Developing clinically translatable chimerism induction protocols in a stringent murine model

by

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Abstract

Allogeneic hematopoietic chimerism induction *via* bone marrow transplantation (BMT) is thus far the most robust method for inducing donor antigen specific tolerance to allogeneic grafts. However, limited by the toxicity of the reagents used in conditioning the recipients, the demand of a high dose hematopoietic stem cells, and the risk of graft *versus* host disease (GVHD), the current conditioning protocols cannot be applied for islet transplantation in patients with brittle type 1 diabetes. We aimed to generate clinical translational chimerism induction protocols for these recipients by using non-obese diabetic (NOD) mice, a mouse model for type 1 diabetes, which is also a stringent model for testing chimerism induction conditioning protocols.

Our lab has previously recognized that recipient T cells in the NOD mice were responsible for the split tolerance after induction of mixed hematopoietic chimerism. Here, we overcame the resistance to tolerance induction and the requirement of toxic reagents and a high dose bone marrow cells in chimerism induction in NOD mice with a maximized T cell depletion approach. This protocol led to full chimerism and tolerance to fully allogeneic donor skin in NOD mice. We further tested this protocol in spontaneously diabetic NOD mice, which have previously been shown to be challenging recipients for chimerism induction. We found that the enhanced resistance to chimerism induction in spontaneously diabetic NOD mice was confounded by age. We showed that a modified protocol with infusion of donor $CD8\alpha^+$ cells generated full chimerism and tolerance to fully allogeneic islets in autoimmune diabetic NOD mice. Lastly, we tried to generate chimerism in recipients that had been

immunized with donor cells. We overcame the high lightened anti-donor immune response in these primed recipients with enzymes that block functions of mouse IgG.

This work has generated a protocol that is potentially translatable for clinical islet transplantation. We also identified that aging has an important role in chimerism induction in NOD mice. Our work on IgG blockade in presensitized recipients supports the further application of this new treatment to bone marrow transplantation in patients with pre-existing antibodies towards donor antigens.

Preface

This thesis entitled 'Developing clinically translatable chimerism induction protocols in a stringent murine model' is an original work by Jiaxin Lin. This research project received research ethics approval from the University of Alberta Research Ethics Board, Project title 'Split tolerance and the NOD mouse', AUP00000370.

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Chapter 4 is in preparation for publication (Jiaxin Lin, Rena L. Pawlick, Louis Boon, A.M. James Shapiro, Colin C. Anderson. A T cell depletion conditioning protocol with donor CD8α cell infusion overcomes age-dependent resistance to chimerism allowing tolerance to fully allogeneic islets in autoimmune diabetic nod mice). J.L. designed and performed experiments, analyzed data, and wrote the manuscript; R.L.P. performed islet isolation and transplantation and survival nephrectomy, and edited the manuscript; L.B. and A.M.J.S.

generated reagents and edited the manuscript; C.C.A. designed experiments, analyzed data, and wrote the manuscript.

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List of abbreviations

AID	Activation-induced deaminase
Aire	Autoimmune regulator
ALS	Anti-lymphocyte serum
APC	Antigen presenting cell
ATG	Anti-thymocyte Globulin(s)
ATS	Anti-thymocyte serum
BCR	B cell receptor(s)
BM	Bone marrow
BMC	Bone marrow cell(s)
BMT	Bone marrow transplantation
BUS	Busulfan
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
ChgA	Chromogranin A
CSF	Colony stimulating factor
cTEC	Cortical thymic epithelial cell
CTLA	Cytotoxic T-lymphocyte–associated antigen
CTV	Cell trace violet
CyBor	Cyclophosphamide and bortezomib
CYP	Cyclophosphamide
DC	Dendritic cell
DN	Double-negative
DSA	Donor antigen specific antibody (or -ies)
DST	Donor specific transfusion
EndoS	Endoglycosidase of *S. pyogenes*
Fc	Fragment crystallizing
FcγR	Fcγ receptor(s)
FcR	Fc receptor(s)
FOXP3	Forkhead box P3
GAD	Glutamate decarboxylase
G-CSF	Granulocyte colony stimulating factor
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GLP	Glucagon-like peptide
GVHD	Graft *versus* host disease
HDAC	Histone deacetylase
HIPs	Hybrid insulin peptides
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
i.p.	intraperitoneally

i.v.	intravenously
IA-2	Islet antigen-2
ICOS	Inducible T-cell costimulator
Idd	Insulin-dependent diabetes
IdeS	Immunoglobulin G-degrading enzyme of *S. pyogenes*
IEL	Intraepithelial lymphocyte
IFN	Interferon
IGRP	Islet-glucose-6-phosphatase catalytic subunit-related protein
IL	Interleukin
INS	Insulin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IVIG	Intravenous immune globulin(s)
LFA	Leukocyte functional antigen
LPS	Lipopolysaccharide
LSF	Lisofylline
mAb	Monoclonal antibody(or-ies)
MAIT	Mucosal-associated invariant T
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MiHA	Minor histocompatibility antigens
mTEC	Medullary thymic epithelial cell
NK	Natural killer
NKT	Natural killer T
NOD	Non-obese diabetic
OD	Optical density
OKT3	Ortho Kung T3
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PD	Programmed death
PD-L	Programmed cell death ligand
pMHC	Peptide-MHC
PRR	Pattern recognition receptor
PTPN	Protein tyrosine phosphatase non-receptor
RAG	Recombination-activating gene
RTE	Recent thymic emigrant
SEB	Staphylococcal enterotoxin B
SIRPα	Signal regulatory protein α
SLE	Systemic lupus erythematosus
STZ	Streptozotocin

T1D	Type 1 diabetes
TBI	Total body irradiation
TCD	T cell depleting/depletion
TCR	T cell receptor(s)
TDT	Terminal deoxynucleotidyl transferase
TGF	Transforming growth factor
TI	Thymic irradiation
TLR	Toll-like receptor(s)
TRA	Tissue restricted antigen
Treg	Regulatory T cell

Chapter 1

Introduction

1.1 Transplantation, rejection and immunosuppression

1.1.1 Current status of organ transplantation

Transplantation of tissues or solid organs is a lifesaving procedure for patients with failure of major organs. With the advances of surgical technologies, the development of better medications, and the improvement of clinical management programs, the short term and long term outcomes of grafts have been greatly improved. As such, transplantation has become increasingly common. About fourteen thousand transplantation procedures were reported worldwide in 2016, according to WHO.[1] In Canada, Canadian Organ Replacement Register estimated close to three thousand transplant surgeries were performed in 2017.[2] While the numbers of both transplantation and organ donation have stably increased in the past several decades, there has been a widening gap between the demand and supply of organs, which is partially due to the increase in prevalence of end-stage heart, kidney and liver diseases.[2] This organ shortage leads to a steady rise in the number of eligible patients on the transplant waitlist as well as an increase of average waiting time for grafts. Unfortunately, a significant proportion of qualified patients even die while waiting for a transplant.[3] In addition to the limited organ source, another limitation for transplantation is rejection after organ replacement. The loss of grafts increases the demand for retransplantation. Such rejection of donor organs is mainly a result of host destructive allospecific immune responses towards the allografts.

1.1.2 The role of alloantigens in graft rejection

Alloantigens within the grafts are divided into antigens from allogeneic major histocompatibility complex (MHC) and minor histocompatibility antigens (MiHA). MHC was first found to be important for alloantibody mediated acute rejection of allografts in 1948 by Peter Gorer and Nobel prize winner George Snell.[4] Later, in 1956, the first human MHC, also known as human leukocyte antigen (HLA), was found by Jean Dausset, who shared the Nobel Prize in Physiology or Medicine in 1980.[5] MHC is extraordinarily polymorphic and plays a critical role in the immune system. The degree of MHC allele mismatches between the patient and donor have been positively and strongly correlated with the chance of rejection after allogeneic organ transplantation. Vice versa, the level of matches between recipient and donor MHC are positively associated with improved graft survival and the possibility of reducing immunosuppression.[6-8] However, due to the presence of homozygosity for a given MHC locus, the extent of MHC mismatches and matching cannot be used interchangeably. MiHA, on the other hand, are antigens derived from non-MHC donor proteins that are presented on recipient type MHC. MiHA can then be recognized by the recipient T cells. Although the alloimmunity towards MiHA is not as strong as towards MHC, it is also not a minor immune response. Grafts from MHC identical but MiHA mismatched donors can be acutely rejected by recipients. Today, more than 50 MiHA have been identified and the list of MiHA keeps growing. [9-11]

1.1.3 The role of allorecognition pathways in graft rejection

During the process of allograft rejection, alloreactive T cells are central players in the process of organ rejection with the involvement of other immune cell types, alloantigen specific antibodies and complement. It is estimated that about one to ten percent of T cells recognize a specific allogeneic MHC haplotype.[12,13] Therefore, in theory, the frequency of alloreactive T cells can be much higher, depending on the number of MHC mismatches between donor and recipient. These alloreactive T cells can recognize donor MHC molecules that are loaded with self-peptides or donor MHC proteins regardless of the presented peptides.[14]

The initiation of T cell recognition of the alloantigens is currently classified as direct, indirect and semi-direct recognition.[15] In the setting of direct recognition, it is believed that donor antigen presenting cells (APCs) migrate to recipient secondary lymphoid tissues after transplantation and present donor peptides on donor MHC to host T cells. This 'passenger leucocyte theory' is mainly supported by the fact that depletion of donor leucocytes prior to transplantation resulted in great improvement of short term graft survival.[16] In the case of indirect recognition, recipient APCs pick up and process donor antigens that are derived from donor MHC or non-MHC proteins. Recipient T cells can then be activated by recipient APCs presenting donor antigens with self MHC. It is believed that chronic allograft rejection is mainly attributed to indirect rejection.[15] Last but not least, the semi-direct pathway refers to the acquisition and presentation of intact donor peptide/donor MHC by recipient APCs without an intracellular antigen processing process. Recipient T cells can then be activated

by these ‘cross-dressed’ recipient APCs. Recently, groups of Morelli and Benichou discovered that this ‘cross-dressing’ is mediated by donor-derived exosomes.[17,18] These two studies suggested that the donor APC derived exosomes and ‘cross-dressed’ recipient APCs play an important role in initiating an alloimmune response.

1.1.4 Autoimmunity plays a role in organ rejection

Besides pathogenic alloimmune response, autoimmunity can also be involved in the rejection of an allograft. In some patients, the failure of organs is due to inappropriate immunological destruction of their own cells. Examples include autoimmune hepatitis, systemic lupus erythematosus (SLE), and type 1 diabetes (T1D). The recurrence of pre-existing autoimmunity may also contribute to the deterioration of grafts.[19] On the other hand, even in recipients that do not suffer autoimmune disorders, de novo autoimmunity towards self-antigens can occur after transplantation of certain types of organs. The best-studied example is the appearance of autoreactive antibodies after allogeneic lung transplantation.[20]

1.1.5 Chronic immunosuppression is required after transplantation but with undesired consequences

To improve graft survival and reduce the incidence of rejection, organ/tissue recipients are managed with life-long immunosuppressants. Immunosuppression can be achieved by depleting or inhibiting the generation of lymphocytes, precluding migration and blocking the activation of immune cells.[21] With the development of new immunosuppression

combinations and strategies, acute rejection episodes are largely reduced. However, while chronic immunosuppression is maintained after transplant, chronic damage to allografts remains a huge challenge in the transplantation field.[22] Furthermore, the long term inhibition of the immune system increases the chance of opportunistic infections and malignancies.[23,24] In addition to the immune system dysregulation, function of other organ systems can be affected by the non-immune toxicity of these medications as well. All of these adverse effects of immunosuppressants can negatively impact the quality of life and more importantly may also lead to the deterioration of graft function. Therefore, rather than achieving impeccable management of immunosuppressants for the purpose of minimizing the detrimental side effects, the infinite acceptance of allografts without the chronic immunosuppression in the absence of rejection episodes is desired.

1.1.6 Allograft acceptance without chronic immunosuppression is possible if immunological tolerance to the donor is achieved

The spontaneous graft acceptance after cessation of immunosuppression is achievable in some individuals, which is often referred as clinical operational tolerance. Such tolerance is observed in the cases of kidney and more commonly liver transplant after weaning from immunosuppression.[25] It is reported that about 20-30% adult and up to 60% pediatric liver recipients have a successful withdrawal of immunosuppressants.[26,27] However, this phenomenon seems to be organ-specific, as it is rarely seen after transplantation of other

organ types except for kidney and liver. The mechanisms underlying operational tolerance are not clear. It is thought to be multifaceted.[26,27]

From a clinical practice perspective, there is currently a lack of consensus on the selection criteria of candidates and optimal time points for immunosuppressant withdrawal studies. Without further manipulation of immune system to achieve donor-specific tolerance, ceasing immunosuppression too early may increase the risk of acute cellular rejection. As the term 'clinical operational tolerance' is defined based on the lack of rejection episodes during clinical practice, it does not fully reflect immunological tolerance. The destructive alloimmunity towards grafts can still occur in the absence of rejection episodes. One good example of the presence of alloimmunity is the de novo generation of pathogenic donor-specific antibodies that is highly associated with the cessation of immunosuppression in recipients of liver and kidney transplants.[28,29] Therefore, achieving immunological tolerance to the donor graft is the holy grail in the field of transplantation immunology.

1.2 A brief survey of the immune system and immunological tolerance

In order to manipulate the immune system to acquire immune tolerance to donor antigens, we need to know how immunological tolerance to self is achieved in a healthy individual. The immune system is an extraordinary self-defense system that protects living organisms from invasion by other organisms. The fundamental question in immunology is how the immune system discriminates self from non-self. The immune system in mammals comprises a complex network of cellular and humoral components. According to the

characteristics of the involved cells, the diversity of antigen-specific receptors on these cells, and the presence of immune memory, the immune system can be divided into innate immunity and adaptive immunity.

1.2.1 Self-nonself discrimination in the innate immune system

It is now known that the innate and adaptive immune system achieve self-nonself discrimination differently. The innate defense mechanisms are thought to be evolved from our non-vertebrate ancestors and are conserved in the history of evolution and germline selected to respond to non-self but not healthy self components. The innate immune cells possess germline coded receptors, namely pattern recognition receptors (PRRs) that recognize common structures of microbes, termed pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include proteins, such as bacterial flagellins, nucleic acids, such as viral RNA, and glycans, such as bacterial lipopolysaccharide (LPS). As these PAMPs are not normally present in the host, the self-non-self discrimination mediated by innate immune cells mainly employs nonself recognition.

While some of the innate immune cells are designed not to see self-antigens, it is not the case for natural killer (NK) cells. The unresponsiveness of NK cells to self requires an education process. During the development of NK cells in the bone marrow (BM), NK cells are selected to recognize all self-MHC-I and only those that express the appropriate inhibitory receptors, i.e. the Ly49 receptors, are licensed and become functionally competent NK cells. Nonetheless, each individual NK cell appears to be licensed by different MHC alleles,

because in the 'hybrid resistance' scenario, the NK cells from F1 hybrid animal could reject bone marrow cells (BMCs) from either homozygous parent. This process is referred to as the NK cell "licensing" hypothesis, proposed by Kim and Yokoyama.[30] Therefore, tumor cells or infected cells would be attacked by NK cells due to the down-regulation of MHC-I. NK cells not only sense 'missing self', but also stress-inducible ligands on target cells. The expression of ligands for the activation receptor NKG2D on stressed or transformed cells can overcome the inhibiting signals from MHC-I specific receptors.[31]

Besides NK cells, it is believed that macrophages are also capable of discriminating self-nonself *via* the interaction between signal regulatory protein α (SIRPα) and CD47.[32] Lakkis' group showed that monocytes were also able to exert innate allorecognition *via* the binding of SIRPα and CD47 in the absence of host T and NK cells. The mismatch of the SIRPα between donor and recipient can trigger infiltration of recipient monocytes into donor grafts.[33] However, how monocytes and macrophages achieve self-tolerance and the role of SIRPα-CD47 system in allograft rejection requires further exploration.

1.2.2 Generation of diversified antigen receptors in the adaptive immune system

While the innate immunity can mount strong and fast response towards a broad spectrum of pathogens, pathogens evolve various mechanisms to escape such recognition. With a limited diversity of receptors, innate immunity cannot match the increasing threat from microbiomes. This problem can be well solved by adaptive immunity. Adaptive immune cells,

including T and B lymphocytes, possess highly diversified receptors, e.g. T cell receptors (TCRs) and B cell receptors (BCRs).

The diversified receptor repertoires of T and B cells are generated through several mechanisms. The random recombination between *Variable* (V), *Diversity* (D), and *Joining* (J) gene segments of BCR and TCR initiated by the *recombination-activating gene* (RAG) coded RAG1/2 complex is a fundamental process in the generation of the receptor diversity.[34] The terminal deoxynucleotidyl transferase (TDT) mediated deletion or addition of nucleotides at the ends of V(D)J gene segments during the recombination process further increases the diversity of variable regions.[35] Finally, the pairing between heavy and light chain for BCR, and two subunits for TCR heterodimers also contributes to the level of diversity. For B cells, the diversity of BCR is even increased, as the successfully rearranged BCR genes can further undergo somatic hypermutation triggered by activation-induced deaminase (AID).[36] The highly diversified TCR or BCR enable T and B cells to recognize an enormous diversity of antigens. The expression of a unique receptor on individual T or B cell defines the unique specificity of each T or B cell clone.

While adaptive immunity prevents the escape of pathogens from recognition, some of the adaptive immune cells are unavoidably recognizing self-antigens. Hence, to avoid autoimmunity, tolerance to self must be achieved in the adaptive immune system. Immunological tolerance refers to the unresponsiveness of the immune system towards an otherwise immunogenic substance in a given organism, while the immune system remains able to mount sufficient responses to other antigens. Two mechanisms, i.e. central and

peripheral tolerance mechanisms, are employed in the development and tolerance of T and B cells for deleting or keeping auto-reactive cells in-check.

1.2.3 T cell central tolerance

According to the composition of TCR, antigen recognition patterns, development and activation requirements, T cells can be divided into conventional αβ-T cells and unconventional T cells. Non-conventional T cells include γδ-T cells, natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells and intraepithelial lymphocytes (IELs).[37] As unconventional T cells are armed with low diversity TCR and less likely to respond to self-antigens, studies on T cell self-tolerance mechanisms are mainly focused on conventional αβ-T cells.

Central tolerance of conventional T cells occurs in the thymus during the process of T cell development.[38] Only precursor cells with successful TCR gene rearrangement will proceed further through education processes, which are positive and negative selection. To ensure a maximal representation of 'immunological self' in the thymus, self-antigens are mainly presented in three ways. First, the ubiquitously expressed housekeeping antigens are constitutively presented by cortical thymic epithelial cells (cTECs). Second, tissue-restricted antigens (TRAs) are presented by medullary thymic epithelial cells (mTECs) under the regulation of transcriptional regulators, like autoimmune regulator (Aire). Lastly, dendritic cells (DCs) and B cells can carry peripheral antigens to the thymus and present them to developing T cells. Thymic DCs can also cross-present mTEC-derived TRA.[39]

The fate of the developing αβ-T cells depends on the affinity of the interaction between TCR and self-peptide-MHC (self-pMHC) interaction.[39,40] Inadequate signals from TCR during positive selection lead to the death of immature T cells by neglect. The positive selection, therefore, ensures the surviving T cells are equipped with TCR that can at least weakly interact with self-pMHC complex on the surface of TECs. It is believed that the presence of such weak pMHC-TCR contacts in the periphery provides T cells tonic signals, which appear important for T cell homeostasis.[41,42]

The surviving T cells also undergo negative selection. In this process, high-affinity interaction between TCR on T cells and self-pMHC displayed on TECs and thymic DCs causes activation-induced cell death of developing T cells, which is known as clonal deletion. Besides clonal deletion, anergy also plays a role in thymic negative selection.[38] Only TCR signals above the threshold of positive selection but below that of negative selection induce survival and further differentiation of immature T cells. Eventually, the majority of the αβ-T cells become CD4 or CD8 single-positive conventional T cells depending on their MHC-I or MHC-II restriction. Some T cells that possess TCR with relatively high affinity to self-pMHC are diverted to $CD4^+$ Forkhead box P3 $(FOXP3)^+$ regulatory T cell (Treg) lineage in an Aire dependent process.[43]

1.2.4 T cell activation and peripheral tolerance

While the central tolerance mechanism is important for both establishment and maintenance of tolerance to self, autoreactive T cells can still escape from this process and

be exported out of the thymus. An obvious example suggesting the presence of autoreactive cells in the periphery is defects of Tregs, more specifically the *FoxP3* gene, results in fetal autoimmune disorders in Scurfy mice and immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans.[44] Therefore, it is critical to prevent an anti-self response from these cells. Owing to peripheral tolerance mechanisms, self-reactive T cells are deleted or silenced (anergy, exhaustion or regulation) in the periphery.[45]

Though ignorance by keeping the targets of autoreactive cells in immune-privileged sites *via* passive physical barriers is sometimes classified as one of the peripheral tolerance mechanism, this scenario does not fit the definition of immunological tolerance for adaptive immunity.[45] Self-reactive cells can be activated once they see the targets under optimal conditions. Furthermore, the evolutionary benefits of immune-privileged sites are questionable.[46]

To get an understanding of T cell peripheral tolerance, one should know in what conditions T cells are activated or what is required to activate T cells. Historically, there has been several models explaining T cell activation and peripheral tolerance. The central theme here is whether or not TCR signaling alone activates or tolerizes T cells in the periphery. A study performed by Jenkins and Schwartz in 1987 demonstrated that chemically modified APCs can induce unresponsiveness but not activation of T cells.[47] Their study suggested that rather than activating T cells in the periphery, seeing cognate antigen alone tolerizes T cells, which is now commonly accepted.

