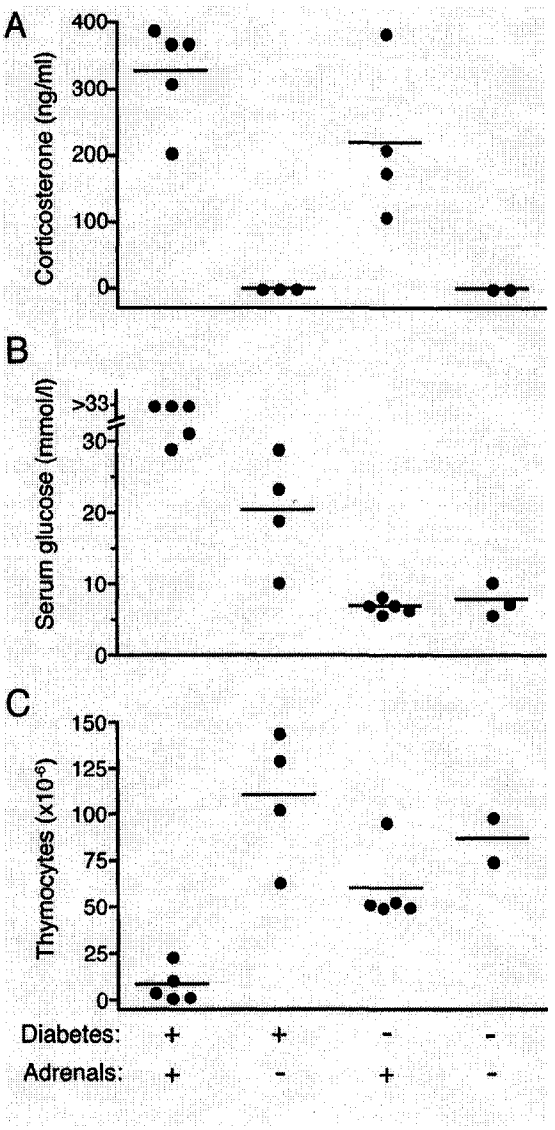

Figure A.3.3. Adrenalectomy prevents diabetes-induced thymus hypocellularity. STZ-induced diabetic B6 mice were transplanted with syngeneic islets and then allowed to maintain normoglycemia for 3 weeks. Some mice were then made diabetic by nephrectomy of the islet-bearing kidney (diabetes +) combined with bilateral adrenalectomy (adrenals –) or sham adrenalectomy (adrenals +); others received a sham nephrectomy (diabetes –) combined with bilateral adrenalectomy or sham adrenalectomy as nondiabetic controls. Five days after surgery, serum was collected for corticosterone measurement (*A*), blood glucose was determined (*B*), and thymus cellularity was assessed (*C*). Each dot represents an individual animal in the different groups.

A.3.3.2. βTC-tet cell engraftment in STZ-induced diabetic mice

The diabetes-induced early loss of lymphoid cellularity suggested that diabetes might cause a rapid depression of immunity. We therefore examined whether STZ or the diabetes it induces could inhibit the rejection of an allogeneic graft. The βTC-tet cell is an insulinoma originating from a double Tg C3H (H-2^k) mouse (17). Growth of βTC-tet cells and their secretion of insulin reverse hyperglycemia in diabetic mice and eventually cause hypoglycemia. These cells have been employed in studies suggesting that STZ is an immunosuppressant; βTC-tet cells engrafted in STZ-treated but not normal allogeneic recipients (3, 4, 17). We examined whether the STZ-induced immunosuppression that allows βTC-tet engraftment is also attributable to the diabetic state. We injected βTC-tet cells into STZ-treated (diabetic) B6 mice, either

combined simultaneously with a syngeneic islet transplant or a sham operation. **Figure A.3.4** shows that βTC-tet cells engrafted in all control STZ-treated immunodeficient B6-RAG-KO mice made normoglycemic with a syngeneic islet transplant, and the majority of STZ-treated diabetic B6 mice. Engraftment did not occur in any normal control B6 mice. In those B6 mice cured of their diabetes with an islet transplant, only 1 of 9 allowed engraftment of the βTC-tet cells ($p=0.01$ compared to STZ-treated diabetic B6 mice without an islet transplant). Thus, the immunosuppression in STZ-treated diabetic B6 mice that allows βTC-tet engraftment appears largely attributable to the diabetic state rather than STZ directly.

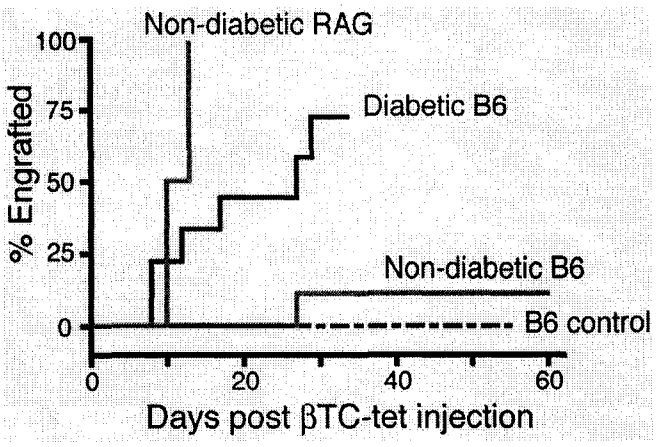

Figure A.3.4. Diabetes allows engraftment of allogeneic βTC-tet cells. βTC-tet cells were injected s.c. into STZ-treated diabetic B6 mice, either combined simultaneously with a syngeneic islet transplant (non-diabetic B6, n=9) or a sham operation (diabetic B6, n=9). Immunodeficient B6-RAG-KO mice treated with STZ and then cured of diabetes by syngeneic islet transplantation (non-diabetic RAG, n=4) and normal B6 mice (B6 control, n=5) were used as controls. The fraction of recipients with tumour engraftment (blood glucose <4 mmol/L) is shown.

A.3.3.3. STZ-induced diabetic immunosuppression blocks immunity to islet allografts; recovery of immunity subsequent to homeostatic T cell proliferation

As we found that immunity to allogeneic insulinoma cells could be reduced by diabetes-induced immunosuppression, we wished to test if islet graft survival could be similarly affected. Experimental islet transplantation is most often performed in the STZ-induced diabetic mouse model and allogeneic islets are usually rejected. However, using BALB/c and B6 mice a small fraction of STZ-induced diabetic recipients have been reported to spontaneously accept fully allogeneic islets (27, 28). We surmised that this acceptance may have been dependent on host immunosuppression triggered by STZ and/or diabetes rather than "spontaneous". To test this possibility we examined another fully allogeneic strain combination in which graft acceptance can be more easily achieved. We gave STZ-induced diabetic CBA/J mice a syngeneic islet transplant and allowed them to remain normoglycemic for 2-3 weeks. Both these

nondiabetic as well as conventional STZ-induced diabetic CBA/J mice (without a syngeneic islet transplant) were challenged with BALB/c islets. The syngeneic islets were removed within 10 days post allogeneic islet transplantation. Surprisingly, we found that the majority of the diabetic CBA/J mice accepted BALB/c islets long-term; however, all nondiabetic mice rejected the BALB/c grafts (**Figure A.3.5**, ***A***). The STZ- or diabetes-induced immunosuppression appeared to be temporary, as delaying the BALB/c islet transplant to 9 days post STZ administration in diabetic mice, largely restored rejection (**Figure A.3.5**, ***A***). The recovery from immunosuppression may have resulted from homeostatic T cell proliferation, as there was a dramatic increase in splenic T cell numbers from day 5 to day 9 post STZ ($p<0.001$) (**Figure A.3.5**, ***B***). To more directly test for homeostatic proliferation we assessed *in vivo* BrdU uptake by lymphocytes in control and STZ-induced diabetic CBA/J mice. B cells had decreased BrdU uptake in diabetic mice (data not shown). In contrast, T cells (both CD4 and CD8) showed a large increase in BrdU incorporation in diabetic mice, and this increase was most apparent in cells with a memory phenotype ($TCR^+CD44^{hi}CD62L^{lo}$) (**Figure A.3.5**, ***C***).