The fate of a mature T cell depends on the net sum of signal one from TCR plus signal two. Depending on the sources, signal two can be classified into two types. The first type of signal two to a given T cell is that provided by another cell that also recognizes the same antigen *via* their TCR. This second signal can either 'help' or 'suppress' the activation of a conventional T cell. The requirement of facilitation from a second antigen specific cells adds another layer of restriction for T cell activation. The first two signal model for lymphocyte activation was proposed by Bretscher and Cohn in 1970.[48] By this theory, naive T cells can be helped by effector T cells if the helper and helpee recognize peptides derived from the same antigen on the same APC. Given that the majority of autoreactive T cells are tolerized in the thymus, the achievement of tolerance to these rare naïve self-reactive T cells can be simply due to a lack of help. This model fits well for the activation of $CD8^+$ T cells and B cells, as $CD4^+$ T cells do provide help to both.[49-52] However, it requires extra efforts to explain the generation of the first helper cells.[53] In contrast to 'help' provided by a second cell, the T cell can undergo epitope specific suppression by another T cell. Tregs were discovered later on and they can also be integrated into this model. It is now evident that self-reactive conventional T cells can be suppressed by Tregs, which also recognize the same antigens.[54]

The second type of signal two is provided by the APCs. It was postulated as 'co-stimulation' by Lafferty and Cunningham in 1975, and later was advanced by Janeway in 1989 and Matzinger in 1994.[55-58] In Janeway's models, an APC that is stimulated by PAMPs during infections can selectively co-stimulate its cognate T cells. However, this model cannot

explain allograft rejection of certain sterile internal organs, as no PAMPs are present in neither donor nor recipient.

Respectively, in Matzinger's model, it is the 'danger signals' that trigger APCs to provide co-stimulation signals to its cognate T cells. Common danger molecules are those released by cells that are damaged, stressed or infected. In the 'danger model', instead of distinguishing self-non-self, the immune system does a harmless-danger discrimination. Although the danger theory tried to unify innate and adaptive immunity with its pervasiveness, it does not successfully explain transplantation rejection. A well-healed allograft in an immune deficient mouse was rejected after the reconstitution of the adaptive immunity, suggesting that allograft can be rejected in the absence of danger.[59,60]

Other than co-stimulation, the second signal can also be negative, as a sum of cumulative co-inhibitory and co-stimulatory signals. This idea was postulated by Sinclair in 1971 for B cells and was later modified to explain T cell tolerance.[61-63] In these 'co-stimulation/co-inhibitory' models, if self-antigens are presented in the absence of co-stimulation or in the presence of the dominant co-inhibition under physiological conditions, the activation of self-reactive T cells is prohibited. Ever since the identification of the first co-inhibitor, FcγRIIb, dozens of co-stimulatory and co-inhibitory molecules and their ligands were identified and the list keeps growing.[62,64] It is now clear that each co-signaling molecule and their ligands have their unique structures and expression patterns. The dynamic spatiotemporal regulation of these co-signaling receptors determines the functional outcome of T cells upon TCR engagement.[64]

1.2.5 B cell tolerance mechanisms

B cells are mainly divided into an innate-like B-1 cell and conventional B-2 cell populations. B-1 cells normally reside in peritoneal and pleural cavities. They express a limited diversity of germline-encoded BCRs that are not self-reactive and naturally secrete low levels of antibodies without T cell help.[65] In contrast, the BCRs for B-2 cells are highly diversified. The diversity of B-2 cells can even increase after B cell somatic hypermutation. Similar to T cells, there are central and peripheral tolerance mechanisms for these highly diversified B cells.[66,67]

The central tolerance of immature B cells occurs in the bone marrow. After successful rearrangement of BCR, B cells will undergo apoptosis, if their BCRs interact strongly with membrane-bound self-antigens in the bone marrow microenvironment.[68] Some of these autoreactive B cells can survive if rearrangements of immunoglobulin light chain genes was triggered and BCRs that do interact with autologous targets were successfully expressed. This process refers to receptor editing.[69] B cell clones that recognize soluble self-antigens or interact with membrane-bound self-antigens with low affinity become anergic.[70]

Anergic B cells and B cells that do not respond to self-antigens in the bone marrow survive central tolerance mechanism then enter the periphery. B cells that strongly interact with peripheral self-antigens shortly after exported from bone marrow will undergo clonal deletion.[71,72] In addition to clonal deletion and anergy, clonal ignorance can happen as some immature auto-reactive B cells that fail to encounter their cognate self-antigens can survive both central and peripheral tolerance mechanisms. This could be a result of the rare

expression or inaccessibility of their cognate antigens.[73,74] After exposing to self-antigens in the circulation, surviving immature B cells are positively selected to enter peripheral lymphoid tissues for maturation if their BCR signaling is above the selection threshold.[75] As activation, antibody production and somatic hypermutation of B-2 cells requires help from $CD4^+$ T cells that recognize the same antigens, the self-tolerance of auto-reactive B cells is further ensured if self-tolerance of $CD4^+$ T cells is well established and maintained.[76] The expression of B cell inhibitory receptors, such as CD22, CD72, Siglec-G, and FcγRIIb, are also important in B cell self-tolerance.[77]

1.3 Type 1 diabetes mellitus is an autoimmune disorder

Autoimmune disorders such as T1D, occur when self-tolerance is not properly established or maintained. T1D is an organ specific autoimmune disorder, which results from immune mediated destruction of insulin-producing beta cells in the islets of Langerhans. Besides the reduction of insulin-producing cells, ongoing inflammation also inhibits the function of residual beta cells. Without a sufficient number of functional pancreatic beta cells, there is a lack of insulin to maintain glucose and lipid homeostasis. Poorly controlled blood glucose leads to complications like diabetic ketoacidosis, atherosclerosis, retinopathy, neuropathy and nephropathy and significantly shortens the lifespan of T1D patients.[78,79] It is estimated that in 2017 around 0.7% of Canadians lived with T1D, according to Public Health Agency of Canada (PHAC). For unknown reasons, the incidence of T1D keeps increasing in Canada and other parts of the world.[80,81]

1.3.1 Genetic susceptibility of T1D

Genetic susceptibility is considered the primary risk factor for T1D. The concordance rate of T1D between monozygotic twins (~65%) is about ten times higher than between fraternal twins by the age of 65 years.[82] Moreover, the disease incidence in the first-degree relatives of T1D patients is 15-20 times higher compared to the general population.[83,84] HLA genes were the first genes to be associated with T1D in the 1973.[85] Later on, some HLA class II molecules, including DRB1, DQA1, DQB1, and DPB1, were found to be the main contributors for the development of T1D.[86] Besides MHC genes, more than 40 non-MHC genetic loci, including *insulin* (*INS*), have also been associated with T1D.[87] The majority of these non-MHC T1D susceptibility genes are those involved in immune responses, such as *protein tyrosine phosphatase non-receptor* (*PTPN*)*-2, PTPN-22*, *cytotoxic T-lymphocyte–associated antigen* (*CTLA*) *-4*, *Interleukin* (*IL*)*-21*, *IL-2*, *IL-2R*, and *IL-7R*.[87]

1.3.2 Environmental factors of T1D

Besides genetic susceptibility, environmental factors also play a role in T1D development.[88] Infection was originally suspected to be the etiology of T1D in 1927.[89] It has been proposed that viruses can lead to beta cell infection, activate beta cell specific T cells *via* perturbation of T cell education in the thymus or *via* molecular mimicry.[90] The molecular mimicry hypothesis refers to autoreactive T cells recognizing virus/bacterial antigens, which share significant sequence similarity with self-antigens, that can be activated during infection and react to self-antigen after the initial infection is resolved. To date, several candidates have

been identified, including rotavirus, mumps virus, Coxsackie virus B as well as rubella virus.[91] It has been found that T cells from patients at high risk of T1D can respond to two sequentially similar peptides from Coxsackie virus B and human glutamate decarboxylase (GAD) respectively.[92] More recently, enterovirus infection has been detected in islets of newly diagnosed T1D patients.[93,94] However, there is not yet enough evidence to support any of these viruses as a causative pathogen of disease.

The changes in lifestyle in the western world have been considered as another environmental factor for the prevalence of both autoimmune disorders and allergy. It is postulated in the hygiene hypothesis that such increases of autoimmune dysregulation result from a reduced frequency of infection. The hygiene hypothesis suggests that exposure to some infectious agents helps the immune system to achieve a better determination between harmless and harmful irritants.[95] Similar to this idea, it was later found that gut commensal flora are involved in regulation of the immune system. A lower abundance of gut microbiome was observed in children with high risk of T1D before clinical diagnosis of disease.[96,97]

1.3.3 Immunopathogenesis of human T1D

As most of the immunological changes happen prior to clinical diagnosis, the evidence of T1D immunopathogenesis mainly came from post-mortem investigation of pancreas from patients who died soon after the clinical onset of the disease. The first observation of immune system's involvement in the pathogenesis came from Gepts in 1965. He found that peri- and intra-islet infiltration by mononucleated cells were common in a series of recent-onset

patients.[98] The immune cell subsets in the islets were later on dissected in an autopsy of a young girl with newly onset diabetes in 1985. Majority of the infiltrating cells were found to be T cells with a minor population of macrophages.[99] A more detailed analysis of the recruited immune cells came from a post-mortem study on 29 newly-onset T1D patients. Most of the infiltrating cells were found to be $CD8^+$ T cells with the presence of minor populations of $CD4^+$ T cells, $CD20^+$ B cells, $CD138^+$ plasma cells and $CD68^+$ macrophages. Importantly, the composition of these immune cells was associated with the stages of insulitis and the insulin content in the islets, suggesting a differential role of each immune subset in the progression of insulitis and disease development.[100] Controversially, NK cells have also been found in infiltrated islets with the co-existence of beta cell Coxsackie B4 viral infection.[101]

In parallel to post-mortem studies, other supportive evidence of autoimmunity as the etiology of human T1D came from blood analysis of T1D patients. Circulating antibodies targeting pancreatic islet antigens were first found in T1D patients in 1974, suggesting the humoral autoimmune response.[102] Indirect evidence from T cell and blood biomarker studies also suggest immune dysregulation in T1D patients. Studies of Tregs in the peripheral blood from T1D patients showed phenotypical and functional defects of $CD4^+CD25^+FoxP3^+$ T cells with no alteration in frequency.[103] In line with the impairment of Tregs, autoantibodies against IL-2, which is important for Treg function, were found in T1D patients recently.[104] On the other hand, increase frequencies of IL-17 producing effector T cells and circulating follicular helper T cells were also noticed in T1D patients. In this study, IL-21 producing effector T cells from T1D patients were also higher.[105]

With the understanding of T1D as an autoimmune disease, identifying the antigens that are recognized by autoreactive T and B cells has been a major aim in T1D research. Proinsulin, GAD, islet antigen 2 (IA-2) and islet-glucose-6-phosphatase catalytic subunit-related protein (IGRP), have been shown to be targeted by T cells and antibodies from patients with T1D.[106] Recently, WE14 derived from Chromogranin A (ChgA), a beta cell prohormonal secretory granule protein, was also shown to selectively activate T cells from T1D patients.[107] Moreover, proinsulin peptides can naturally be covalently cross-linked to other peptides, such as WE14 in beta cell secretory granules. These hybrid insulin peptides (HIPs) are antigenic to autoreactive T cells isolated from T1D patients.[108]

1.3.4 Non-obese diabetic mouse as a T1D animal model

While investigation of samples from patients with T1D and epidemiology studies have generated invaluable knowledge for T1D, an animal model that well reflects human T1D would provide a profound and comprehensive understanding of T1D. There are mainly two types of T1D animal models, which are induced or autoimmune diabetic models. The primary animal model for autoimmune diabetes is non-obese diabetic (NOD) mice. NOD mice mirror many facets of human T1D. The most important similarity between NOD mice and human T1D patients is the existence of spontaneous diabetes. Like human T1D, the development of diabetes in NOD mice is accompanied by the autoantibodies and autoreactive T cells targeting beta cell antigens. The pancreas from young NOD mice also exhibit immune cell infiltration in the islets and beta cell loss even before the onset of disease.[109]

On the genetic level, gene mapping for T1D susceptibility loci in NOD mice has revealed more than 50 candidates.[110] Resembling the human T1D risk MHC alleles, MHC-II I-A^{g7} located on *insulin-dependent diabetes 1* (*Idd1*) locus, confers the most of the risk for disease in NOD mice.[111] Other genes such as *IL-2* (on *Idd3*), *IL-21*(on *Idd3*), *CTLA-4* (on *Idd5.1*) and *PTPN-22* (on *Idd18.2*), also contribute to the increased risks of T1D in both human and NOD mice.[110]

Extensive studies on NOD mice have contributed significantly to our understanding of the diabetogenic autoimmunity in T1D.[112] In 1986, it was shown that the transfer of splenocytes from autoimmune diabetic NOD mice into young NOD mice led to an accelerated progression of diabetes of the hosts, suggesting T1D in the NOD mice was mediated by autoreactive cells.[113] In the same year, it was shown that depleting T cells prevented NOD mice from developing disease, indicating that T cells are required for T1D development in NOD mice.[114] Later studies showed that depletion of either $CD4^+$ or $CD8^+$ T cells significantly reduced diabetes incidence in NOD mice.[115,116] Thus, both $CD4^+$ and $CD8^+$ T cells were required for the development of diabetes in NOD mice. To reveal the contribution of $CD4^+$ and $CD8^+$ T cells in the pathogenesis of T1D, purified $CD4^+$ and $CD8^+$ splenocytes from diabetic NOD mice were transferred into different NOD.SCID mice. Only recipients of $CD4^+$ cells from diabetic NOD mice led to overt diabetes, suggesting that splenic autoreactive $CD4^+$ T cells can cause T1D independent of $CD8^+$ T cells.[117] On the other hand, the recruitment and proper function of diabetogenic $CD8^+$ T cells requires assistance of beta cell specific $CD4^+$ T cells. In terms of B cells, NOD.$\mu MT^{-/-}$ mice, which

lack mature B cells, can still become diabetic. However, the prevalence of diabetes is significantly reduced compared to wildtype NOD mice, suggesting an essential role of B cells in T1D pathogenesis.[118] When NOD.μMT$^{-/-}$ mice were reconstituted with bone marrow cells from wildtype NOD mice, the development of diabetes was restored.[119] It was shown that female NOD mice with defective I-A^{g7} expression specifically on B cells were resistant to spontaneous diabetes, suggesting important antigen-presenting properties of B cells in the development of T1D.[120] Resident macrophages in islets have also been shown to participate in diabetogenesis in NOD mice. Depletion of islet resident macrophages with anti-colony stimulating factor (CSF)-1 receptor slowed down the disease progression.[121]

The generation of diabetogenic T cell clones in NOD mice is a result of defects in central and peripheral tolerance. Clonal deletion is one of the T cell central tolerance mechanisms. A superantigen, staphylococcal enterotoxin B (SEB), which strongly binds to MHC-II I-A^{g7} and Vβ8^{+} TCR without specific recognition of peptide, has been used to study clonal deletion in mice. It was shown that SEB induced a significant reduction of Vβ8^{+} CD4 single positive thymocytes in B6.H-2^{g7} mice, but not in NOD mice, suggesting a defect in the deletion mechanism in the thymus.[122] Immature T cells with high affinity to self-antigens were not sufficiently eliminated in the thymus if they were on the NOD background.[123] Thus, the resistance of autoreactive T cells to negative selection, depends on the non-MHC genes. The defect in removal of forbidden clones of T cells was found to be associated with a failure to induce apoptosis of the targeted cells.[124] As for peripheral tolerance, Tregs in NOD mice are critical for keeping autoreactive T cells in check. Depletion of Tregs in NOD mice with

antibodies targeting CD25 accelerated the development of disease.[125] IL-2 is important for the development, homeostasis and function of Tregs. The *IL-2* and *IL-21* genes were linked to the pathogenesis of autoimmune diabetes in NOD, both of which are located in the *Idd3* locus. This is similar to what has been found in T1D patients. It was shown that the susceptibility alleles of *IL-2* led to a twofold reduction of *IL-2* expression in NOD mice.[126] Indeed, Tregs from NOD mice were less suppressive when compared to those from B6.H-2^{g7} mice in *in vitro* T cell suppression assays. However, this was not due to the defects of Tregs in NOD mice but rather the effector T cells' resistance to suppression *via* overproduction of IL-21.[127]

Peripheral double-negative (DN) T cells characterized as TCR$\alpha\beta^{+}CD3^{+}CD4^{-}CD8^{-}$ $NK1.1^{-}$, are another population of suppressive T cells.[128] It has been shown that infusion of NOD splenic DN T cells delayed the development of diabetes in NOD.SCID mice transferred with diabetogenic splenocytes.[129] However, the proportion of DN T cells in the periphery was lower in NOD mice as compared to B10.Br mice.[130] This reduced frequency of DN T cells in NOD mice was associated with their *Idd2* and *Idd13* loci.[131,132]

1.3.5 Reversal of T1D

Despite almost a century of research ever since the discovery of insulin by Banting, Best, Collip, and Macleod, injection of exogenous insulin remains the primary treatment option for T1D.[133] However, glycemic control *via* insulin injection is difficult to achieve. Intensive blood glucose control can effectively reduce the risk of developing microvascular diseases,

but it is associated with the increase of severe hypoglycemia.[134] Although new technologies have been developed to reach a better control of blood glucose, efforts were also made to identify populations at high risk of T1D, develop interventions to prevent, postpone or stop the progression of disease, and lastly reverse diabetes even at its late stage.

Numerous reagents have been tested on NOD mice and have been shown to prevent diabetes in this model. While progression of diabetes seems easily disrupted in NOD mice that are not yet diabetic, only few interventions can reverse overt hyperglycemia in newly-onset diabetic NOD mice.[135-164] Similarly, clinical trials have been launched for prevention of T1D in individuals with high risk for T1D based on the findings from NOD mice. However, an intervention that permanently prevents T1D has not yet found in these clinical studies.[165] In trials for preventing T1D in individuals with high risk of disease, delay of the disease course can be found in studies with anti-CD3 treatment.[165,166] However, reversal of T1D in the long run has not yet been achieved in such populations.[165]

1.3.5.1 Reversal of newly onset diabetes in NOD

To date, only limited strategies have been shown to restore euglycemia in NOD mice with recent-onset diabetes. Here, I reviewed studies that reversed diabetes in new-onset diabetic NOD with blood glucose higher than 250mg/dl before treatments. Knowing the importance of autoreactive T cells in the pathogenesis of T1D, most of these successful approaches employed methods targeting T cells. The first encouraging data was from Chatenoud and Bach, showing that depleting T cells *via* anti-CD3 antibodies allowed long-

term reversal of overt diabetes in NOD mice.[135] However, anti-CD3 mAb did not just deplete T cells, it also led to a transient massive T cell activation and cytokine storm. The cytokine releasing syndrome mediated by the humanized anti-CD3 monoclonal antibodies (mAb), Ortho Kung T3 (OKT3), was also observed, which precludes the use of OKT3 for the treatment of autoimmune diseases. It was then found that the fragment crystallizing (Fc) of the anti-CD3 antibodies binding to Fc receptors (FcR) on APCs was responsible for massive cytokine production, which can be avoided by using a non-FcR binding anti-CD3 mAb.[167] However, Chatenoud and Bach found that the Fc portion of anti-CD3 mAb was required for the optimal effect of this treatment.