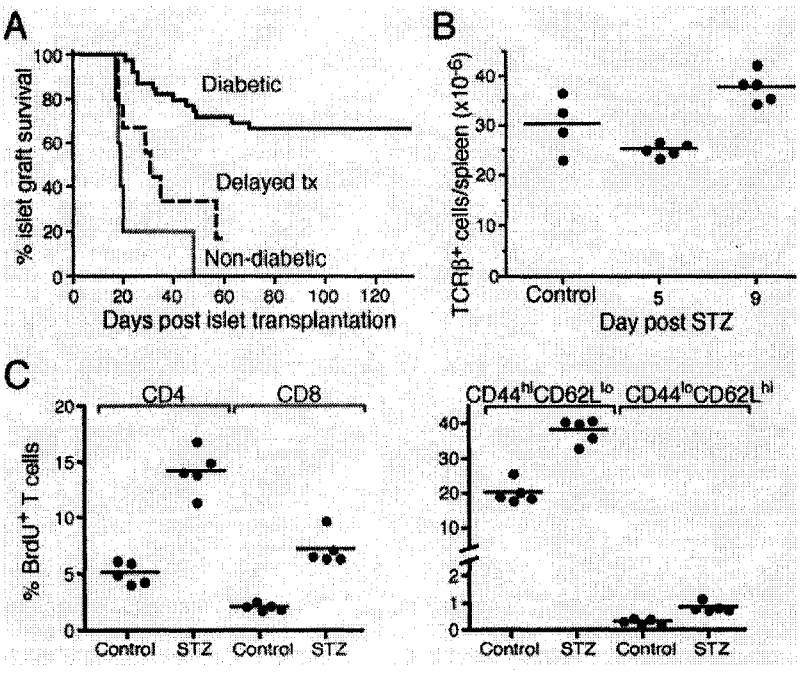

Figure A.3.5. Diabetes-induced immunosuppression leads to islet allograft acceptance while subsequent homeostatic T cell expansion is associated with islet rejection. (*A*) Conventional STZ-induced diabetic CBA/J mice given a BALB/c islet graft 2 days post STZ (Diabetic, solid black line, n=39; 15 were nephrectomized between days 70-76 to confirm graft function) or 9 days post STZ (Delayed tx, dashed black line, n=9) administration were compared to CBA/J mice cured (2 days post STZ) of diabetes by a syngeneic islet transplant (Non-diabetic, solid grey line, n=5) prior to challenge with BALB/c islets. The syngeneic islets were removed 10 days after (n=1, rejection of BALB/c islets at day 21), or on the same day (n=4) as when allogeneic BALB/c islets were transplanted. (*B*) Splenic T cell numbers 5 and 9 days post STZ administration in CBA/J mice as defined by TCRβ expression (as in **Figure A.3.1**, *A*; thymocytes did not increase, data not shown), compared to control mice given acetate buffer. (*C*) Incorporation of BrdU in splenic $TCR\beta^+$ gated cells that were either $CD4^+$ or $CD8^+$ (left; $p<0.0001$ for both subsets) or in $TCR\beta^+$ gated cells that were either of the memory ($CD44^{hi}CD62L^{lo}$; $p<0.0001$) or naïve ($CD44^{lo}CD62L^{hi}$; $p=0.0003$) phenotype (right), in STZ-induced diabetic versus nondiabetic control mice.

We further tested for diabetes-induced immunosuppression using islet grafts mismatched only for the male antigen H-Y. Although both diabetic and nondiabetic recipients accepted the male islets long-term (>120 days), the nondiabetic recipients exhibited stronger anti-donor CTL priming and much more extensive peri-islet infiltration of grafts (**Figure A.3.6**). Together these data indicated that instances of "spontaneous" islet allograft acceptance or weak anti-donor responses can be attributed to STZ- and/or diabetes-induced immunosuppression.