To further improve the efficacy of non-FcR-binding CD3 mAbs, anti-CD3 treatment was combined with other treatments. Similar to the murine version of non-FcR-binding CD3 mAbs, it was shown that the administration of Otelixizumab, a humanized non-FcR-binding CD3 antibody, induced remission of diabetes in 50% of NOD.huCD3ε mice, which express the human version of CD3ε. Combining anti-CD3 mAb with oral givinostat, which protects beta cells from proinflammatory cytokine induced apoptosis by inhibiting histone deacetylase (HDAC), improved the reversal rate up to 80%.[164,168] Likewise, exendin-4, a long-acting agonist of glucagon-like peptide (GLP) -1 receptor that enhances insulin secretion of beta cells, was also shown to synergistically improve the effect of anti-CD3 on reversing diabetes in NOD mice.[142] The synergistic effect of exendin-4 and T cell depletion was also shown in another study using anti-lymphocyte serum (ALS).[137]

Other methods have also been tested together with T cell depletion in order to make the interventions more specific for beta cell autoantigens. Combining intranasal proinsulin administration or supplementation of food-grade commensal bacterium that was engineered to secrete proinsulin with anti-CD3 treatment significantly improved efficacy in curing recent-onset diabetic NOD mice.[139,154] Interestingly, this combination of insulin and anti-CD3 treatment increased the frequency of circulation $CD4^{+}FoxP3^{+}$ T cells, which suggests a deletion independent immune modulatory effect of the treatment.[139] In line with these findings, supplementation of T cells with regulatory properties was beneficial when combined with T cell depletion in newly diabetic NOD mice. A combination of anti-thymocyte serum (ATS) and DN T cells, which had been primed with GAD65 *ex vivo*, resulted in an over 80% reversal of diabetes in newly diagnosed NOD mice, as compared to 20% diabetes reversal with ATS alone.[161] In agreement with this observation, Bluestone and colleagues found that reversal of autoimmune diabetes can be achieved *via* injection of antigen pulsed tolerogenic APCs. Insulin-coupled fixed APCs induced long-lasting reversal of diabetes in new-onset diabetic NOD mice without other intervention.[140]

As previously discussed, reduced IL-2 was suspected to be responsible for the impairment of Treg function in NOD mice. It was proposed that supplementation of IL-2 should be able to correct this defect. Indeed, five days injection of low-dose IL-2 right after the confirmation of diabetes restored normal blood glucose in 60% of the diabetic NOD mice within one week. Investigation of the islet infiltrating T cells showed that Treg frequency was significantly increased after a short course of IL-2 treatment. Islet infiltrating Tregs from IL-

2 treated mice also upregulated the expression of CD25, CTLA-4, inducible T-cell costimulator (ICOS), and glucocorticoid-induced tumor necrosis factor receptor (GITR), suggesting an enhancement of Treg function.[149] Cobbold and Waldmann showed that $FoxP3^+$ $CD4^+$ T cells can be induced by blocking TCR co-receptor CD4 in the presence of antigen stimulation.[169] Based on this observation, Tisch and colleagues tried to induce disease remission of spontaneously diabetic NOD mice by blocking both CD4 and CD8. A rapid and long term remission of diabetes was found in almost all new-onset diabetic NOD mice after a two-dose administration of non-depleting antibodies specific for CD4 and CD8. Consistent with Waldmann's finding, the therapeutic effect of CD4 and CD8 blockade in diabetic NOD mice depended on the presence of transforming growth factor (TGF)-β.[156] Akidgel showed that infusion of human cord blood stem cell-modulated autologous Tregs also cured diabetes in autoimmune diabetic NOD mice.[148]

Other than boosting, inducing or transfer of Tregs, naïve and effector T cells are also suitable targets for immunomodulatory therapies. As previously discussed, overproduction of IL-21 was associated with effector T cells' resistance to suppression mediated by Tregs. Boursalian and colleagues showed that neutralizing IL-21 *in vivo* did achieve long term remission of disease in 50% of the new-onset diabetic NOD mice. Furthermore, with the help of liraglutide, a GLP-1 receptor agonist, the reversal of diabetes was achieved in almost all spontaneously diabetic NOD mice.[163] IL-7 is another cytokine that is essential for naïve and effector T cell homeostasis. Blocking IL-7 receptor with antibodies restored normal blood glucose in 50% of the newly onset diabetic NOD mice. It is found that IL-7R blockade

induced programmed death (PD)-1 expression on effector T cells and also increased the frequency of Tregs.[153] Demonstrated by another group of scientists, the protection of IL-7R blockade in diabetic NOD mice was mediated by PD-1, as blocking PD-1 resulted in recurrence of disease.[152] Lastly, continuous administration of FTY720, a sphingosine analog, which inhibits lymphocyte trafficking, or Galectin-1, which induces apoptosis of T cells, have also been shown to restore euglycemia in 50-60% of the diabetic NOD mice.[138,146] Given the essential role of B cells in the development of T1D, targeting B cells has also been shown to be useful in diabetes reversal.[144,158]

Proinflammatory cytokines, such as interferon (IFN)-γ and IL-1β, are key to the pancreatic beta cell dysfunction in autoimmune diabetes.[170] Besides targeting T cells, protection of the residual beta cells from cytokine mediated destruction or inhibition would effectively improve blood glucose control. It was found that IL-1β blockade further improved the efficacy of anti-CD3 treatment in curing new-onset diabetic NOD mice.[150] In line with this, serum IL-1β levels were found higher in newly onset diabetic patients. This increase of IL-1β in these patients was associated with the dysregulation of toll-like receptor (TLR)-4 signaling pathway.[171] In accord with the finding in human T1D, dysregulation of TLR4 expression on bone marrow-derived macrophages was found in NOD mice upon LPS stimulation. Interestingly, LPS induced down-regulation of TLR4 in pre-diabetic NOD, but upregulation in NOD that became diabetic.[172] Therefore, modulating the TLR4 signal is of interest for T1D treatment. Showed by Ridgway and colleagues, agonistic TLR4/MD-2 antibodies led to a cure of diabetes in over 70% of spontaneously diabetic NOD mice without

recurrence of disease for more than 100 days. Mechanistically, this TLR4 antibody treatment was associated with a decreased expression of costimulatory molecules, including CD40, CD80, and CD86, on APCs. Surprisingly, elevation of serum IL-2 and IL-4 alone with an increase of circulating Helios^{+}Nrp-1^{+}Foxp3^{+} Tregs were found after treatment, highlighting the immune modulatory properties of the TLR4 agonist antibodies.[160] In line with this finding, studies aiming at promoting an anti-inflammatory environment has been shown to be promising. Lisofylline (LSF), an anti-inflammatory agent, in combination with exendin-4 restored euglycemia in all NOD mice newly diagnosed with diabetes. All cured mice remained diabetes-free for more than 20 weeks.[141] Lastly, new-onset diabetes can also be cured with tyrosine phosphorylation inhibitors or complete Freund's adjuvant (CFA), which have potent immune modulatory effects.[136,145,147,151,173]

As mentioned above, the autoimmune diabetes in NOD mice is the result of defects in both central and peripheral T cell tolerance mechanisms. Although resetting T cell peripheral tolerance by depleting or suppressing autoreactive T cells can reverse hyperglycemia and autoimmunity, without preventing the de novo generation of diabetogenic T cells *via* correcting T cell education in the thymus the long term therapeutic effects of such approaches remain questionable. Studies on diabetes reversal in newly onset NOD mice *via* allogeneic or genetically edited autologous hematopoietic stem cell transplantation (HSCT) took advantage of both central and peripheral T cell tolerance mechanisms and have generated encouraging data.[143,162] Zeng and colleagues showed that successful engraftment of

allogeneic HSCT with bone marrow cells from an MHC-mismatched and diabetes resistant strain after anti-CD3 treatment reversed diabetes in NOD mice at the time of disease onset.[143]

Programmed cell death ligand (PD-L)-1 is a ligand for the co-inhibitory molecule PD-1. Its expression is broadly found on both hematopoietic and non-hematopoietic cells. The cross-linking of PD-1 and PD-L1 modulates the positive selection of thymocytes and the regulation of T cell activation in the periphery.[174] Thus, the PD-1/PD-L1 pathway can be a target for T1D therapy. Indeed, it was found that the long-lasting diabetes reversing effect after anti-CD3 or insulin-coupled fixed APCs can be abrogated by blocking PD-L1.[140] In line with this finding, Fiorina and colleagues showed that transplantation of genetically engineered NOD hematopoietic stem cells, which overexpressed PD-L1, was able to reverse overt diabetes in new-onset diabetic NOD mice.[162]

1.3.5.2 Reversal of newly onset human T1D

While some early interventions lowered daily insulin doses for 6 to 12 months in patients with new-onset diabetes, many of the clinical trials did not demonstrate therapeutic effects of T1D.[165] To date, the most promising interventions that led to the reversal of hyperglycaemia in patients with early-stage diabetes are administration of anti-CD3 antibodies and autologous HSCT.[165] A short course of Otelixizumab targeting CD3 reduced daily insulin dose over 48 months in younger T1D patients.[175] By using another anti-CD3 antibody, reversal of diabetes was found in a small percentage of patients.[176] The most encouraging data came from autologous HSCT. Oliveira and colleagues showed that all of

the new-onset diabetes patients were free from insulin therapy after autologous HSCT for over 7 months. About half of the autologous hematopoietic stem cell recipients remained insulin-free for over 3.5 years.[177] Interestingly, transplantation of bone marrow cells from young NOD mice that are not yet diabetic into new-onset diabetic NOD mice did not lead to a restoration of normal glycaemia.[178] Thus, more research is needed to confirm the therapeutic effects of this approach.

1.3.5.3 Reversal of late-stage T1D

As T1D cannot be prevented or reversed permanently, a cure for late-stage diabetes is necessary for prevention of chronic complications of diabetes and saving lives of patients with brittle T1D. It has been shown that inducing mixed allogeneic hematopoietic chimerism alone with gastrin and epidermal growth factor that augment beta cell regeneration restored normal blood glucose in NOD mice that had been overtly diabetic for more than two weeks.[155] While this approach appears promising, its clinical application relies on the development of a safe allogeneic HSCT protocol for curing autoimmunity and treatment that stimulates beta cell neogenesis and replication. Moreover, human beta cell regeneration is beyond reach at this point.[179,180]

Alternatively, supplementation with exogenous beta cells *via* either allogeneic pancreatic or islet transplantation can cure late-stage T1D.[181,182] Although recipients regain sustained normoglycemia and are less likely to develop microvascular complications of T1D, there are some drawbacks of both pancreatic and islet transplant. First, due to the complicated

anatomy of the pancreas, pancreatic transplantation remains a very technically challenging procedure with high risks of surgical complications.[183] In contrast to pancreatic transplant, islet transplantation is a less invasive and safe procedure. However, it requires a highly professional and experienced team to acquire sufficient numbers of islets from one donor.[184] Secondly, like most of other allogeneic organ transplantation, chronic administration of immunosuppressants is necessary to prevent rejection. However, chronic immunosuppression is associated with increased risk of infections and malignancy. In addition, some immunosuppressants such as calcineurin inhibitors, are potentially diabetogenic.[184] Furthermore, allogeneic pancreatic or islet grafts can still undergo rejection chronically. Lastly, recurrence of autoimmunity towards beta cells is another concern despite the presence of chronic immunosuppression.[19] Therefore, inducing tolerance to alloantigens and restoration of tolerance to beta cell antigens are both required to obtain a long term graft survival of donor pancreas or islets without immunosuppression.

1.4 Prevention of allo-islet graft rejection *via* tolerance induction

Tolerance induction for alloantigens has been broadly studied for promoting long term acceptance of allografts. Most of the strategies utilize T peripheral tolerance mechanisms, including induction or infusion of immune regulatory cells, such as Tregs and co-stimulation blockade.[185] However, whether or not these treatments can lead to a long-lasting donor specific tolerance remains unknown. For example, the phenotypic stability, antigen specificity and the *in vivo* half-life of the infused Tregs are considered important for the

induction of stable tolerance to allografts.[186] Induction of tolerance to donor organs *via* establishing mixed hematopoietic chimerism is the only approach that employs both central and peripheral tolerance mechanisms. It is believed that this is the most robust method for inducing donor specific tolerance.[187]

Various tolerance induction protocols have been shown to improve survival of allogeneic islets in chemically induced diabetic mice that are not on NOD background. However, the therapeutic efficacy of these protocols are largely compromised when applied to spontaneously diabetic NOD mice.[188-192] Thus, it is believed that NOD mice are resistant to transplantation tolerance induction as compared to non-autoimmune strains. To date, only the establishment of allogeneic hematopoietic chimerism by using HSC from the same islet donor has been shown to induce tolerance to allo-islet grafts in autoimmune NOD mice. Tolerance induction *via* other approaches only led a slight or moderate prolongation of allogeneic islet survival. Of note, NOD mice usually reject allogeneic islets quickly as compared to STZ induced diabetic BALB/c or B6 mice when no other intervention is involved. This accelerated rejection in NOD mice is partially attributed to the pre-existing autoimmunity as preventing rejection of syngenic NOD islets is also difficult.[193-195]

In tolerance induction approaches that did not utilize chimerism induction, costimulation blockade was the first and major one that has been used to promote transplantation tolerance in NOD mice with an allogeneic islet graft. Infusion of donor lymphocytes followed by a course of anti-CD154, the ligand for CD40, prevented rejection of BALB/c islets in streptozotocin (STZ) induced diabetic B6 mice.[188] However, BALB/c

islets were rapidly rejected in spontaneously diabetic NOD mice treated with the same protocol. Moreover, this resistance to tolerance induction *via* co-stimulation blockade in NOD mice was not limited to islets but also skin.[189] The rejection of allogeneic islets can be delayed but not prevented with a higher dose of anti-CD154 mAb or combined anti-CD45RB and CD154 mAbs.[196,197] Similarly, blocking both CD28 and CD154 resulted in indefinite acceptance of allogeneic islets in STZ induced diabetic B6 mice, but did not improve allo-islet graft survival in autoimmune diabetic NOD mice. When costimulation blockade was combined with antibodies blocking common γ-chain, the rejection of allogeneic islets was moderately postponed.[190] Likewise, blockade of ICOS, a CD28-superfamily costimulatory molecule, delayed the rejection of allogeneic islets in chemical induced diabetic B6 mice but not in spontaneously diabetic NOD mice.[192] Lastly, the combination of CD28 blockade with CTLA4-Ig and toxin-conjugated anti-B cell mAbs also prolonged the survival of donor islets in autoimmune NOD mice.[198]

Besides costimulation blockade, resetting the host immune system *via* T cell depletion with anti-TCR or prolonged ATG treatment moderately prolonged allo-islet survival.[199,200] Inducing Tregs with rapamycin and granulocyte colony stimulating factor (G-CSF) after insulin peptide immunization slightly delayed allogeneic graft rejection in autoimmune diabetic NOD mice.[201] Lastly, improved allogeneic islet survival was also observed in a study on FT720 that inhibits T-cell trafficking.[202]

In terms of clinical islet transplantation, anti-leukocyte functional antigen (LFA)-1 antibody efalizumab, which inhibits T cell activation and migration, was shown to prolong

the insulin independent period post islet transplant.[203] Except for trying to widen the insulin-free window post islet transplantation, there are currently no interventions that have been reported to promote an indefinite acceptance of allogeneic islet grafts in human. Furthermore, there are currently no reliable clinical markers that can provide evidence for a safe reduction or withdrawal of immunosuppressants after allogeneic islet transplantation.[204]

1.5 Chimerism induction for inducing transplantation tolerance

As protocols that employed merely peripheral tolerance did not generate long-standing tolerance to donor islets in autoimmune diabetic recipients, procedures that take advantage of both central and peripheral tolerance mechanisms remain the only reasonable approaches for inducing robust tolerance to donor antigens. To achieve central tolerance, constant exposure of immature lymphocytes to donor antigens, including MHC and MiHA, is prerequisite. To date, the generation of mixed hematopoietic chimerism *via* simultaneous tissue and hematopoietic stem cell transplantation is the only approach that meets this requirement. Importantly, the induction of chimerism in T1D recipients does not only lead to tolerance of donor islets, but also reshapes the immune system towards self-tolerance.[205]

The hematopoietic chimerism was first found by Owen in 1945. In this study, dizygotic bovine twins that shared blood supplies by vasculature anastomoses in the womb led to naturally acquired hematopoietic chimerism. In this setting, blood cells from the sibling fraternal twins can be found in the circulation of their twin counterparts. Moreover, this phenomenon persisted into adulthood, suggesting the exchange and successful engraftment

of hematopoietic stem cells from their twin donors during fetal development was associated with robust tolerance to donor antigens.[206] In 1953, Medawar and colleagues showed that the injection of cells from adult mice into the newborns of another murine strain led to acceptance of donor skin.[207] In 1955, Main and Prehn went on and tested this method in adult recipients. They showed that tolerance to (BALB/c × DBA/2) F1 skin in DBA/2 mice can be induced by successful engraftment of donor bone marrow in irradiated DBA/2 recipients.[208]

According to the sustained levels of donor chimerism, chimerism can be classified as full donor chimerism, mixed chimerism, microchimerism and transient chimerism.[209] To date, inducing tolerance to donor kidney *via* chimerism induction has been tried in three clinical centers on limited numbers of patients. Each protocol has its own successes and drawbacks, especially when considered for application to islet transplantation. Scandling and Strober from the Stanford group showed that sustainable mixed chimerism was achieved with total lymphoid irradiation and ATG in patients that received kidney and stem cells from HLA-matched donors.[210] However, the same protocol did not lead to successful engraftment of donor BM when tried on HLA-mismatched donor recipient combinations. Transient chimerism was induced in the patients given HLA-mismatched BM by using the Stanford protocol. Withdrawal of immunosuppressants was tried in two recipients, but failed.[211] As islets are only currently available from cadaver donors, matching for all HLA between donor and recipient is very difficult. Leventhal's and Ildstad's groups in Northwestern University and University of Louisville achieved full chimerism in patients that received HLA-mismatched human kidney and BM transplantation. In the initial report, chimerism was

induced without incidence of graft *versus* host disease (GVHD) and immunosuppressants were successfully withdrawn in these recipients.[212] However, in an eight-year follow-up report, two cases of GVHD were found.[213] The toxicities from irradiation and other chemotherapeutic reagents used in this conditioning protocol may also limit its application in T1D patients. Furthermore, a high dose of HSCs was used in some patients in the Stanford and Northwestern studies, which is not feasible for islet transplantation as only a limited number of HSCs can be isolated from one cadaver donor.[214] Lastly, Cosimi and colleagues from Massachusetts General Hospital showed that transient mixed chimerism in HLA-matched and HLA-mismatched human kidney transplantation also led to operational tolerance of donor grafts.[215] However, the tolerance induced by transient chimerism seems to apply only to kidney and lung, but not heart and islets in non-human primate studies.[216-218]

In summary, the application of these protocols to islet transplantation is limited by the toxicity of conditioning protocols, the risk of life-threatening GVHD, the requirement of some degree of MHC matching, and the need of a high dose of HSCs. Therefore, developing a clinically translatable conditioning protocol for chimerism and tolerance induction for islet transplantation is needed.

1.6 NOD mice as a model for chimerism induction

Numerous chimerism induction protocols have been published in rodents. However, only the above protocols have been successfully translated into human studies.[209] One of the reasons for the low translation rate is that these protocols were not developed in stringent

models. NOD mice are a good model for testing the efficacy of chimerism induction conditioning protocols due to their generalized resistance to tolerance induction.[219-221] Successful protocols that established chimerism in murine strains that are not on NOD background often are not successful in NOD mice.[222]

Several components are required for a successful conditioning protocol, including immune modulation and creation of space for donor HSCs. Methods that inhibit the host immune system in the induction period are needed to prevent early rejection and allow the engraftment of donor HSCs. Several approaches have been used for this purpose, including T cell depletion, co-stimulatory blockade, and conventional immunosuppressants. As previously mentioned, T cell depletion *via* anti-CD3 treatment, ALS or ATG can be safely used in human. Moreover, T cell depletion *via* ALS and ATG can be used for GVHD prevention and treatment.[223,224] Generation of mixed chimerism *via* blocking co-stimulatory pathways has been achieved in many rodent models. CD40-CD40L and CD80/86-CD28 are the two pathways that are often targeted in chimerism induction. While anti-CD40L is potent in inducing tolerance in small animals, the human version of anti-CD40L antibody is thrombogenic and not clinically available.[225] The interaction between Fc portion of anti-CD40L and FcγRIIa on human platelets was found to be responsible for the thrombogenicity of humanized anti-CD40L antibodies.[226] Recently, it was shown that the non-FcR binding anti-CD40L antibody can prolong the survival of allogeneic renal graft in non-human primates without evidence of thromboembolism. It would be interesting to know if the Fc portion of anti-CD40L antibodies is required for the establishment of hematopoietic

chimerism. Blocking CD80/86 with CTLA4-Ig has also been tried for inducing transient chimerism in non-human primates in substitution of CD40L blockade. However, the efficacy of CTLA4-Ig requires further exploration.[227] Lastly, conventional immunosuppressants have also been combined with T cell depletion and/or co-stimulation blockade for chimerism induction. For example, rapamycin, or sirolimus, inhibits T cell activation and proliferation *via* disrupting the IL-2 signal transduction.[21] It has been shown to work synergistically with costimulation blockade in inducing chimerism and foster tolerance.[228] However, the use of rapamycin in chimerism and tolerance induction for islet transplantation is limited by its toxicity to pancreatic beta cells.[229,230]

HSCs are multipotent stem cells that can differentiate into multiple lineages of blood cells. In quiescent state, HSCs are thought to reside in their specific niche.[231] To achieve chimerism, creating space for donor hematopoietic stem cells is required. Recently, Shizuru and colleagues showed the combined blockade of the CD47 and anti-c-Kit, a marker for HSCs, depleted host HSCs, created a niche for donor HSCs and enabled long-term chimerism.[232] Traditionally, irradiation and busulfan, a chemotherapeutic reagent that is toxic to hematopoietic stem cells are often used for creating space for donor HSCs. Both modalities have off-target toxicities. To minimize the toxicity during niche creation, the trend is to develop an irradiation free conditioning protocol by using HSC specific depleting antibodies or minimum doses of myelo-reductive chemotherapeutic reagents.[209] The degree of MHC and MiHA mismatches and the dose of bone marrow cells are also critical for the success of

establishing chimerism. However, the source of donor islets and the number of HSCs are both limited in islet transplantation.

To date, allogeneic chimerism has been achieved in NOD mice by using various conditioning regimens. In general, irradiation[205,233-243], costimulation blockade [222,235,236,239,241,244-249], a high dose of rapamycin [222,244-246,249], or a mega-dose of BMC [143,250] from a fully MHC[143,205,222,234,237,241,246,249-251] or more often partial MHC plus MiHA mismatched donor, are required to overcome the resistance to chimerism induction in NOD mice (Table 1-1).

Table 1-1 Irradiation-free hematopoietic chimerism induction protocols in NOD mice

ref#	Donor	DM	BMC (doses)	Treatment (doses)	Chimeric rate and chimerism level (%)*
244	BALB/c	No	40	Fludarabine (1), CYP (1), ↑αCD40L (5), Rapamycin (9)	6/6, 26%
		No	40	↑DST, fludarabine (1), CYP (1), ↑αCD40L (X5), Rapamycin (9)	7/8, 53%
		Yes	40	↑DST, fludarabine (1), CYP (1), ↑αCD40L (5), Rapamycin (9)	15/15, <30% (7), >75% (8)
250	FVB	No	200	αCD3 (1), DLI 20×10^6 $CD8^+$ T cells (1)	5-10%
		No	200 (2)	αCD3 (1), DLI 20×10^6 $CD8^+$ T cells (2)	90%, 45-70%
		No	200	αCD3 (1), DLI 10×10^6 $CD8^+$ T cells (2)	100%, 40-60%
		No	100	αCD3 (1), DLI 5×10^6 $CD8^+$ T cells (2)	63%, 35-50%
245	BALB/c	No	40	↑DST, CYP (1)	0/4
		No	40	↑DST, CYP (1), Rapamycin (9)	0/7
		No	40	↑DST, CYP (1), ↑αCD40L (5)	5/7, 15%
		No	40	↑DST, CYP (1), ↑αCD40L (5), Rapamycin (9)	11/11, 30%
		No	40	↑DST, CYP (1), ↑αCD40L (5), Rapamycin (9), DLI 10×10^6 splenocytes (2)	9/9, 40%
		Yes	40	↑DST, CYP (1), ↑αCD40L (5), Rapamycin (9)	14/14, 20%
		Yes	40	↑DST, CYP (1), ↑αCD40L (5), Rapamycin (9), DLI 20-30×10^6 splenocytes (2)	14/14, 30%
246	BALB/c	No	40	Fludarabine (1), CYP (1), αCD40L (5), Rapamycin (9)	4/6, 90%
		No	40	Fludarabine (1), CYP (1), ↑αCD40L (5), Rapamycin (9)	5/5, 80%
		Yes	40	Fludarabine (1), CYP (1), αCD40L (5), Rapamycin (9)	0/12
		Yes	40	Fludarabine (1), CYP (1), ↑αCD40L (5), Rapamycin (9)	15/18, 50%

ref#	Donor	DM	BMC (doses)	Treatment (doses)	Chimeric rate and chimerism level (%)
	C3H	No	40	Fludarabine (1), CYP (1), αCD40L (5), Rapamycin (9)	6/6, 95%
		Yes	40	Fludarabine (1), CYP (1), αCD40L (5), Rapamycin (9)	0/6
		Yes	40	Fludarabine (1), CYP (1), ↑αCD40L (5), Rapamycin (9)	1/9, 30%
143	FVB	No	100	αCD3 (1), DLI 100×10^6 CD4TDSPL (2)	8/8, >30%
		Yes	100	αCD3 (1), DLI 100×10^6 CD4TDSPL (2)	4/16, >30%
		No	100	αCD3/CD8(1), DLI 100×10^6 CD4TDSPL (2)	8/8, >30%
		Yes	100	αCD3/CD8(1), DLI 100×10^6 CD4TDSPL (2)	30/31, >30%
222	C3H	No	20	DST(1), αCD40L (7), CTLA4-Ig (7), busulfan (1), ↑rapamycin (28)	26/27, 1-30%
		No	40	DST(1), αCD40L (7), CTLA4-Ig (7), busulfan (1), ↑rapamycin (28)	4/4, 20-40%
	FVB	No	20	DST(1), αCD40L (7), CTLA4-Ig (7), busulfan (1), ↑rapamycin (28)	4/4, <5%
252	B6	No	20	αCD3/CD8 (2), DLI 20×10^6 CD4TDSPL (1)	4/4, 60%
		No	50	αCD3/CD8 (2), DLI 20×10^6 CD4TDSPL (1)	4/4, >99%
249	C3H	No	20	αCD4/CD8/CD40L (1), busulfan (1), ↑rapamycin (28)	20/23, 40-80%
		No	20	DST(1), αNK (3), αCD40L (7), CTLA4Ig (7), busulfan (1), ↑rapamycin (28)	13/16, <30%
253	C3H	No	20	αNK (1), CYP (1), busulfan (1) , ↑rapamycin (24)	0/5
		No	20	DST, αNK (1), CYP (1), busulfan (1) , ↑rapamycin (28)	0/5
		No	20	αNK/CD4/CD8 (1), CYP (1), busulfan (1) , ↑rapamycin (24)	3/5, 10-40%

DM: chimerism induction after onset of diabetes mellitus; BMC: bone marrow cells ($\times10^6$); DST: donor specific transfusion, 20×10^6 donor splenocytes; ↑DST: donor specific transfusion, 100×10^6 donor splenocytes; fludarabine: 400mg/kg; busulfan: 20mg/kg; rapamycin: 1-2mg/kg; ↑rapamycin: 3mg/kg; CYP: cyclophosphamide, 200mg/kg; αCD40L: 0.2-0.25mg; ↑αCD40L: 0.5mg; CTLA4-Ig: 0.25mg; DLI: donor lymphocyte infusion; CD4TDSPL: donor CD4 T cell depleted splenocytes; * chimerism levels at 2-8 weeks post bone marrow transplantation.

1.7 Overview and objectives of my thesis

Generating allogeneic hematopoietic chimerism is thus far the most robust method for inducing donor specific tolerance to allogeneic grafts and re-establishing self-tolerance in recipients with autoimmune diseases. It can potentially be used for inducing tolerance to donor islets in T1D patients without chronic use of immunosuppressants and recurrence of autoimmune diabetes post transplantation. However, its application is limited by the toxicity of the current conditioning protocols, the requirement of some degree of MHC-matching between donor and recipient, the demand of the high dose of HSCs, as well as the fear of possible GVHD. Developing a clinically feasible conditioning protocol on NOD mice, a human T1D model, has been very challenging due to their generalized defects in response to transplantation tolerance induction. The goal of this work has been to generate a translatable conditioning protocol that fosters both hematopoietic chimerism and transplantation tolerance to fully allogeneic islets without the presence of GVHD.

Our lab has previously identified NOD T cells as the major barrier causing a state of split tolerance when using an irradiation free chimerism induction conditioning protocol. However, both rapamycin and co-stimulation blockade were included in this regimen. In **Chapter 3**, we hypothesized a conditioning protocol with maximized T cell depletion can overcome the requirement of irradiation, rapamycin and co-stimulation blockade for chimerism induction. We combined donor specific lymphocyte infusion followed by cyclophosphamide, a combination of T cell depleting (TCD) antibodies targeting CD4, CD8

and CD90, a low dose of busulfan and a moderate dose of bone marrow cells. With stem cells from a fully allogeneic donor, full donor chimerism was induced without signs of GVHD.

Chimerism induction in NOD mice that are spontaneously diabetic is even more difficult compared to young NOD mice that are not yet diabetic. Although the exact reason for this enhanced resistance to tolerance induction in autoimmune diabetic NOD mice is unknown, in **Chapter 4** we hypothesized age may be a contributing factor and examined this possibility. In an attempt to generate stable chimerism in these aged recipients, we examined whether infusion of donor splenic $CD8\alpha^+$ cells into conditioned diabetic NOD mice would overcome their resistance to chimerism. Donor splenic $CD8\alpha^+$ cells have been shown to improve bone marrow engraftment.[254]

Preexisting enhanced immune response towards donor antigens has been another obstacle for both islet transplantation and chimerism induction. Presensitization to alloantigens is common due to previous transplantation, blood transfusion, and certain virus infections. Alloantigen specific antibodies have been showed to be the major reason for bone marrow graft failure in these presensitized recipients. We hypothesized that we could overcome this barrier with enzymes that inhibit the function of antibodies *in vivo*. Endoglycosidase of *S. pyogenes* (EndoS) inhibits binding of the Fc of antibodies to the FcR on effector cells. Immunoglobulin G-degrading enzyme of *S. pyogenes* (IdeS, or imlifidase) cleaves the Fc portion from antibodies.

Collectively, my studies examined whether allogeneic chimerism and transplantation tolerance to donor islets is achievable in young-non-diabetic and age-diabetic NOD mice

with a clinically feasible conditioning protocol. We further examined a novel approach modifying allo-specific antibodies to overcome the allosensitization in bone marrow transplantation (BMT).

Chapter 2

Materials and methods

2.1 Animals

Adult NOD/ShiLtJ (H-2^{g7}; termed NOD), FVB/NJ (H-$2K^{q}$; termed FVB), C3H/HeJ (H-$2K^{k}$; termed C3H), B6.NOD-(D17Mit21-D17Mit10) (H-2^{g7}; termed B6.H-2^{g7}), C57BL/6J (H-2^{b}; termed B6), B6.SJL-Ptprc a Pepcb./Boy (H-2^{b}, termed B6.CD45.1), NOD.B10Sn-$H2^{b}$/J (H-2^{b}; termed NOD.H-2^{b}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). NOD.Rag2pGFP mice (H-2^{g7}) were generated by crossing B6.Rag2pGFP mice to NOD mice. F1 mice were backcrossed to NOD mice for over 14 generations and selected for the expression of transgene and NOD MHC. The diabetes incidence of NOD.Rag2pGFP mice were similar to NOD mice. (Unpublished observation) B6.Rag2pGFP were kindly provided by Pamela Fink (University of Washington, Seattle, WA, USA).[255,256] All mice were bred and housed in a specific pathogen-free facility at the University of Alberta. All care and handling of animals were conducted in accordance with the guidelines of the Canadian Council on Animal Care. All recipient mice used for chimerism induction were females. Chimerism induction recipient mice used in **Chapters 3 and 5** were at 8 to 10 weeks of age. The age of recipient mice used in **Chapter 4** for chimerism induction was indicated in Fig 4-1. As for donor mice, both male and female mice were used at 8 to 14 weeks of age. MHC genotypes of the mice used are shown in Table 2-1.

Table 2-1 Haplotypes of mice used

	MHC-I			MHC_II	
Strain	H2-K	H2-D	H2-L	I-A	I-E
NOD	d	b	-	g7	-
NOD.Rag2pGFP	d	b	-	g7	-
B6.H-2^{g7}	d	b	-	g7	-
C3H	k	k	-	k	k
FVB	q	q	q	q	-
B6	b	b	-	b	b
NOD.H-2^{b}	b	b	-	b	b

2.2 Diagnosis of diabetes and control of hyperglycemia

In **Chapter 4**, female NOD mice older than 12 weeks were checked for blood glucose every week (OneTouch Ultra 2, LifeScan, Canada). Diabetes was confirmed when two consecutive non-fasting blood glucose readings were higher than 300 mg/dl. Diabetic NOD mice were treated with subcutaneous implantation of one to two insulin pellets (LinBit, LinShin Canada) or daily subcutaneous injection of insulin (1U, Novolin, NPH).

Irradiation-free chimerism induction protocol for Chapters 3 and 4

Donor specific transfusion (DST) with 20×10^6 allogeneic splenocytes from the BMC donor strain was performed intraperitoneally (i.p.) on day -10 with respect to the date of BMT. Cyclophosphamide (CYP, 150 mg/kg) was given on day -8 by a single i.p. injection. Busulfan (BUS, 20 mg/kg) was administered i.p. on day -1. *In vivo* depletion mAbs were given as indicated in Figure legends: anti-CD4 (GK1.5, 0.25 mg), anti-CD8α (53.6.7, 0.25 mg), anti-CD90.2 (30H12, 0.3 mg), and anti-asialo GM1 antibody (20 μl). All mAbs were injected i.p.

Allogeneic bone marrow cells (BMC, 10-40×10^6) were given intravenously (i.v.) *via* the lateral tail vein on day 0. The standard treatment protocol is shown in Fig 2-1.

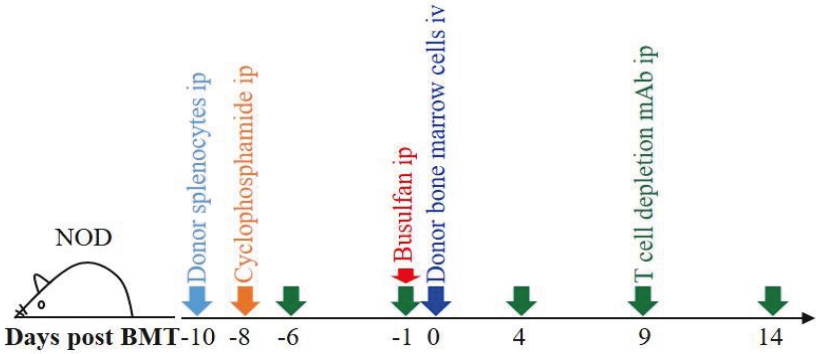

Figure 2-1 Schematic for the basic treatment protocol used in Chapters 3 and 4.

2.3 Determine the short-term survival of donor BMC in primed recipients

To determine the short-term survival of donor BMC in primed recipients, NOD mice that had been sensitized to B6.CD45.1 splenocytes, were T cell-depleted (anti-CD4, Gk1.5, 0.25mg, anti-CD8, YTS169.4, 0.25mg, anti-CD90, YTS154, 0.3mg, i.p.) two days prior to BMT and i.v. injected with EndoS and/or imlifidase 4 hours prior to BMT. B6.CD45.2 BMC (80×10^6) were i.v. injected. Splenocytes and BMC were analyzed at 4 hours post BMC injection. (Fig 2-2)

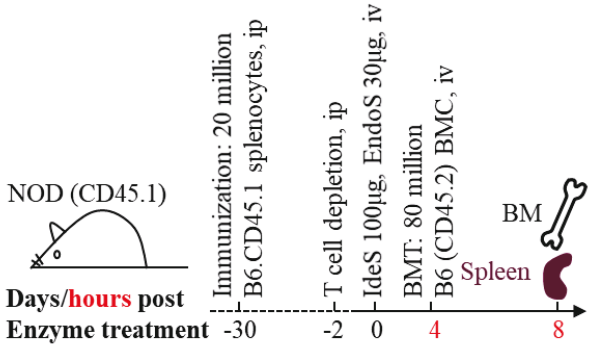

Figure 2-2 Schematic for determining the short-term survival of donor BMC in primed recipients

2.4 Chimerism induction protocol for primed recipients

For long-term chimerism induction, NOD or B6.H-2^{g7} mice that had been sensitized to FVB splenocytes were treated with imlifidase and EndoS i.v. on day -6 with respect to the date of BMT. Cyclophosphamide (150mg/kg, i.p. or i.v.) and bortezomib (1mg/kg, i.v.) were given on day -4. T cell-depleting antibodies (anti-CD4, Gk1.5, 0.25mg, anti-CD8, YTS169.4, 0.25mg, anti-CD90, YTS154, 0.3mg) were administered i.p. on day -2, 2, 6, 11, and 16. A repeated dose of imlifidase and EndoS and 6 Gy total body irradiation (TBI, Gammacell 1000 Elite) was given at four hours prior to BMT on day 0. FVB bone marrow cells (80×10^6) were given intravenously (i.v.) *via* the lateral tail vein on day 0.

2.5 Reagents for *in vivo* experiments

Monoclonal antibodies targeting CD4/8/90.2 used in **Chapter 3 and 4** were purchased from Bio X Cell, West Lebanon, NH, USA. Anti-asialo GM1 antibody were purchased from Wako Chemicals, USA and reconstituted with 1 ml PBS for each vial. Monoclonal antibodies targeting CD4/8/90 used in **Chapter 5** were provided by Dr. Loius Boon from Bioceros B.V. (Utrecht, Netherlands). The YTS 169.4 mouse anti-CD8α mAb producing cells were developed by Prof. H Waldmann and Dr. SP Cobbold (Department of Pathology, University of Cambridge) and obtained *via* Cambridge Enterprise Limited (Hauser Forum, 3 Charles Babbage Road, Cambridge CB3 0GT). Cyclophosphamide and busulfan were purchased from Sigma (MO, USA). Bortezomib was purchased from ApexBio (TX, USA). Imlifidase and EndoS were generated by Hansa Biopharma (Lund, Sweden) and used with permission.

2.6 Cell sorting

Where indicated FVB CD8α$^+$ cells were injected into diabetic mice at d19. Donor splenic CD8α$^+$ cells were purified with a mouse CD8α positive selection kit (Catalog #18753, EasySep™). Approximately 98% of sorted cells were stained with PE conjugated CD8α mAb (clone 53-6.72).

2.7 Definition of chimerism and health status

Recipients were considered chimeric when at least 5% of MHC-I$^+$ cells in the lymphocyte gate were donor-derived at day 28 post-BMT. In **Chapter 3**, stable chimerism was defined as the persistent presence of chimerism as assessed every four weeks post-BMT with the level of donor cells being no less than 20% of the level that was detected at day 28 post-BMT for at least 20 weeks. Body weight and blood glucose of recipient mice were monitored weekly. Mice with two consecutive blood glucose readings above 300 mg/dl were considered diabetic as assessed with a glucose meter (OneTouch, LifeScan, Canada).

2.8 Islet isolation, transplantation and survival nephrectomy

The pancreas from a FVB mouse was distended *via* infusion of cold HBSS containing 0.125 mg/mL Liberase TL Research Grade enzyme (Roche Diagnostics, Canada) into the common bile duct. Resected pancreases were placed in HBSS containing 0.125 mg/mL Liberase and incubated for 14 minutes a 37°C shaking water bath. Histopaque-density

gradient centrifugation (1.108, 1.083, and 1.069 g/mL, Sigma-Aldrich) was then used to isolate islets from digested pancreas.

The volume of isolated islets was converted into islet equivalents as described by Ricordi in 1991.[257] One islet equivalent is considered equivalent to an islet of 150 μm of diameter. Around five hundred donor islet equivalents were transplanted under the renal capsule at the day of bone marrow transplantation. Insulin pellets were removed prior to islet engraftment. Islet graft function was assessed *via* measuring non-fasting blood glucose, twice per week. Reversal of diabetes was confirmed when two consecutive readings of blood glucose were below 200 mg/dl. Islet grafts were considered rejected when blood glucose exceeded 300 mg/dl on two consecutive readings.

For long term chimeric recipients, at three months post-BMT, a recovery nephrectomy of the islet bearing kidney was performed and blood glucose was then monitored.