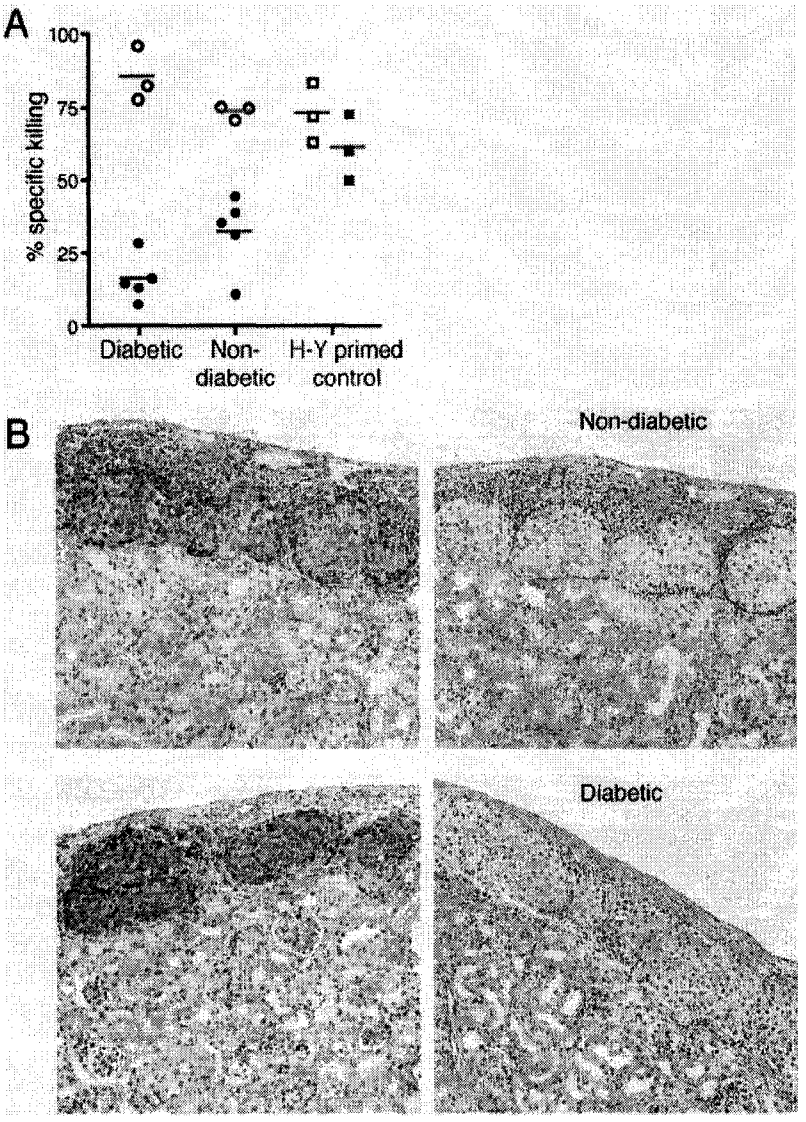

Figure A.3.6. Diabetes-induced immunosuppression weakens anti-donor responses. (*A-C*) Both diabetic and nondiabetic female B6 mice, generated as in **Figure A.3.5** for CBA/J mice, were challenged with B6 male islet grafts. Syngeneic islets (non-diabetic group) were removed the same day as male islets were transplanted. (*A*) Greater than 120 days post male islet challenge anti-H-Y (closed symbols) and anti-third party (C3H, open symbols) CTL responses were assessed; p=0.04 between diabetic versus non-diabetic mice in the H-Y-specific CTL response, and p=0.11 in the third party-specific response. Female B6 mice primed with male spleen cells were used as a positive control (H-Y primed). (*B*) Insulin (brown) and hematoxylin (left) and hematoxylin and eosin (right) staining of representative male islet grafts in diabetic (bottom) and non-diabetic (top) recipients (magnification 200×).

A.3.3.4. Lack of tolerance when "costimulation" targeted therapy is given to nondiabetic recipients

We next examined whether diabetes alone could affect the outcome of islet transplantation, and whether this could be a critical issue for testing islet transplantation tolerance protocols. Using the conventional approach to test for tolerance (*i.e.* diabetic recipients), we have recently shown that antibodies targeting CD40L and ICOS induce tolerance, with both first and second set donor islets being accepted long-term (16). While the roles of CD40L and ICOS have not been fully elucidated, they are important potential therapeutic targets, as they appear to play a critical role in either costimulating lymphocyte responses or controlling the class of response generated. To determine if the tolerance induced was due solely to the regimen of antibodies targeting CD40L and ICOS or required additional immunosuppressive effects of diabetes, we gave second set islet transplants to nondiabetic recipients. STZ-induced diabetic B6 mice received BALB/c islets under the cover of temporary treatment with anti-CD40L and anti-ICOS antibodies. Mice with grafts surviving greater than 100 days were re-challenged with a second donor islet transplant. Extending our previous studies (16), we found that anti-CD40L plus anti-ICOS treated recipients accepted allogeneic islets long-term, and generally accepted a second set donor islet graft long-term when made diabetic by removal of the first islet transplant prior to re-challenge. In contrast, if we left the "tolerant" recipients normoglycemic by removing the first islet graft only after the re-challenge with a second donor islet graft, then the second islet graft was rejected (**Table A.3.1**). Together these data indicate that the diabetic state can greatly influence the outcome of islet transplantation and the ability to assess the level of tolerance obtained.

Table A.3.1. Second set donor islet survival depends on the diabetic state.