2.9 Skin graft

Two pieces of 1 cm^2 full thickness trunk skin from FVB and B6.H-2^{g7} were transplanted onto the dorsum of recipient mice with 1 cm distance in between. The skin grafts were secured with sutures to the recipient graft bed and then bandaged for seven days. The grafts were inspected daily and considered rejected at the time when approximately 90% surface area was necrotic.

2.10 Immunization tests of immunocompetence

As an additional test of immunocompetence, mice that had remained chimeric for 13-15 months were immunized with ovalbumin (OVA) and serum anti-OVA antibodies were assessed by ELISA. Stable chimeric FVB→NOD and naïve NOD mice were immunized with 100 µL OVA/CFA containing 50 µL of 2 mg/mL OVA (Sigma-Aldrich, USA) and 50 µL complete Freund's adjuvant (CFA, OZ Biosciences, France) subcutaneously on the hind legs. Mice were bled *via* submandibular vein 21 days post-immunization and serum was stored at -80 °C. To detect OVA-specific mouse IgG, 96-well flat bottom plates (Corning, USA) were coated with 1 µg OVA in 100 µL 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) at room temperature for two hours. After washing three times with washing buffer (0.05% Tween 20 in PBS, pH 7.4), plates were blocked with assay buffer (1% FBS in PBS, pH 7.4; for two hours) and then incubated for two hours with 100 µL of two-fold serial dilutions of serum in assay buffer. Plates were then washed three times and incubated for one hour with 100 µL of 1:5000 dilution of Peroxidase AffiniPure Donkey Anti-Mouse IgG (715-035-150, Jackson ImmunoResearch, USA). Incubation steps were at room temperature with plates placed on a plate shaker. Plates were washed four times and incubated with 100 µL TMB substrate solution (OptEIA reagent set; BD) in the dark. After 10 minutes, 100 µL 0.16 M sulfuric acid was added, and the optical density (OD) at 450 nm was measured using an ELISA plate reader (µQuant Microplate Spectrophotometer and Gen5, Bio-Tek, USA). OD values from the duplicate wells were averaged, and the averages were then subtracted from average OD value of negative control wells (OD~0.05) that was not cultured with serum to

remove background. The average OD value of control wells that were not coated with OVA but had serum added (1/10,000 dilution) from each sample was approximately 0.05.

2.11 Determine the effect of the combination of cyclophosphamide and bortezomib on B cells in primed mice

Four weeks after immunization with FVB splenocytes, NOD mice were treated with cyclophosphamide and bortezomib (CyBor) intravenously. Four days after CyBor treatment, bone marrow transplantation with 20 million FVB BMC was done. Splenocytes and bone marrow cells were collected five days after BMT for analysis. Sera were collected before CyBor treatment and five days post BMT. (Fig 2-3)

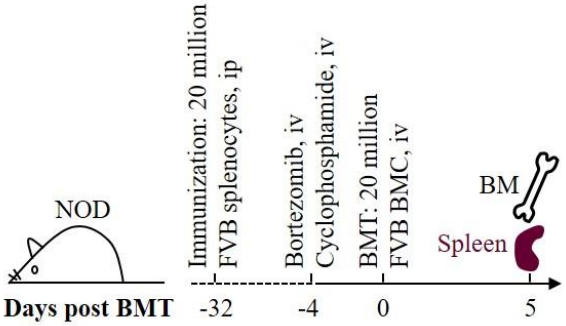

Figure 2-3 Schematic for determining the effect of cyclophosphamide and bortezomib combination on B cells in primed mice

2.11 *In vivo* EndoS mediated monoclonal DSA inhibition assay

NOD (Fig 2-4A) or B6.H-2^{g7} (Fig 2-4B) mice were i.v. injected with vehicle, anti-MHC-I H-$2K^b$ (10μg) alone, or a mixture of EndoS (30μg) and anti-MHC-I H-$2K^b$ (10μg or 100μg). EndoS and anti-H-$2K^b$ were mixed right before injection. Five million 1:1 mixture of CFSE labeled NOD and CTV labeled B6 BMC were i.v. injected into the pretreated NOD mice. Similarly, a 1:1 mixture of CFSE labeled B6.H-2^{g7} and CTV labeled NOD.H-2^b BMC were injected into pretreated B6.H-2^{g7} mice. Blood was collected at 1, 2, and 3 hours post cell administration and analyzed by flow cytometry. Splenocytes and BMC from one hind limb were collected from each mouse and analyzed at four hours post BMC injection.

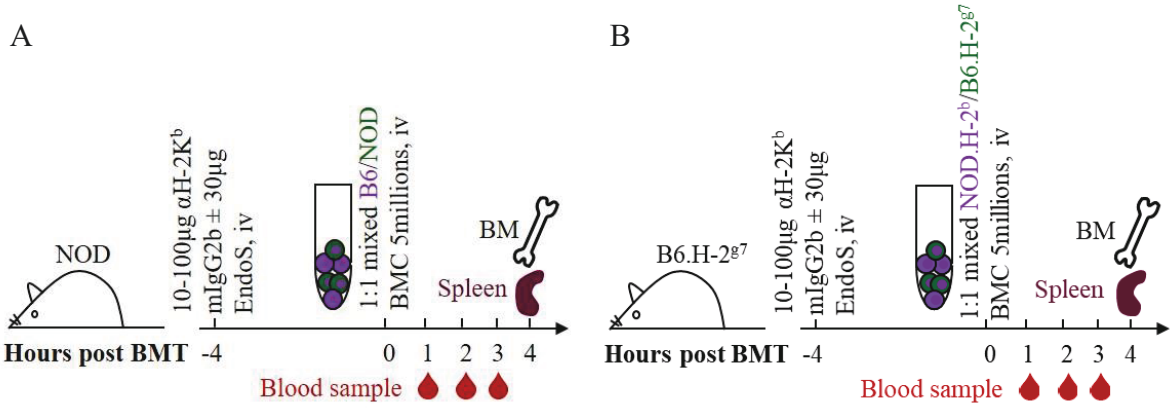

Figure 2-4 Schematics for *in vivo* EndoS mediated monoclonal DSA inhibition assay

2.12 Serum DSA detection assay

NOD mice were immunized by i.p. administration of 20×10^6 FVB splenocytes. Sera were collected prior to and at 4 to 6 weeks post priming as well as at 4 hours post imlifidase and EndoS - treatment. FVB splenocytes (2×10^5) were treated with FcR blockade (anti-

mouse CD16/CD32 rat IgG_{2b} antibodies, clone 2.4G2, BE0307, Bio X cell) for 5 minutes, followed by incubation with a titrated amount of sera in 100µL for 30 minutes. Cells were washed twice and incubated with Fluorochrome conjugated secondary antibodies in 100µL for 30 minutes. The following secondary antibodies were used: FITC conjugated $F(ab')_2$ fragment from rabbit anti-mouse IgG Fc antibody (1:200, 315-096-046, Jackson ImmunoResearch), APC conjugated goat anti-mouse IgG_1 Fc antibody (1:100, 115-135-205, Jackson ImmunoResearch), FITC conjugated goat anti-mouse IgG_3 Fc antibody (1:100, 115-095-209, Jackson ImmunoResearch) and Alexa Fluor 488 conjugated goat anti-mouse IgG_3 heavy chain antibody (1:100, A21151, Thermo Fisher Scientific). Cells were washed twice and analyzed by flow cytometry. HBSS with 2% FBS was used for cell washes and reconstitution.

2.13 Antibodies and flow cytometry

Fluorochrome-labeled antibodies against H-$2K^d$ (SF1-1.1.1), H-$2K^k$ (36-7-5), H-$2K^q$ (KH114), TCRβ (H57-597), CD4 (RM4-5 or RM4-4), CD8β (H35-17.2), CD11b (M1/70), CD11c (N418), B220 (RA3-6B2), CD49b (DX5), CD122 (TM-β1), FoxP3 (FJK-16s), Vβ11 (RR3-15), Vβ6 (RR4-7), and Vβ17a (KJ23) were purchased from BD Pharmingen (San Diego, CA, USA), BioLegend (San Diego, CA, USA) or eBioscience (San Diego, CA, USA). An LSR II (Becton Dickson, Sunnyvale, CA, USA) flow cytometer was used for data acquisition, and data analysis was performed using FlowJo VX (Treestar software, Portland, OR).

2.14 Statistical analysis

Statistical tests were used where appropriate and indicated in Figure legends. All statistical analyses were done using Prism (GraphPad Software, San Diego, CA, USA) with statistical significance defined as $p < 0.05$.

Chapter 3

Stability of chimerism in NOD mice achieved by rapid T cell depletion is associated with high levels of donor cells very early after transplant

A version of this chapter has been published:

Lin J, Chan WFN, Boon L, Anderson CC. Stability of chimerism in non-obese diabetic mice achieved by rapid T cell depletion is associated with high levels of donor cells very early after transplant. *Front Immunol* (2018) 9:837. doi: 10.3389/fimmu.2018.00837.

3.1 Introduction

Mixed hematopoietic chimerism is the state of coexistence of donor and recipient derived hematopoietic cells in the host. Establishment of such chimerism, *via* BMT, is a robust method for generating donor specific tolerance to donor tissue/organs without the need for lifelong immunosuppression,[208,258-263] and it can be used to treat severe autoimmune diseases.[264,265] However, its clinical application is dampened by the toxicity of current recipient conditioning regimens.

Although significant efforts have been made to generate reduced intensity and nonmyeloablative conditioning protocols in murine models, the success of such protocols typically depends on the inclusion of TBI, TI, anti-CD40L mAb, or a very high dose of BMC.[143,250,266-269] Of note, anti-CD40L mAb is known to cause thromboembolic complications in humans.[225] A mega dose of BMC from one deceased donor is currently clinically unachievable,[214] which would be relevant in the cases when cadaveric bone marrow and organs, such as islets, are the only option. Also, more stringent transplant settings, in which donor and recipient are fully MHC and MiHA mismatched, are often not tested.

More importantly, low-intensity conditioning protocols that induced mixed chimerism in B6 mice were not usually successful in autoimmune-prone, tolerance induction resistant recipients, such as NOD mice.[219-221] The difficulty in inducing chimerism in NOD mice is manifested not only by a lower success of initial chimerism but also by the inability to maintain multilineage chimerism.[222] In general, this obstacle in NOD mice can be overcome if irradiation,[205,233,234,236-243] costimulation blockade,[222,236,239,241,244-249] high doses of rapamycin,[222,244-246,249] or mega dose BMC[143,250] from a fully MHC[143,205,222,234,237,241,246,249-251] or more often partial MHC[233,236,238-240,244-247] plus MiHA mismatched donor, are applied.

T cell depletion is another commonly used method for temporally inhibiting the host immune system. However, it was often used as adjuvant therapy with irradiation, costimulation blockade, or the combination of both.[237,239,241,243,247-249] In a rare success, Zeng and colleagues induced fully mismatched chimerism in NOD mice conditioned with anti-CD3/CD8 and donor lymphocyte infusion.[143,250,251] However, the transfer of a very high dose BMC currently prevents the translation of this approach to a clinical setting. We previously showed that an irradiation-free mixed chimerism protocol in NOD mice is achievable with antibodies to T cells and CD40L together with busulfan and high dose rapamycin. We determined that recipient T cells were a critical barrier for generating chimerism in NOD recipients;[249] however, the level of T cell depletion and its relationship to chimerism was not assessed. In addition, this protocol prevented donor islet rejection but did not generate tolerance to donor skin grafts. Recently, we also developed a T cell depletion and rapamycin-based protocol that is irradiation and costimulation blockade free;[253] however, donor chimerism waned over time.

Chimerism can be stable or transient in both animal models and in humans; and the loss of chimerism can increase the susceptibility of particular organs to rejection.[270] The ability to identify early after BMT those recipients who will later loose chimerism would provide the opportunity to implement approaches that promote the stability of chimerism. We, therefore, sought to generate a more clinically feasible protocol generating stable chimerism for autoimmune-prone recipients, and determine whether stability of chimerism is associated with events occurring early after BMT. We tested the hypothesis that maximizing recipient T cell depletion would eliminate the need for high dose BMC or agents lacking clinical translatability (e.g., anti-CD40L and high dose rapamycin) and would generate robust donor

specific tolerance across fully allogeneic barriers. We found that an extensive T cell depletion conditioning protocol, consisting of DST-CYP and multiple T cell depleting antibodies achieved the goal of donor specific tolerance and that very early levels of chimerism and donor T cells were significantly correlated with the later stability of chimerism.

3.2 Results

3.2.1 DST-CYP preferentially prevents the expansion of alloreactive host T cells in NOD mice

To create an efficient T cell depletion based conditioning protocol, we employed DST-CYP with T cell depleting monoclonal antibodies. CYP administration following DST is also called cells-followed-by-CYP system, which is proposed to eliminate actively dividing alloreactive T cells.[271] Although this system has been widely shown to be valid in different strains of mice, it was rarely tested in the tolerance resistant NOD mouse model of type-1 diabetes, especially for chimerism induction.[238,245,253] Lee and colleagues showed that DST-CYP along with Treg transfusion prolonged the survival of allogeneic islets in autoimmune diabetic mice. However, it appeared that DST-CYP depleted alloreactivity in a non-specific fashion.[272] Whether DST-CYP could preferentially block the generation of allostimulated effector cells in NOD mice has not been reported. Here we examined this by conditioning naïve NOD mice with vehicle, DST-vehicle, vehicle-CYP, or DST-CYP and comparing the frequencies of effector memory ($CD44^{high}$ $CD62L^{low}$) T cells in peripheral blood lymphocytes (PBL) before and after treatment (Fig 3-1A). Preconditioning with fully allogeneic splenocytes alone led to an over 1.5 fold expansion of effector memory $CD4^+$ T cells and seven-fold expansion of effector memory $CD8^+$ T cells in PBL (Fig 3-1B). In contrast, CYP

alone reduced the frequency of effector memory $CD4^+$ and $CD8^+$ T cells by approximately 50% in PBL, while DST-CYP completely prevented the expansion of memory/effector cells caused by the DST (Fig 3-1B). Results from splenocyte analysis on day 7 showed that DST alone tended to increase the absolute numbers of $CD4^+$ and $CD8^+$ T cells, and significantly increased the numbers of effector memory $CD4^+$ and $CD8^+$ T cells compared to the vehicle treated group (Fig 3-1C and 3-1D). DST-CYP prevented this increase and significantly reduced the absolute numbers of $CD4^+$ and $CD8^+$ T cells as well as their effector memory subsets (Fig 3-1C and 3-1D). In addition, NOD mice treated with CYP alone showed a trend towards reduction of T cells and effector memory T cells (not statistically significant; Fig 3-1C and 3-1D). Thus, while DST-CYP causes some generalized T cell depletion, it preferentially and effectively prevents the expansion of allostimulated effector memory T cells in NOD mice.

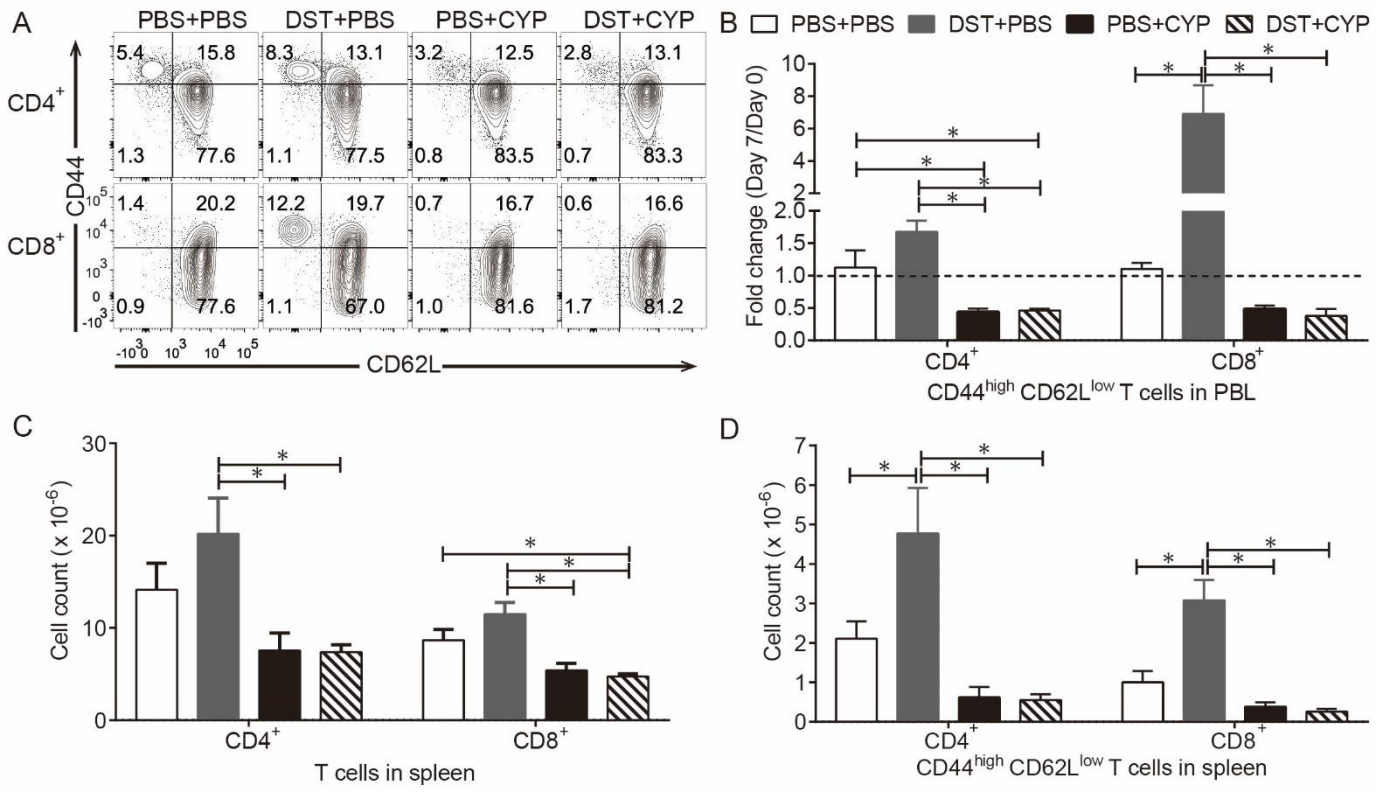

Figure 3-1 DST-CYP is effective in autoimmune recipients.

Naïve female NOD mice were given 20×10^6 C3H splenocytes or vehicle (PBS) i.p. on day 0 and a dose of CYP, or vehicle i.p. on day 2. PBL were taken on day 0 and day 7 for analysis. All mice were euthanized on day 7 and splenocytes were harvested for analysis. (A) Representative analysis of PBL on day 7, $CD4^+TCR\beta^+$ gate for the upper panel and $CD8^+TCR\beta^+$ gate for the bottom panel. (B) Shown is fold change of $CD44^{high}$ $CD62L^{low}$ cells in $CD4^+TCR\beta^+$ gate (left) and $CD8^+TCR\beta^+$ gate (right) on day 7 compared to day 0 (mean ± SEM). (C) Shown are absolute numbers of $CD4^+$ T cells (left) and $CD8^+$ T cells (right) in the spleen on day 7 (mean ± SEM). (D) Shown are absolute numbers of $CD44^{high}$ $CD62L^{low}$ $CD4^+$ T cells (left) and $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells (right) in the spleen on day 7 (mean ± SEM). Data were pooled from four independent experiments (4-6 mice per group). *$p < 0.05$, Two way ANOVA with Tukey's multiple comparison test.

3.2.2 Combination of DST-CYP and dual anti-T cell mAb treatment peri-BMT induces chimerism that lacks stability and is donor dependent

We then asked if the combination of DST-CYP and T cell depleting mAbs induces chimerism in NOD mice by using fully allogeneic donors. NOD mice were preconditioned with DST from C3H mice, CYP, antibodies against CD4/8, busulfan, and a donor bone marrow transplant. By using this protocol, mixed chimerism was induced in eight of ten NOD mice with six chimeras having levels of chimerism higher than 75% at four weeks post-BMT (Fig 3-2). Despite the high-level chimerism at four weeks post-BMT, only two chimeras were able to maintain substantial chimerism long-term (Fig 3-2B) with multiple-lineages of donor cells, including T, B, NK and dendritic cells (data not shown). Four chimeras quickly lost their chimerism at eight weeks post-BMT. Although we found no obvious signs of GVHD, such as chronic weight loss, dermatitis, or hyperglycemia in chimeric recipients, two recipients died at 9 and 14 weeks post-BMT. However, these results already strongly supported the hypothesis that by employing DST-CYP and T cell depleting mAbs mixed chimerism could be induced in NOD mice without irradiation, costimulation blockade or rapamycin.

As C3H only represents one fully allogeneic donor, we sought to test this protocol with a second fully allogeneic donor, FVB, to test the stringency of the current protocol. Donor and recipient MHC disparities are shown in Table 3-1. Surprisingly, none of the ten NOD mice became chimeric, even in the two recipients given a double dose of BMC in a single injection (Fig 3-2A row 2). Thus, the combination of DST-CYP and anti-CD4/8 mAbs induced multi-lineage chimerism only when using a C3H donor. However, this success could not be extended to the FVB→NOD combination.

A

Donor	αCD90.2	Donor BM (x 10^{-6})	Chimerism	Stable chimerism	Details of chimeras
C3H	-	20	8/10	2/8	Figure 3-2B
FVB (CD90.1)	-	20 or 40#	0/10		
FVB (CD90.1)	+	20 or 40#	14/14	11/13§	Figure 3-2F
C3H	+	20	9/9	3/7§	Figure 3-2G

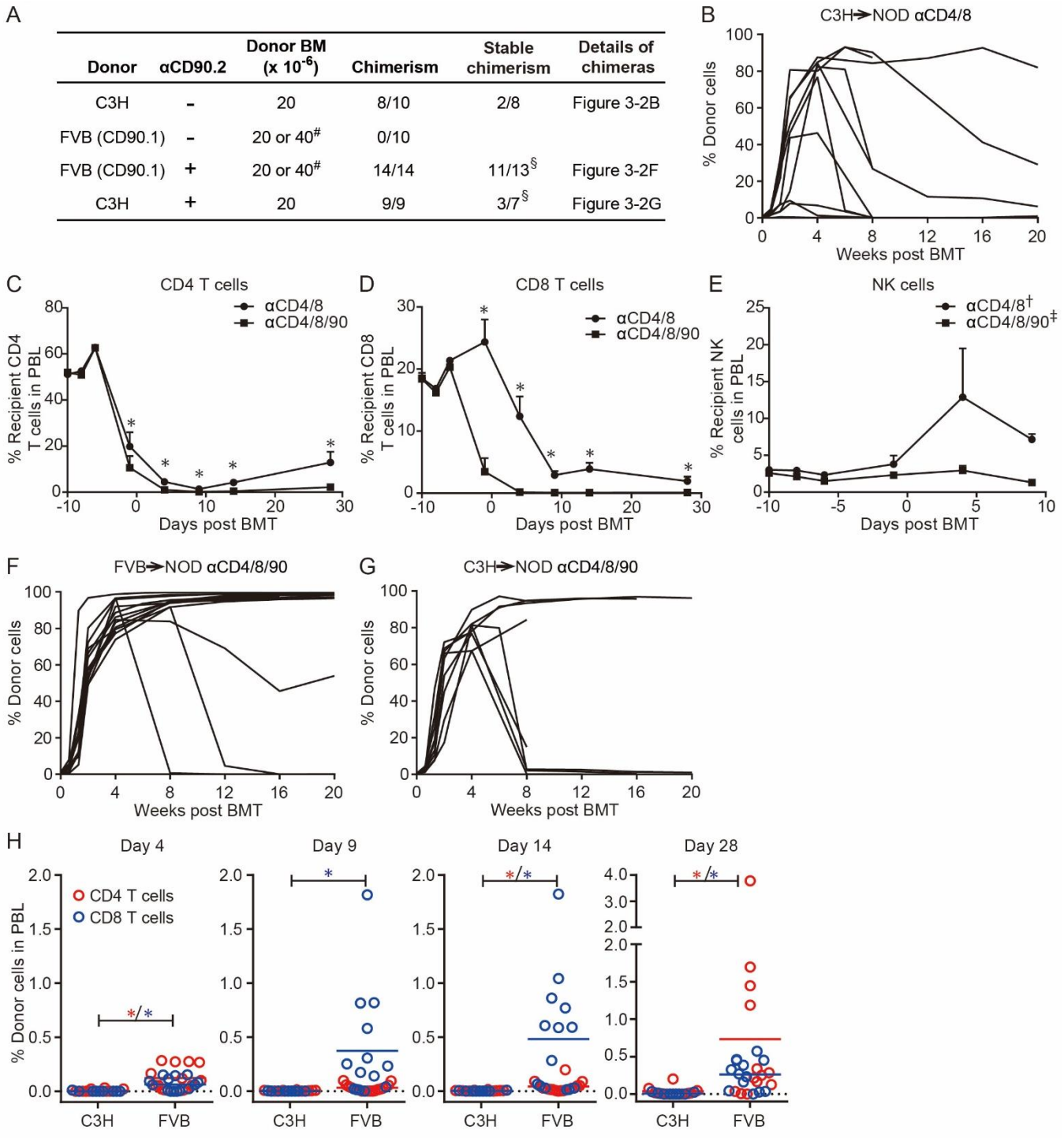

Figure 3-2 Robust T cell depletion in NOD mice preconditioned with DST-CYP allows chimerism using fully allogeneic donor hematopoietic cells.

(A) NOD recipients were conditioned with DST (day -10), CYP (day -8), a combination of T cell depletion mAbs (anti-CD4/8 ± anti-CD90, day -6, -1, 4, 9, 14; '-' indicates no injection), BUS (day -1) and BMC (day 0, 20 or 40×10^6). # two recipients from each group given 40×10^6 BMC, the remainder received 20×10^6; cells were from the same donor strain as the DST). The success of chimerism was determined at 4 weeks post-BMT. § One FVB→NOD and two C3H→NOD chimeras were excluded as they were found dead prior to analysis of the stability of chimerism. (B, F-G) Shown are the proportion of donor cells in lymphocyte gate in PBL over time. (C-E) PBL were harvested before each treatment to evaluate the $CD4^+$, $CD8^+$ T cell and NK cell components in FVB→NOD recipients conditioned with anti-CD4/8 or anti-CD4/8/90 mAbs. Shown are percentages of recipient $CD4^+$, $CD8^+$ T cell and NK cells in the lymphocyte gate (mean ± SEM). NOD recipients were treated with anti-CD4/8 (n = 10; † n = 2) or anti-CD4/8/90 (n = 14; ‡ n = 6) mAbs. *$p < 0.05$, Mann–Whitney U test for each time point. (H) PBL were harvested at the indicated time points to evaluate the donor $CD4^+$ and $CD8^+$ T cells in C3H→NOD or FVB→NOD recipients conditioned with anti-CD4/8/90 mAbs. Shown are percentages of donor $CD4^+$ and $CD8^+$ T cell in the lymphocyte gate (mean). Data were pooled from at least two independent experiments. *$p < 0.05$, Mann–Whitney U test.

3.2.3 A triple anti-T cell mAb protocol facilitates the depletion of recipient T cells and the induction of high-level chimerism

As $CD8^+$ T cells[273] and NK cells[274] are both important barriers to chimerism induction, we hypothesized more efficient $CD8^+$ T and NK cell depletion would prevent bone marrow rejection in the FVB to NOD combination and induce chimerism. We included anti-CD90 mAbs in the new protocol, as CD90 is expressed not only on T cells but also on a subset of NK cells.[275] As shown in Fig 3-2C, the combination of anti-CD4/8/90 mAbs (termed triple antibody protocol) modestly, although significantly, increased depletion of $CD4^+$ T cells compared to anti-CD4/8 mAbs (termed duo antibody protocol). More strikingly, the triple antibody protocol accelerated the depletion of $CD8^+$ T cells compared to the duo antibody protocol (Fig 3-2D). The triple antibody protocol also appeared to have superiority in depleting NK cells after the infusion of the second dose of antibodies as compared to the duo antibody protocol (Fig 3-2E).

With the success of host T cell depletion after the inclusion of anti-CD90 mAbs, chimerism was induced in 14 of 14 NOD recipients using FVB BMC (Fig 3-2A row 3; Fig 3-2F). Though one mortality was found at eight weeks post-BMT without obvious signs of GVHD, 11 of 13 remaining chimeras maintained stable multi-lineage chimerism (Fig 3-3A-B). These data are in agreement with our previous finding that NOD T cells are the major cells that mediate split tolerance in chimerism induction[249] and a robust T cell depletion based regimen could overcome split tolerance. Strikingly, 10 of the 11 stable chimeras developed nearly complete chimerism (Fig 3-2F and 3-3). The generation of complete chimerism is considered to be more difficult to achieve compared to mixed chimerism, with the establishment of a higher level of chimerism in NOD mice requiring a higher dose of

BMC,[234] a higher dose of irradiation,[241] and more costimulation blockade[241] when fully allogeneic donor cells are used. Moreover, complete chimerism had not previously been achieved in NOD mice conditioned with an irradiation free protocol and given fully allogeneic BMC.[143,222,246,249,250,253]

As shown in Fig 3-2B, only two NOD mice conditioned with the duo antibody protocol and infused with C3H BMC developed stable mixed chimerism. We asked if the success of the triple antibody protocol with FVB BMC could be applied to C3H BMC recipients. In this case, all nine NOD mice became chimeric at four weeks post-BMC when treated with the triple antibody protocol. However, the success of chimerism induction with anti-CD90 mAbs in C3H BMC recipients was not as striking as in FVB BMC recipients. On the one hand, the inclusion of anti-CD90 mAbs did not increase the rate of stable chimerism, as only three NOD mice became stable chimeras (Fig 3-2A and 3-2G) with multiple lineages of donor cells (data not shown). Four mice had unstable chimerism (chimerism declined by 8 weeks post-BMT; one was found dead at 11 weeks), and another two were found dead at 6 and 10 weeks post-BMT without obvious signs of GVHD (Fig 3-2G) prior to the determination of chimerism stability. On the other hand, targeting CD90 improved the levels of donor cells in stable C3H→NOD chimeras, as complete chimerism was maintained in three C3H→NOD chimeras treated with the triple antibody protocol (Fig 3-2G). In contrast, complete chimerism was not observed in any of the C3H→NOD chimeras treated with the duo antibody protocol (Fig 3-2B).

The success of BMT results not just from less rejection by recipient immune cells but also the promotion of BMC engraftment mediated by donor cells, within which donor $CD8^+$ T cells have been shown to play a role.[250,276] Indeed, though recipient T cells, as well as NK

cells, were depleted equally in both C3H→NOD and FVB→NOD chimeras, donor cells from FVB (CD90.1) were not susceptible to the anti-CD90.2 mAb we employed for depletion. We therefore asked if the difference of success in chimerism induction with FVB and C3H donors was associated with the presence of donor passenger T cells. We observed a significantly increased frequency of donor $CD8^+$ T cells in FVB→NOD but not C3H→NOD chimeras at early time points post-BMT (Fig 3-2H). Although this early existence of donor T cells was associated with the success of FVB→NOD chimerism generation, it was not an absolute requirement for the current protocol, as chimerism could in some cases be established even when donor passenger T cells were depleted in C3H→NOD chimeras.

Taken together, the inclusion of anti-CD90 enhanced recipient T cell depletion greatly facilitated the induction of stable high-level chimerism using a donor that had more class I mismatches (FVB). However, this facilitation was much less apparent when using a donor (C3H) that had more class II mismatches and T cells susceptible to the anti-CD90. These findings are consistent with the major effect of anti-CD90 being a more efficacious depletion of $CD8^+$ T cells (Fig 3-2D).

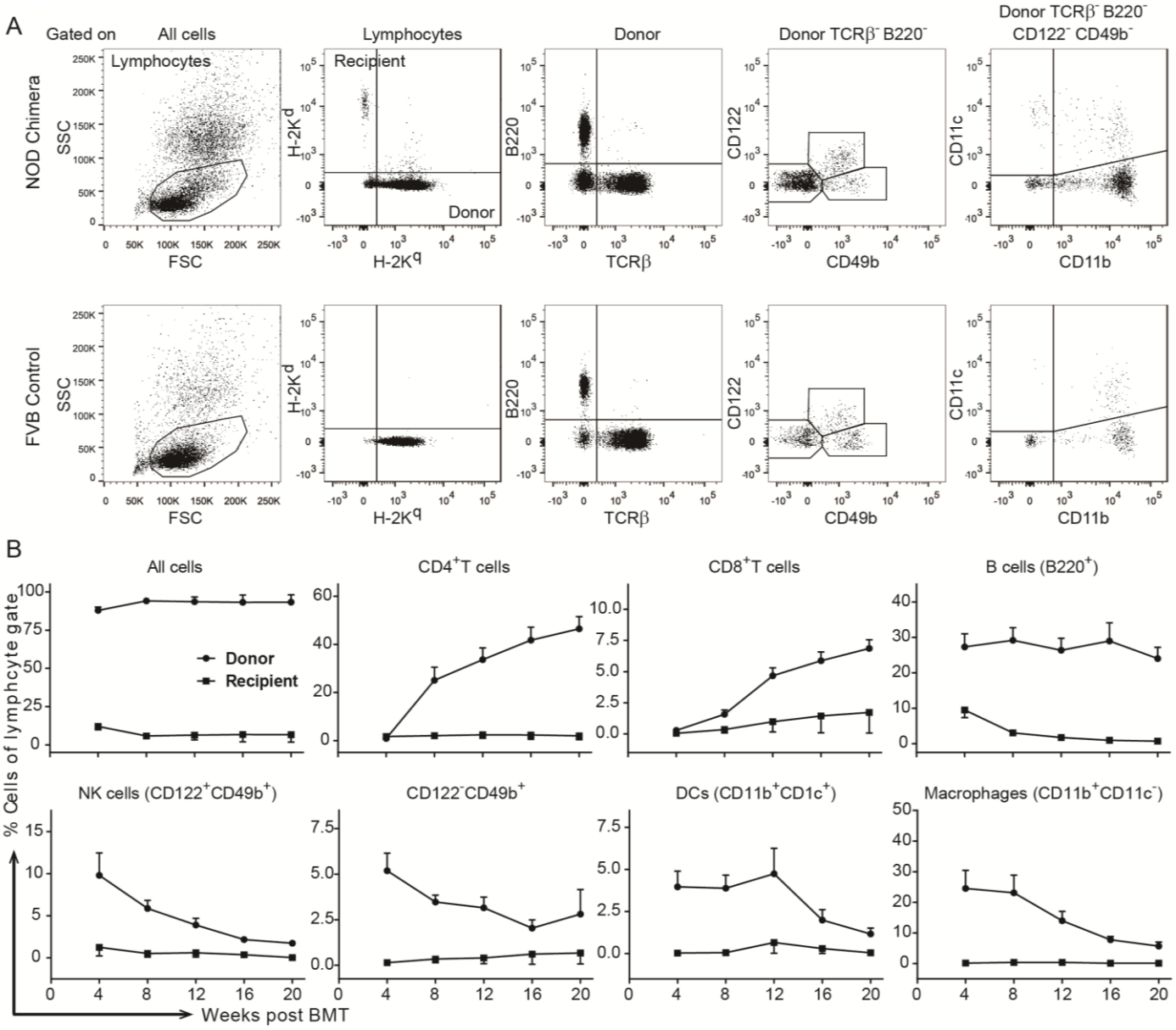

Figure 3-3 Stable high-level multilineage donor chimerism was maintained in FVB→NOD chimeras conditioned with a robust T cell depletion protocol.

Stable chimeras (FVB→NOD; n = 11) induced by the triple antibody protocol (refer to Figure 3-2B) were analyzed for different lineages of donor and recipient derived cells in PBL over time. (A) Shown is the gating strategy. (B) Shown are the percentages of donor or recipient derived MHC-I$^+$ cells, CD4$^+$ T cells, CD8$^+$ T cells, B cells, NK cells, TCRβ$^-$B220$^-$CD122$^-$

CD49b$^+$ cells, DCs and Macrophages in the lymphocyte gate in PBL over time. Values are shown as the mean ± SEM.

3.2.4 NOD mice with high level chimerism acquire robust donor specific tolerance and recover a substantial but diminished level of immunocompetence

After the success in generating stable multi-lineage chimerism, we sought to examine if NOD chimeras re-established tolerance to self-antigen and displayed donor specific transplantation tolerance. Chimerism induction with allogeneic BMC has been shown to re-establish tolerance to self-antigens in NOD mice. Firstly, and consistent with previous studies,[277] we found that all of the FVB→NOD chimeras remained free of diabetes and lacked islet infiltration (Fig 3-4A and 3-4D). In contrast, all naïve NOD mice, and some NOD mice that were conditioned with a duo or triple antibody protocol without developing chimerism, became diabetic over time (Fig 3-4A). Secondly, the tolerance status in chimeras was bidirectional. On the one hand, the successful engraftment of donor hematopoietic cells represents tolerance to the donor antigens present in donor hematopoietic-derived cells. More importantly, FVB→NOD chimeras accepted FVB donor skin grafts indefinitely (Fig 3-4B-D), which represents the most stringent test of tolerance to a donor and indicates that tolerance extends beyond hematopoietic cells to other donor tissue antigens. And finally, their healthy appearance, and continued increase in body weight (Fig 3-4E), suggested a lack of GVHD in FVB→NOD chimeras with complete chimerism, indicating donor cells were tolerant of host antigens.

In addition to the tolerance status of chimeras, immunocompetence is another important concern, particularly in the case of full chimerism. Full chimerism has been considered by some to be less desirable as there is the potential the recipient will have some immunodeficiency due to the T cells being selected in a thymus that has different MHC alleles (recipient MHC type) than that on the antigen presenting cells (donor MHC type).[259,278] To address if NOD mice with full chimerism were immunocompetent, FVB→NOD chimeras were also transplanted with skin from B6.H-2^{g7} mice (3^{rd} party), which has the MHC genes from NOD and the non-MHC genes from B6 (i.e. mismatched for multiple MiHA as well as gene(s) regulating innate allo-responses[33]). All chimeric and naïve NOD mice were able to reject B6.H-2^{g7} skin (Fig 3-4B and 3-4C), although chimeras rejected B6.H-2^{g7} skin more slowly. Chimeric NOD mice were also immunized with OVA to evaluate anti-OVA antibody production for determining the level of humoral immunocompetence. As shown in Fig 3-4F, NOD chimeras produced substantial IgG against OVA, although the titer that was approximately eight-fold less than in young naïve NOD mice. Together, these data indicate that the chimeras were fully tolerant of the donor with substantial but diminished immunocompetence.

As central tolerance *via* clonal deletion has been proposed as the main mechanism for donor specific tolerance *via* chimerism induction, we investigated whether clonal deletion was occurring in the chimeras. Superantigens encoded by endogenous and exogenous viral genes in mice are known to elicit strong binding of particular T-cell receptor (TCR) variable-beta (Vβ) chains and the MHC class II molecule, which then leads to the deletion of certain $V\beta^+$ T cells.[279] This phenomenon 'mimics' process of clonal deletion during normal T cell development in the thymus. As a result, the frequency of $V\beta11^+$ T cells in C3H and $V\beta17a^+$

T cells in NOD mice are much lower compared to Vβ6^{+} T cells. In our model, the recipient Vβ11^{+} T cells bind to C3H derived superantigen in C3H→NOD chimeras with the presence of I-E^{k} and donor Vβ17a^{+} T cells react with NOD-derived superantigen in FVB→NOD chimeras with the presence of I-A^{g7}.$^{280\text{-}282}$ We found the reduction of these two populations occurred in the chimeras (Fig 3-5A and 3-5B) compared to naïve controls, which indicated that clonal deletion of at least a subset of donor-reactive and host-reactive T cells had occurred.