First graft removed before (diabetic) or after (nondiabetic) the second graft	Second islet graft survival (days)
Diabetic	$>100 \times 6$[1]
Diabetic	65, >70, >70
Nondiabetic	17×2, 22, 23, 85, >100

[1] Our recently reported data (16) from 6 diabetic animals are shown for comparison.

A.3.4. DISCUSSION

The chronic effects of diabetes on immunity are well recognized; in contrast, the acute effects of diabetes have not been established. Drugs such as STZ that are diabetogenic due to their toxicity to beta cells have been associated with a suppression of immunity. Both STZ and alloxan are glucose analogues that are taken up by pancreatic beta cells specifically due to their expression of the GLUT2 glucose transporter, and are able to induce DNA damage and beta cell death (29). The current view appears to be that STZ directly (2-5), rather than diabetes, is rapidly immunosuppressive. However, chronically diabetic mice, many weeks post STZ administration have also demonstrated some immunosuppression, and this could be reversed with islet transplantation (30); the acute effects of diabetes on immunity were not assessed. In contrast, Nichols *et al.* concluded that STZ is directly immunosuppressive because insulin treatment only partially reversed the suppressed parameters of immunity in STZ-treated mice (7). However, insulin injections do not cure diabetes and full control of blood glucose levels was not evident. We instead assessed the role of acute diabetes versus STZ in immunosuppression by fully ameliorating diabetes with an islet transplant. Our data strongly suggest that most if not all of the rapid immunosuppression post STZ administration can be attributed to the acutely induced diabetic state. Firstly, the most dramatic effect of STZ on immune system cellularity occurs in the thymus (7), and we found this hypocellularity can be completely reversed with islet transplantation. We also found that a similar degree of hypocellularity (thymus and spleen) can be generated by causing diabetes via removal of the islet transplant (**Figure A.3.1** and **Figure A.3.2**). In terms of immune function, our data showed that the previously described reduced ability of STZ-treated mice to reject allogeneic βTC-tet tumour cells (3, 4), or in some cases a whole islet transplant, can be reversed by curing diabetes with syngeneic islets (**Figure A.3.4** and **Figure A.3.5**).

Given the above data we consider that alternative interpretations are likely for other studies supportive of a direct immunosuppressive role for STZ. While direct lymphocyte toxic effects of STZ occur *in vitro* (6) they may not occur *in vivo*. This view is supported by the observation that alloxan, unlike STZ, is not toxic to lymphocytes *in vitro* (6) and yet induces the same decrease in thymocyte and splenocyte numbers *in vivo*. Therefore, the capacity that both STZ and alloxan have in common that is more likely to explain reduced cellularity is the capacity to induce diabetes. While, our data do not rule out a direct lymphocytotoxic effect of STZ *in vivo*, they indicate that indirect effects of STZ on lymphocytes,

via diabetes induction, are substantial. The ability of STZ to suppress autoimmunity and islet allograft rejection in NOD mice that were already diabetic at the time of STZ administration (2, 5) has also been attributed to direct immunosuppressive effects of STZ. However, a more likely explanation for the STZ effects in NOD mice is the alteration in presentation of beta cell antigens (8, 31). Since NOD mice are prone to autoimmune diabetes and the response studied was to the target of autoimmunity (beta cells), one cannot differentiate between the immunosuppressive effects of STZ versus an effect of STZ on altering the presentation of beta cell antigens to which the immune response is directed. This difficulty is also relevant when assessing immunity to islet allografts in NOD mice, as the autoreactive T cells that cause diabetes can contribute to allogeneic islet rejection (32), and alteration of presentation of self beta cell antigens could therefore effect the rejection. STZ can prevent autoimmunity by a mechanism that is dependent on beta cell apoptosis (8) and potentially involves Treg cells (5, 8). Furthermore, Rayat *et al.* have shown that the ability of STZ to block anti-islet immunity in NOD mice can occur simply by treating the islets *in vitro* with STZ, strongly suggesting that STZ is acting on beta cells rather than acting directly on recipient lymphocytes (31). Thus, STZ in the NOD model seems to cause a beta cell antigen-specific regulation, rather than a general immunosuppression. These considerations do not apply to islet cell transplants in nonautoimmune mice, as alloantigens, not beta cell-specific antigens, are the target of the rejection response in such animals.

One of the most important implications of the finding that diabetes causes a rapid depression in immunity capable of inhibiting islet rejection relates to the evaluation of new immunosuppressive drugs and tolerance strategies for islet transplantation. In the conventional method of testing new islet transplant tolerance protocols the recipient is acutely diabetic at both the induction phase and the test for tolerance (33-43). The general idea is to give a tolerance inducing treatment temporarily after the first islet transplant and then challenge with a second donor transplant in the "absence of any immunosuppression". However, our data indicate that simply removing the first islet transplant will lead to some immunosuppression during challenge with the second islet transplant (**Table A.3.1**). Thus, the efficacy with which treatment regimens allow acceptance of either first or second donor islet transplants may be significantly overestimated given that the immunosuppressive effects of diabetes are not taken into account. We therefore propose that future tests of tolerance protocols for islets be done both by the above standard method and additionally verified

in nondiabetic recipients. The frequent success in generating acceptance or even tolerance of donor islets but not skin grafts (36, 40, 44-46) may in some instances be due to the absence of diabetes-related immunosuppression in skin graft tests rather than any inherent differences in the immunogenicity of these tissues. Similarly, the tolerance strategy of APC depletion of allografts may have been more successful with islets than other tissue grafts because of diabetes-induced immunosuppression of recipients.

One of the surprising findings in these studies was the ability of STZ to prevent rejection of fully allogeneic BALB/c islets by CBA/J mice (**Figure A.3.5**). However, even in strain combinations considered more difficult in terms of achieving tolerance, there has been some precedent for a low frequency of "spontaneous" islet acceptance, which we now suggest was due to unrecognized diabetes-related immunosuppression (27, 28). In terms of BALB/c islet acceptance in CBA/J mice, in early studies the islets were not given until 2 weeks post STZ treatment (47). In more recent studies in this strain combination, it was not clearly defined how many days after STZ administration islets were transplanted (28, 41, 48). This is an important issue because we found that the ability of STZ-treated CBA/J mice to accept BALB/c islets is greatly decreased if the transplant is given 9 days instead of 2 days after STZ. The loss of immunosuppression over time was associated with significant homeostatic T cell proliferation, and greatly increased numbers of splenic T cells from day 5 to day 9 post STZ administration. Since homeostatic T cell proliferation may block tolerance (49), STZ and/or diabetes may produce the two competing effects of immunosuppression and resistance to tolerance induction. Which effect predominates may be determined by the timing of transplantation in relation to diabetes induction. Diabetes-induced homeostatic proliferation raises the possibility that any intrinsic predisposition for lymphopenia and homeostatic proliferation in the NOD autoimmune background (50) may be exacerbated with the onset of diabetes.

Mechanistically it is not yet clear what aspect(s) of acute diabetes leads to the immunosuppression that we have observed. Both reduced insulin and hyperglycemia could contribute as *in vitro* studies suggest that insulin can promote T cell growth/division (51, 52), while high glucose levels have the opposite effect *in vitro* (52) and *in vivo* (53). Furthermore, activated but not resting T cells express insulin receptors and act as an insulin sensitive tissue (54-58). However, acute diabetes also induces adrenal gland hypertrophy and increased corticosterone levels (23, 24, 59). Glucocorticoids are known to have a similar effect on

thymus cellularity (26) as we have described here for the acute diabetic state. Our data suggest that an absence of corticosterone production prevents diabetes-induced thymus hypocellularity (**Figure A.3.3**), although we do not yet know whether corticosterone is solely responsible for reduced immunity in acutely diabetic mice. However, glucocorticoids may skew T cell responses towards the Th2/Th3 type (60), and could explain the long-term acceptance of allogeneic islets that we observed. Further studies should be directed at testing the role of corticosterone or other potential mediators (*e.g.* opioid receptors) (61) in acute diabetic immunosuppression.

A.3.5. AUTHOR CONTRIBUTIONS

The following data were contributed by BL alone, who designed and performed experiments, analyzed data and wrote the paper (Figure A.4.1, *A*; Figure A.4.2, *C*; Figure A.4.3; and Figure A.4.6, *B*). The following data were contributed by WFC alone, who designed and performed experiments, analyzed data and wrote the paper (Figure A.4.1, *B* and *C*; Figure A.4.2, *B*; Figure A.4.5, *B* and *C*; and Figure A.4.6, *A*). Data in Figure A.4.2, *A* and Figure A.4.4 were the collaborative efforts of BL and WFC. Data in Figure A.4.5, *A* were generated by BL and SJL, who also analyzed data and revised the manuscript. Data in Table A.4.1 were generated between BL and SAN, who also analyzed data and revised the manuscript. RVR and AMS advised on the design of experiments and revised the manuscript. CCA designed the study, analyzed data and wrote the paper.