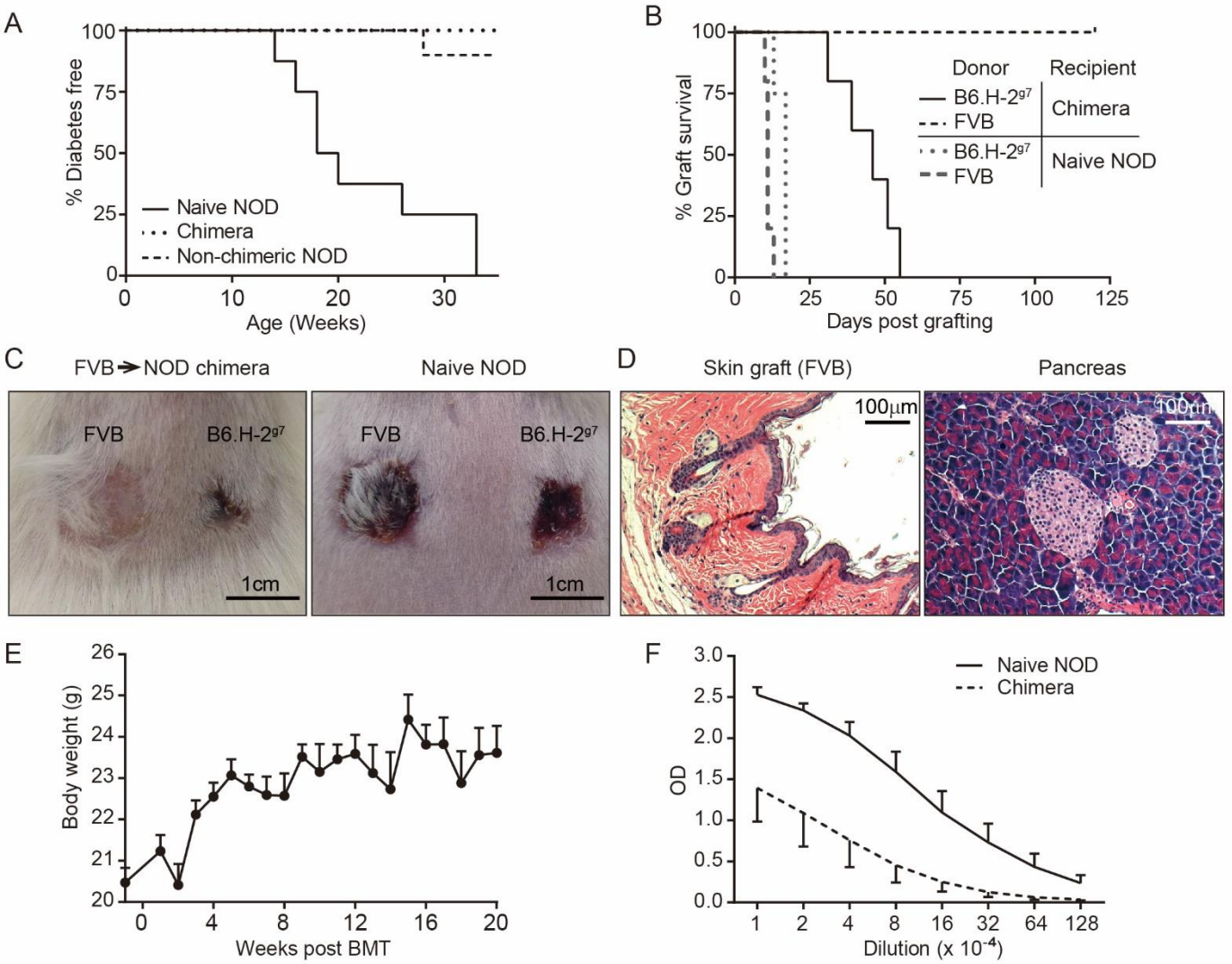

Figure 3-4 Chimeras with high level or complete chimerism acquired full donor specific tolerance, did not develop autoimmune disease, and recovered immunocompetence.

(A) Lack of autoimmune diabetes in chimeras. Recipients (C3H→NOD and FVB→NOD; n = 43) conditioned with duo or triple antibody protocol (refer to Figure 3-2A) and naïve female NOD mice were monitored for blood glucose weekly starting from 8-10 weeks of age. Shown are percentages of mice that were diabetes-free over time (chimeric mice, n = 31; non-chimeric mice, n = 12; naïve NOD, n = 8). (B) FVB→NOD (n = 5) and Naïve NOD mice (n = 5) were engrafted with skin from FVB and B6.H-2^{g7} donors at 6 months after BMT. (C) Shown are representative macroscopic pictures of the acceptance and/or rejection of skin grafts in FVB→NOD chimeras (51 days post skin transplant) and naïve NOD mice (13 days post skin transplant). (D) Pancreas and FVB donor skin (6 months post skin transplantation) from FVB→NOD chimeras that maintained donor chimerism for about 12 months were subjected to hematoxylin and eosin staining. Shown are representative photographs from individual chimeras (n = 4). (E) Shown is the body weight of FVB→NOD chimeras (refer to Figure 3-2F; mean ± SEM). (F) FVB→NOD (n = 4) and Naïve NOD mice (n = 5) were immunized with OVA at 12 months after BMT. Serum was collected three weeks post immunization and for anti-OVA IgG detection. Shown are OD value (mean ± SEM) for each serum dilution.

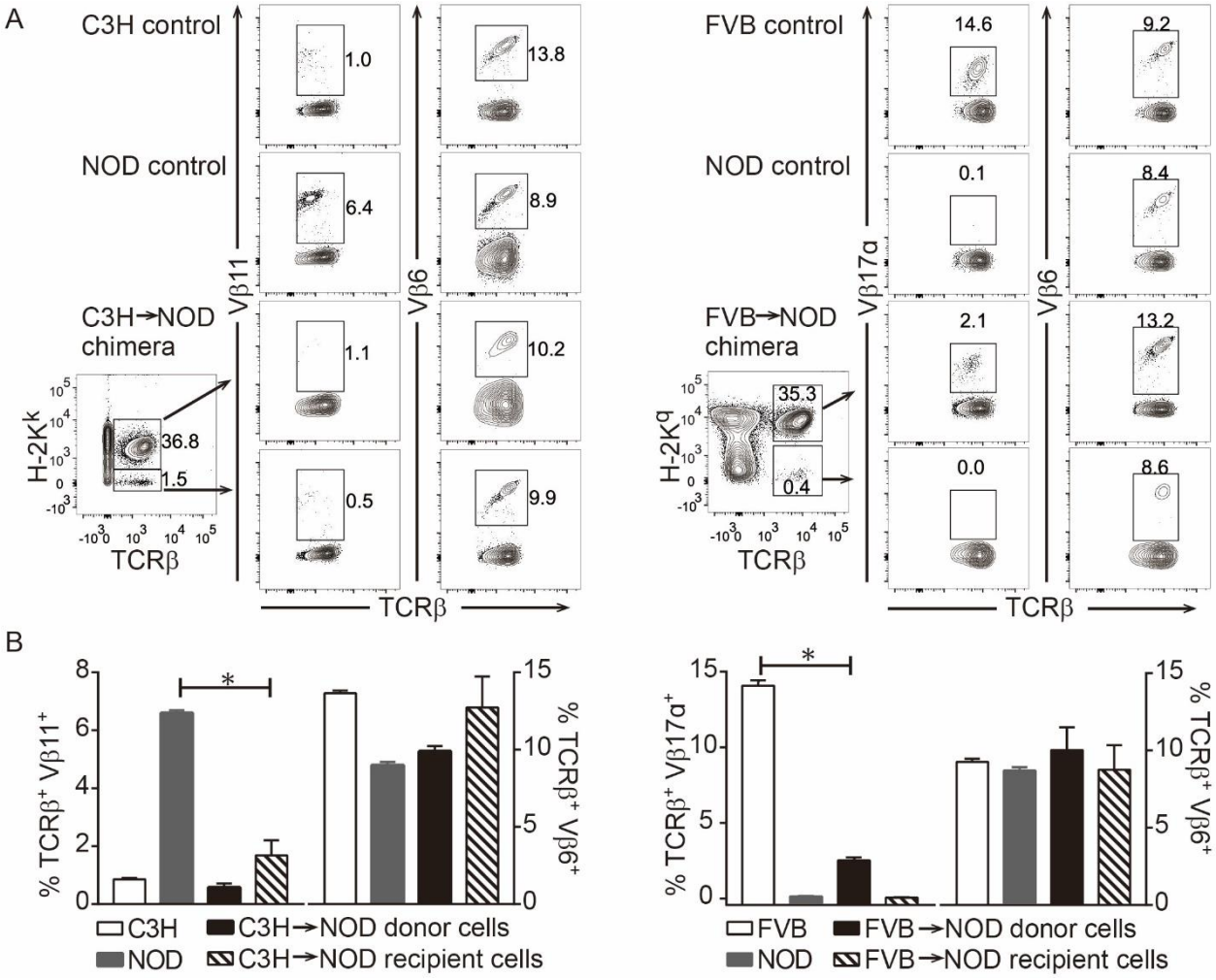

Figure 3-5 Clonal deletion of alloreactive T cells in chimeras

(A-B) Frequency of recipient anti-donor ($V\beta11^+$) T cells in C3H→NOD chimeras and donor anti-recipient ($V\beta17^+$) T cells in FVB→NOD chimeras were evaluated at 8 to 12 weeks post BMT. (A) Shown is the gating strategy. (B) Shown are the frequencies of $V\beta11^+$, $V\beta6^+$, and $V\beta17^+$ T cells in the PBL. Values are shown as the mean ± SEM. Numbers of animals used for analysis in the left panel: naïve C3H n = 6, naïve NOD n = 7, C3H→ NOD chimeras n = 4; right panel: naïve FVB n = 4, naïve NOD n = 4, FVB→ NOD chimeras n = 8. *$p < 0.05$, Mann–Whitney U test between designated tested groups.

3.2.5 **The eventual loss of chimerism is associated with lower levels of chimerism and donor T cells early after BMT**

FVB→NOD and C3H→NOD chimeras treated with the triple antibody protocol developed high-level chimerism that was either maintained or the chimerism was lost between 6 to 12 weeks post-BMT (Fig 3-2F-G). Strikingly, the decrease of chimerism in these unstable chimeras was sudden and sharp despite the presence of a very high level of chimerism in the previous 2-4 weeks (Fig 3-2F and 3-2G). Being able to predict which recipients will lose chimerism later on would provide the opportunity for early interventions on an individual basis. We therefore sought to determine if there might be some intrinsic differences between the stable and unstable chimeras at early time points post-BMT that would be detectable and associated with the fate of chimerism in the long term. In an attempt to address this issue, we compared the chimerism and donor T cell levels at days 4, 9, 14, and 28 in mice that maintained stable chimerism to those whose chimerism level had dropped more than 80% from the level at day 28 (Fig 3-6). We found that chimeric NOD mice that maintained stable chimerism had significantly higher donor chimerism at days 9 and 14 post-BMT (Fig 3-6A) and this significant difference was also apparent when donor T cells were excluded from the analysis (data not shown). Higher levels of donor T cells at early time points post-BMT (from day 9) were also found in stable chimeras compared to chimeric mice that would later lose their chimerism (Fig 3-6B). Thus, despite the continued rise of chimerism levels for a period and eventually exceeding 60% donor cells in all recipients, some recipients subsequently lost chimerism, and this was significantly associated with a lower level of chimerism and donor T cells very early after BMT.

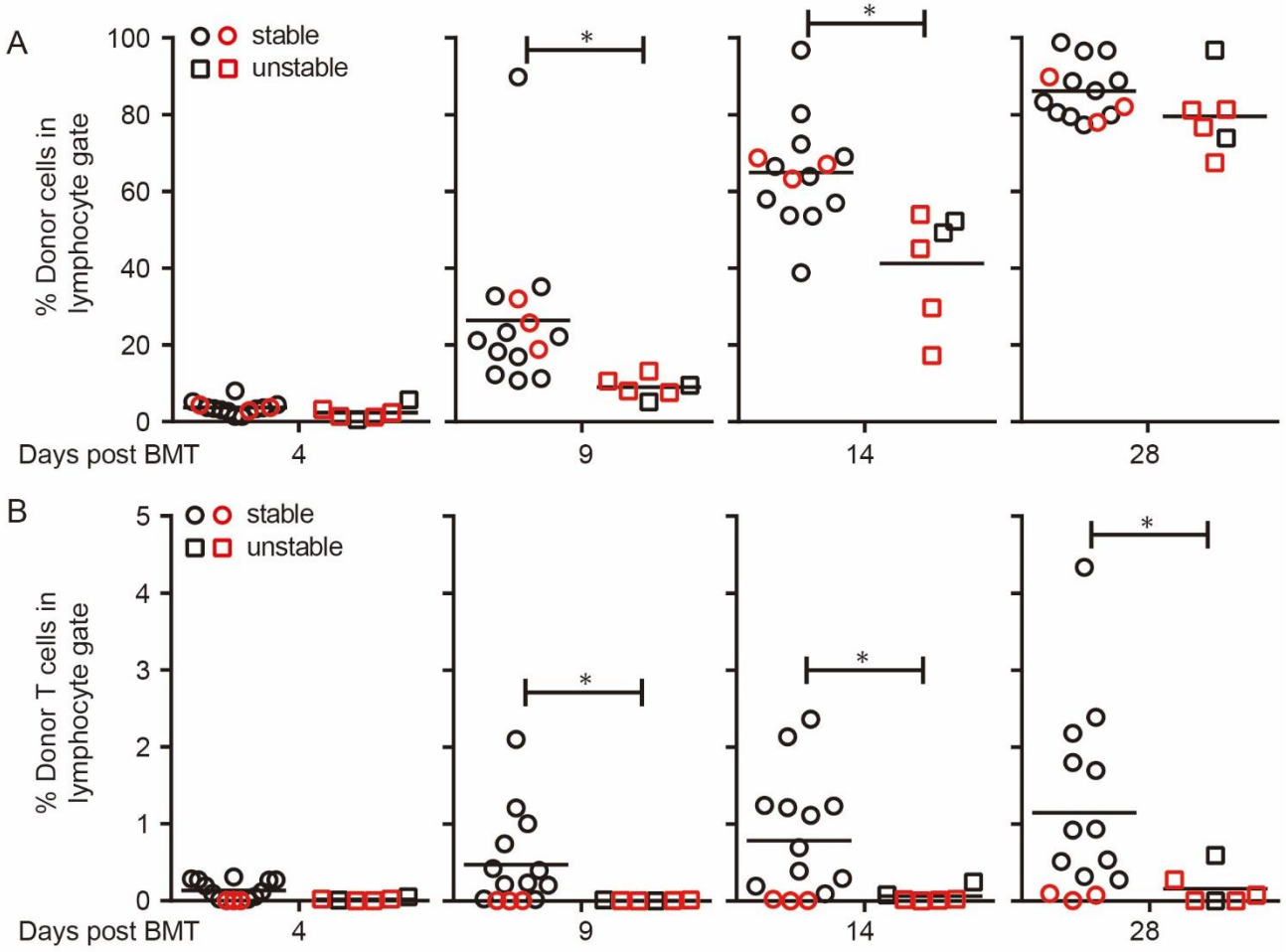

Figure 3-6 Loss of chimerism is significantly associated with a lower early level of chimerism and donor T cells.

NOD recipients were conditioned with DST (day -10; using C3H or FVB splenocytes), CYP (day -8), anti-CD4/8/90 mAbs (day -6 and every 5 days until day 14), BUS (day -1) and BMC (day 0, 20×10^6; using BMC from same donor strain as the DST). PBL were harvested before each treatment to evaluate the donor cell component in the lymphocyte gate. Comparison of early levels of chimerism (A) and donor T cells (B) in recipients that maintained stable chimerism (n = 14) and those had unstable chimerism (n = 6). Black: FVB→NOD; Red: C3H→NOD. *$p < 0.05$, Mann–Whitney U test.

A

Donor	DST	CYP	BUS	Chimerism	Stable chimerism	Details of chimeras
C3H	-	+	+	2/3	0/2	Figure 3-7B
C3H	+	+	-	0/3		

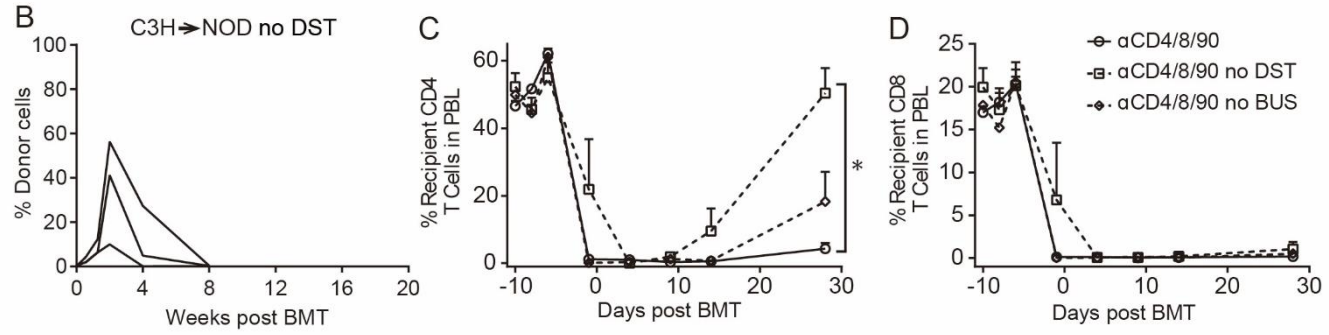

Figure 3-7 DST and BUS are required for chimerism induction.

NOD recipients were conditioned with anti-CD4/8/90 mAbs, and BMC (day 0, 20×10^6; using C3H BMC). The injections of DST (day -10; using C3H splenocytes), CYP (day -8), and BUS (day -1) were as indicated in (A). (B) Shown are the proportion of donor cells in lymphocyte gate in PBL over time. (C-D) PBL were harvested before and after each treatment to evaluate the $CD4^+$ and $CD8^+$ T cell components. Shown are percentages of recipient $CD4^+$ and $CD8^+$ T cells in lymphocyte gate (mean ± SEM). Data was pooled from two independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test was used to compare the mean of each group at each time point post BMT, $*p < 0.05$

3.3 Discussion

Mixed hematopoietic chimerism is considered the most robust method for inducing donor specific tolerance to prevent organ rejection. However, its clinical application has been

impeded by the toxicity and complexity of current recipient conditioning regimens. We and others have been focusing on developing reduced intensity host condition protocols in murine models. However, several issues remain in the current conditioning protocols, which prevent the translation of these protocols into clinical applications. The limitations include the use of irradiation, high multiple doses of chemotherapeutics, and a mega dose of BMC as well as thrombogenic anti-CD40L mAb. Moreover, allogeneic chimerism induction is more difficult to achieve in tolerance defective recipients that develop autoimmune disease, such as diabetes-prone NOD mice, when compared to non-autoimmune strains.[205,234,236,239,250] We showed here that a robust T cell depletion by an optimized DST-CYP and T cell depleting mAb combination leads to success in generating chimerism in NOD mice even when using a clinically relevant amount of fully allogeneic BMC, without the inclusion of irradiation, costimulation blockade, and rapamycin.

We demonstrated that the key factor in our current protocol is the administration of DST-CYP and anti-CD4/8/90 mAbs. First, we confirmed that DST-CYP led to the preferential inhibition of allostimulated T cells, which was likely due to the killing of these cycling cells. Moreover, NOD recipients conditioned with the triple protocol without DST only developed transient chimerism, with lower levels of donor cells at four weeks post-BMT (Fig 3-7A and 3-7B). As the inhibition of donor-specific T cells is not sufficient without DST, the alloreactive naïve and memory T cells that survived CYP treatment might be activated after BMT. Such alloreactive T cells, mainly $CD4^+$ T cells, then increased in frequency in lymphopenic hosts (Fig 3-7C) and rejected all the donor cells rapidly.

T cell depletion mediated by either monoclonal or polyclonal antibodies has been used for solid organ transplantation and hematopoietic chimerism induction for several decades.[283]

Although the employment of such antibodies could eliminate over 90% of T cells in the periphery in most cases, the depletion of T cells is less efficient in spleen, thymus, and tissues. In addition, memory T cells are resistant to antibody-mediated depletion compared to naïve T cells.[284] Moreover, T cell depletion creates a space and resource enriched microenvironment for residual T cells, which then undergo lymphopenia-induced proliferation (LIP) and are more likely acquire the phenotype of effector/memory T cells.[284,285] Such T cells are more resistant to tolerance induction. Therefore, it is important that we employed DST-CYP before T cell depleting mAbs because dividing T cells driven by specific antigens have been shown to be more sensitive to CYP compared to T cells undergoing LIP.[286] Thus, DST-CYP before the application of T cell depleting mAbs reduces the overall donor-reactive T cells and prevents the enrichment of such host T cells in LIP post T cell depletion and BMT.

Although using T cell depleting mAb for chimerism induction in NOD mice is not new, T cell depletion was mainly used before and shortly after BMT with irradiation and/or costimulation blockade.[237,239,241,243,247-249] We employed extended T cell depletion post-BMT as it provides a prolonged window for the development of donor hematopoietic cells and the education of both donor and recipient T cells. Indeed, the ability to induce chimerism was lost if fewer doses of T cell depleting mAbs were included in the triple protocol, and chimerism was not rescued by adding extra NK depleting mAbs or BMC (Table 3-1). In addition, CYP is a cytoreductive reagent that not only reduces T cells in the periphery but also decreases immature thymocytes in the thymus.[287] The combination of CYP and sufficient T cell depleting mAbs further postpones the recovery of the host T cell repertoire.