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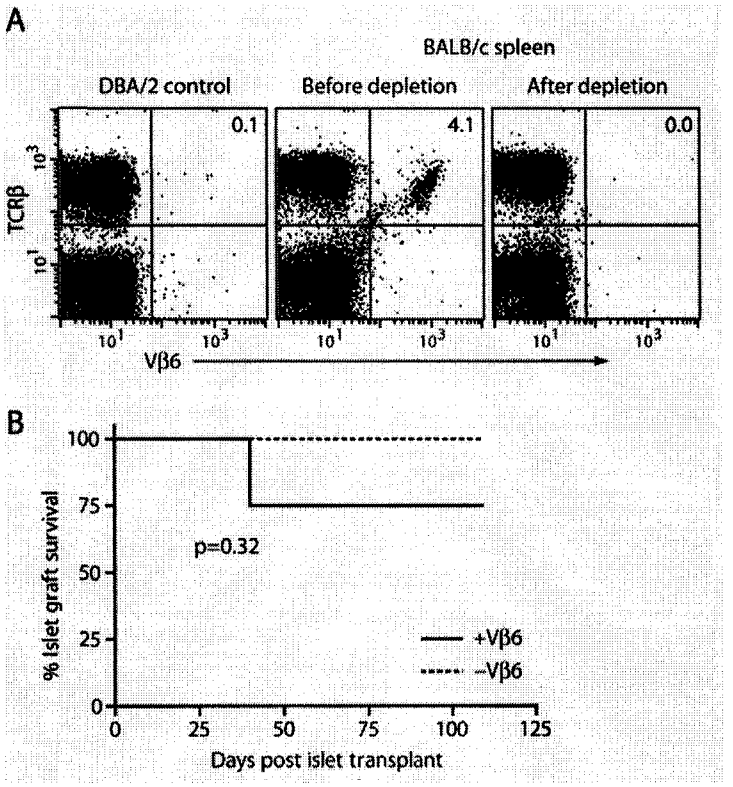
A.4. TESTING THE ROLE OF BALB/c Vβ6$^+$ T CELLS IN DBA/2 ISLET REJECTION



(*A*) Spleens from WT BALB/c donor mice were harvested and their cells depleted of the Vβ6$^+$ population by magnetic sorting. Cells were labelled with a biotinylated anti-Vβ6 antibody followed by a secondary incubation with streptavidin-conjugated magnetic microbeads. Negative selection was performed on a MACS® LD column. Purity was assessed by staining a cell sample before or after selection with a fluorescently labelled anti-biotin antibody, along with DBA/2 control cells (negative for Vβ6). Plots show the expression of Vβ6 versus TCRβ along with the percentage of Vβ6$^+$ T cells within the lymphoid gate. (*B*) At least 40 × 10^6 purified spleen cells (–Vβ6; n=4) or unmodified spleen cells (+Vβ6; n=4) were injected i.v. into BALB/c-SCID mice bearing DBA/2 islet transplants and graft survival was monitored.

A.5. TESTING DIFFERENT CHIMERISM INDUCTION PROTOCOLS IN NOD MICE

(All unpublished data shown were generated by B. Luo.)

Table A.5.1. Chimerism induction in NOD mice by different protocols.

	Number of stable mixed chimeras (>20 weeks) generated	
Irradiation-based protocol[1]	**B6**	**NOD**
TBI 3 Gy, MR1, SRL[2]	7/7	0/3[3]
Irradiation-free protocol[1]	**B6**	**NOD**
ALS, BUS[4]	NT[5]	0/4
ALS, BUS, CYP[6]	NT	0/5
ALS, BUS, CYP, SRL	NT	0/5
BUS, MR1	NT	0/5
BUS, MR1, SRL	5/5	0/3[7]

[1] BALB/c donors.
[2] TBI, total body irradiation; Gy, Gray; MR1, anti-CD40L antibody; SRL, sirolimus.
[3] Mixed chimerism induced but lost by 10 wks.
[4] ALS, anti-lymphocyte serum; BUS, busulfan.
[5] Not tested.
[6] CYP, cyclophosphamide.
[7] Mixed chimerism induced but lost by 16 weeks.