Regulatory T cells (Tregs) have been shown to be important for chimerism induction.[268] Although they are as sensitive to DST, CYP[288], and T cell depleting antibodies[284] as conventional T cells, Tregs might still play an important role for generating chimerism in our protocol. Specific destruction of donor reactive T cells is essential for infused donor specific Tregs to prolong graft survival.[272] In addition, donor specific Tregs can be induced during the process of chimerism induction and exert immune regulatory function.[289] In fact, using our protocol, the frequency of host Tregs did increase early after BMT despite being reduced in absolute number (Fig 3-8). Although we surmise that the need for recipient Tregs may depend on the efficacy of T cell depletion, this hypothesis has yet to be tested in detail.

Table 3-1 Extended T cell depletion post-BMT is needed for chimerism induction in naïve NOD mice

Donor	αCD4/8/90	Donor BM ($\times 10^{-6}$)	Chimerism
FVB	×2	20 or 30	0/4
FVB or C3H	×3	10 or 20	0/4
C3H	×4	(10 or 20)×2‡	0/2
C3H	×5	20	3/3
FVB	×9†	20	2/2

NOD recipients were conditioned with DST (day -10), CYP (day -8), anti-CD4/8/90 (day -6 and every 5 days afterwards; ×2, ×3, ×4, ×5, ×9 indicate 2, 3, 4, 5, 9 doses; † mice were injected on day -6, -1, 4, 9, 15, 21, 27, 34, 41), anti-Asialo GM1 (day -6 and day -1, 20 µl), BUS (day -1) and BMC (day 0; 10, 20, or 30×10^6; ‡ mice were infused with one more dose

of BMC on day 9; cells were from the same donor strain for DST). Chimerism levels were determined at 4 weeks post BMT.

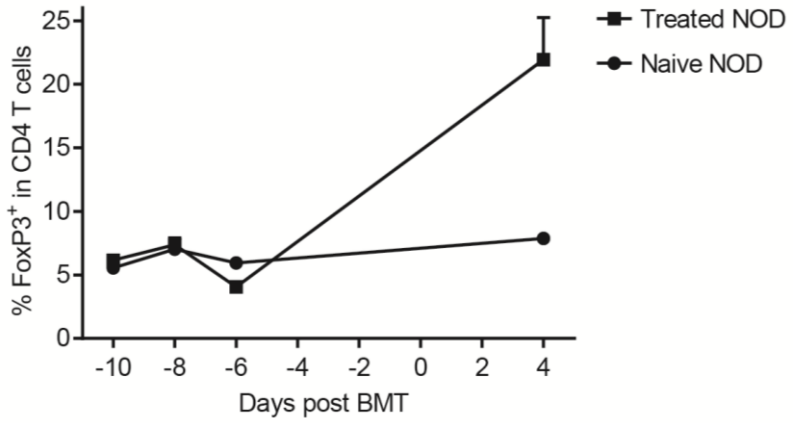

Figure 3-8 Tregs frequency increased after treatment.

Shown are the frequencies (mean ± SEM) of $FoxP3^+$ cells in $CD4^+$ T cells from naive NOD (n=1) and NOD recipients (n=4) conditioned with DST (day -10), CYP (day -8), T cell depletion mAbs (anti-CD4/8 ± anti-CD90, day -6, -1), BUS (day -1) and FVB BMC (day 0, 20×10^6). Data were from one experiment.

By using the DST-CYP triple antibody protocol, we induced chimerism in NOD mice with two different fully MHC and multiple minor antigen mismatched donors, achieving a very high level of chimerism. As the induction of complete chimerism requires a more complex and intensive conditioning protocol, it was surprising that we established complete chimerism in NOD mice with such a simplified conditioning protocol without the help of irradiation, costimulation blockade, nor mega doses of BMC. By using C3H donors,

induction of chimerism seemed to be easier, as the inclusion of anti-CD90 mAbs was unnecessary (Fig 3-2B); however, chimerism was less stable.

Some C3H→NOD mice were found sick at around 6 to 11 weeks post-BMT (Fig 3-2B and G). The morbidity in these mice was characterized as acute weight loss, hunched posture, and paleness but without skin lesions or signs of diarrhea (data not shown), which could be due to the toxicity of the conditioning regimen, GVHD, or the failure of bone marrow engraftment. In contrast, almost all recipients given FVB bone marrow and triple antibodies became stable full chimeras and remained healthy for at least 20 weeks post-BMT. In addition, body weight for FVB→NOD chimeric mice steadily increased (Fig 3-4A). Considering that FVB→NOD mice remained healthy, the morbidity in some C3H→NOD chimeras was unlikely to be due to the conditioning regimen.

GVHD has been associated with the presence of passenger T cells in the BM.[290] However, passenger T cells in BM would be expected to be targeted by the anti-T cell mAbs used in our protocol with C3H donors. In addition, the inclusion of CYP and T cell depleting mAbs might help prevent GVHD, as both CYP[291,292] and anti-thymocyte globulin[293] are effective for GVHD prophylaxis in the clinic. Moreover, GVHD can be avoided in patients with full chimerism with HLA mismatched donor cells.[294] With the anti-CD4/8/90 conditioning regimen, stable FVB→NOD (Fig 3-3A) and C3H→NOD chimeras tended to be complete rather than mixed chimeras, with full donor T cell chimerism (Fig3-3B). All of these chimeras were free of signs of GVHD. Therefore, complete donor chimeras induced by using a robust T cell depletion protocol can be GVHD free.

Another concern is the potential that complete chimeras will have some immunodeficiency. Though the rejection of skin graft from a 3rd party that is MHC

mismatched to either NOD or BMC donor is commonly used for determining immunocompetence of chimeras, it is not a stringent test as the natural frequency of T cells against allo-MHC is high. We showed here that FVB→NOD chimeras were able to reject skin from an MHC matched B6.H-2^{g7} donor and produced antibodies in response to OVA immunization, although the rejection was delayed and the titer of anti-OVA Abs was lower when compared to otherwise much younger naïve NOD mice. However, it is unclear that this would be a substantial issue, as patients with full chimerism with HLA mismatched donor cells have appeared fully immunocompetent in other studies.[294,295] On the other hand, complete chimerism is arguably favorable in autoimmune recipients, as this would more fully eliminate the cells responsible for autoimmunity. We showed here that stable NOD chimeras were free of autoimmune diabetes without substantial insulitis (Fig 3-4D).

Albeit chimerism was induced with 100% success at four weeks post-BMT using the triple antibody protocol, not all the chimeras maintained stable chimerism. Similar to the C3H→NOD chimeras treated with the duo antibody protocol, a slow decline of chimerism in NOD mice is common in the literature. In contrast, unstable chimeras in our study lost their high-level chimerism acutely, which has not been reported previously. On the one hand, transient mixed chimerism has been shown to be invaluable for induction of operational tolerance in allogeneic organ transplantation.[270] Here, we also provide a new strategy for inducing transient high-level chimerism without using irradiation and costimulation blockade. Although donor specific tolerance has not been tested in transient chimeras, these NOD recipients never developed hyperglycemia (Fig 3-4A). Whether or not such transient chimerism can be used for resolving autoimmunity remains unknown. On the other hand, by investigating the difference in early chimerism levels between stable and unstable chimeras,

we found that both a lower early overall level of chimerism and a lower donor T cell level were significantly associated with the instability of long-term chimerism. Though other groups have recorded donor cell levels starting from two weeks post-BMT, none have reported at these earlier time points. Closely monitoring chimerism in this early window after BMT might give us some hints for the important events and cell subsets for stable chimerism induction. It also provides an opportunity for early intervention, and thus better more personalized conditioning protocols.

Although we have successfully eliminated the use of irradiation, anti-CD40L mAbs and rapamycin in our current protocol, and avoided a mega dose of BMC, there are some limitations in our approach. First, we still employed chemotherapeutic drugs, CYP and BUS, which could be improved by examining the efficacy of lower doses or replacement with other bioreagents. For instance, BUS could be replaced with anti-c-kit and anti-CD47 mAbs for creating a niche in host bone marrow[296] without radiation or chemotherapy. Second, anti-CD4/8/90 mAbs are not available for use in humans. Replacement of anti-CD4/8/90 mAbs with ATG, anti-CD52 mAbs (alemtuzumab), or other T cell depleting antibodies might be required for better T cell depletion. Third, the lymphopenia-induced proliferation of T cells has been associated with the development of alternative forms of autoimmunity in humans, such as in patients with multiple sclerosis who frequently develop thyroid autoimmunity post anti-CD52.[297] Whether or not this would be the case after allogeneic bone marrow transplantation remains unclear. Fourth, the preferential sparing of donor $CD8^+$ T cells was associated with stable chimerism in FVB→NOD chimeras. Although this is not clinically relevant, giving donor $CD8^+$ T cells[250] or other donor cells[294] that facilitate BMC engraftment is clinically feasible. Fifth, although chimerism could be induced in naïve NOD mice that

were not yet diabetic using the triple antibody protocol, chimerism in some mice was not stable. Further adjustment of the current protocol or early intervention is needed to improve the chances of stable chimerism. Finally, chimerism induction in spontaneously diabetic NOD mice is more challenging.[143] Whether chimerism could be generated in diabetic NOD mice with our current protocol remains to be examined.

Thus far, we achieved high-level chimerism and transplant tolerance in tolerance induction resistant NOD recipients given clinically feasible amount of donor BMC, *via* robust T cell depletion through the combination of DST-CYP and T cell depleting mAbs without the need for irradiation, costimulation blockade, and rapamycin. This protocol is, to our knowledge, the most clinically feasible to have achieved fully allogeneic mixed chimerism in NOD mice. Furthermore, unlike our previous protocol that successfully generated stable mixed chimerism in NOD mice,[249] the current protocol induced robust donor specific tolerance as evidenced by the acceptance of the most immunogenic donor tissue graft, skin. Achieving such a complete state of tolerance is likely to be even more critical in humans, where infectious agents have the potential to trigger heterologous immunity and graft rejection.[298,299] Lastly, our data point out the importance of the early window post-BMT for developing successful personalized chimerism induction protocols. We provided here a way to induce stable or transient chimerism by maximizing T cell depletion. Transient chimerism is frequently observed in combined kidney and hematopoietic stem cell transplantation.[300] Our findings open the posibility of identifying early-on those patients that might lose their chimerism at later time points. Intervening to increase the stability of chimerism can be anticipated to reduce the posibility of organ/tissue rejection in these selected patients.

Chapter 4

A T cell depletion conditioning protocol with donor CD8α cell infusion overcomes age-dependent resistance to chimerism allowing tolerance to fully allogeneic islets in autoimmune diabetic NOD mice

4.1 Introduction

Hematopoietic chimerism *via* BMT from the same donor would, in theory, be the most effective way to induce tolerance to donor organs and resolve underlying autoimmune disease in T1D patients, who need islet and/or kidney transplantation.[208,258-260,301] Chimerism eliminates the need for lifelong immunosuppressants, reducing undesired complications. Chimerism for organ transplantation has been tested in three clinical centers for kidney transplantation, with promising results, although safety remains a significant issue.[187,211-213,261,302,303] With sufficient numbers of islets and HSC from one donor, this approach is also possible for islet/kidney transplantation for brittle T1D.[304,305] However, the application is limited by whole body irradiation conditioning protocols, the risk of life-threatening GVHD, the requirement of some degree of MHC matching, and the need of a mega dose of HSC.[212,261,303]

Using NOD mice, widely considered the best mouse model of human T1D, our lab has developed a translatable chimerism induction protocol for islet transplantation with the ability to overcome the above limitations.[306] NOD mice are resistant to tolerance induction, which makes them a very stringent model.[222] We found that a robust recipient T cell depletion allows stable fully mismatched chimerism and donor-specific tolerance in young prediabetic NOD mice while using an irradiation free and clinically feasible protocol.[249,306] However, chimerism induction is even more challenging in NOD mice that have already spontaneously become diabetic, as compared to young NOD mice that are not yet diabetic.[143,246,248] While

the exact reasons for such resistance in diabetic NOD mice remain unclear, previous studies have not ruled out the possibility that recipient age may be the determining factor.

Here, we aimed to develop a protocol for inducing chimerism and tolerance to allogeneic islets in NOD recipients that have become diabetic. Our T cell depletion based protocol induced only transient chimerism in old-diabetic NOD mice with significantly prolonged survival of donor islets in chimeric mice. We found that age rather than overt diabetes was associated with the resistance to chimerism. Since preclinical studies have shown that donor CD8α$^+$ cells are able to facilitate engraftment of allogeneic HSC, we tested whether they could overcome the age related resistance to chimerism.[254] We found that the delayed infusion of donor CD8α$^+$ cells facilitated the establishment of a high level and stable multilineage donor chimerism and the acceptance of donor islets without the presence of graft versus host disease in old-diabetic NOD mice.

4.2 Results

4.2.1 Age rather than overt diabetes is associated with resistance to chimerism

We first tested our T cell depletion based chimerism induction protocol in 'young' and 'old-diabetic' NOD mice. Details of the experimental groups are shown in Fig 4-1 A-C. As shown in Fig 4-1D and 4-1E, all four young NOD mice became stable and full chimeras, which is consistent with our previous data.[306] In contrast, in 'old-diabetic' recipients, only 10 of 14 mice became chimeric at four weeks post-BMT; nine of these ten recipients lost their chimerism over time. At twelve weeks post BMT, only one old-diabetic recipient was still

chimeric. (Fig 4-1D) Thus, it is more challenging to induce chimerism in autoimmune diabetic NOD mice as compared to young non-diabetic NOD hosts.

In previous studies, the generation of chimerism was even more difficult in autoimmune diabetic NOD mice when compared to young NOD mice.[143,246,248] As the spontaneously diabetic NOD mice used in these studies were more than four weeks older than young and non-diabetic NOD mice, we hypothesized that the increased age in spontaneously diabetic NOD mice rather than the status of diabetes contributes to the resistance to chimerism induction in these recipients. As shown in Fig 4-1D, the generation of chimerism in 'old' non-diabetic NOD mice was at least as difficult, if not more so, when compared to 'old-diabetic' NOD mice. Only two of six recipients became chimeric with donor cells. Chimerism in these two mice was lost at six and eight weeks post-BMT (Fig 4-1D). Therefore, age rather than the overt diabetes status was associated with increased resistance to chimerism in older NOD mice.

We have previously shown that the levels of chimerism at very early time points, well before reaching peak chimerism levels, were associated with the long term chimerism stability.[306] We then investigated if the early chimerism levels in 'old' or 'old-diabetic' recipients were lower compared to those in 'young' NOD mice. As shown in Fig 4-1E, the chimerism levels in both 'old' and 'old-diabetic' groups were significantly lower as compared to those in 'young' mice as early as day four post-BMT. Chimerism levels steadily increased until full chimerism was reached at day 28 post-BMT in 'young' recipients, consistent with our previous data.[306] The increase of donor chimerism levels in both 'old' and 'old-diabetic'

recipients was also observed during the first four weeks post-BMT. However, the average donor chimerism levels only reached around 40% in chimeric ‘old-diabetic’ NOD mice at day 28 post-BMT. In ‘old’ recipients, the average level of donor cells peaked at day 14 post-BMT at around 40% and started to decline. (Fig 4-1E)

Briefly, we found that transient chimerism can be achieved in diabetic NOD recipients with a T cell depletion based protocol. We determined that age rather than overt diabetes was associated with resistance to chimerism induction. The increased resistance to chimerism induction in ‘old’ and ‘old-diabetic’ NOD mice was associated with lower levels of donor chimerism very early after BMT.

A

Recipients (n)	Age (weeks)	Diabetic	BMT	Islet transplant
Young (4)	8	No	Yes	No
Old (6)	15-20	No	Yes	No
Old-diabetic (14)	12-26	Yes	Yes	Yes/No*

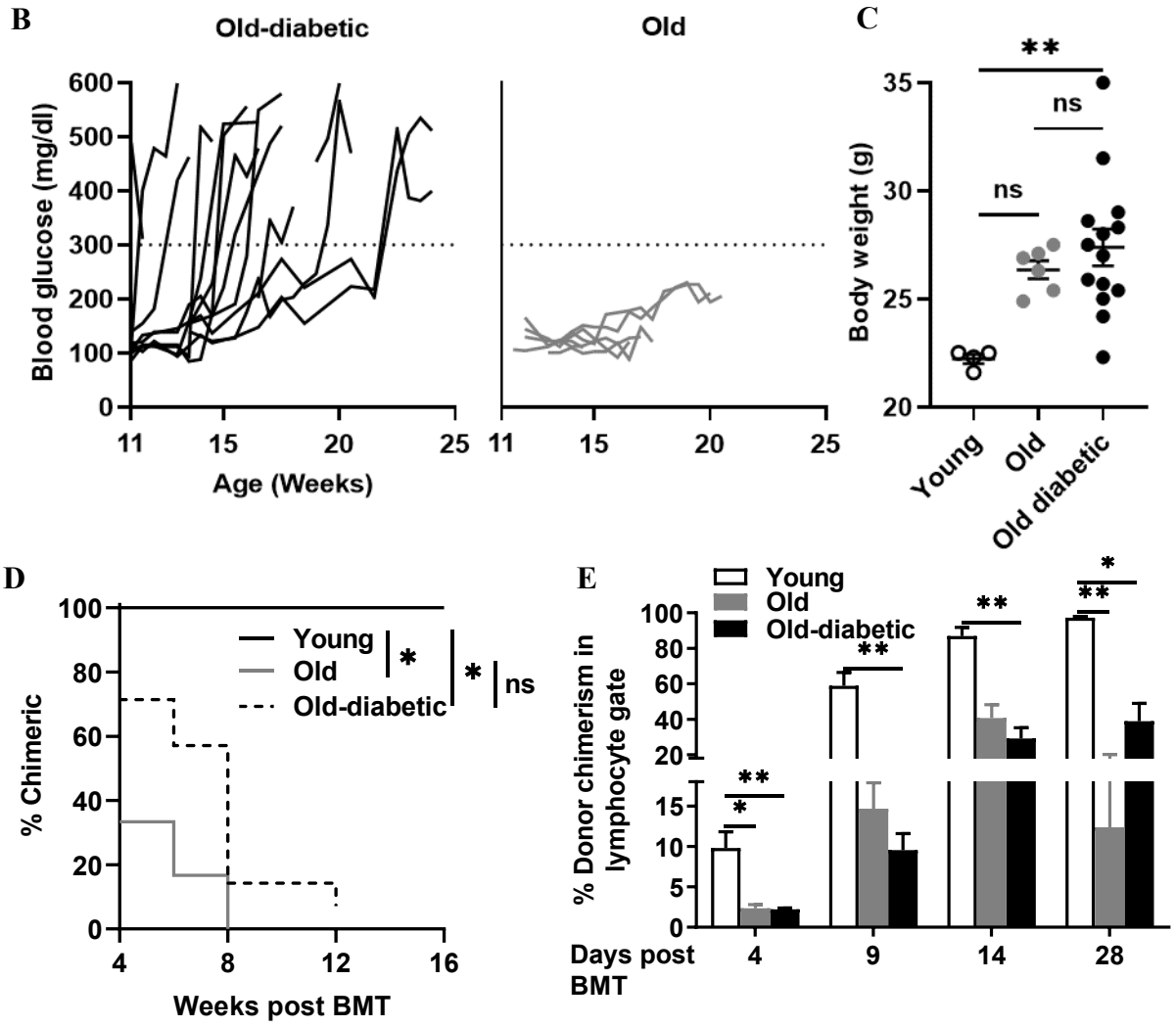

Figure 4-1 Age rather than overt diabetes is associated with resistance to chimerism.

(A) Shown are characteristics of NOD recipients used for chimerism induction. * denotes nine 'old-diabetic' NOD mice received islet grafts and five mice did not receive islet transplantation. (B) Shown are blood glucose levels of 'old-diabetic' NOD mice between 11 weeks of age and the diagnosis of diabetes (left panel) and blood glucose levels of 'old' NOD

mice before chimerism induction (right panel). The dashed line represents the criteria for diabetes diagnosis. (C) Shown are body weights of the three groups of recipients (mean ± SEM). Kruskal-Wallis test with Dunn's multiple comparisons test was used for the comparisons shown and denoted ** $p<0.01$. (D-E) NOD recipients were conditioned with DST (day −10), CYP (day −8), a combination of anti-CD4/8/CD90 mAb (days −6, −1, 4, 9, 14), busulfan (day −1) and bone marrow transplantation (BMT, day 0). (D) Shown are the percentages of recipients that were chimeric at designated time points post BMT. Log-rank (Mantel-Cox) test with Bonferroni correction with * $p<0.05$. (E) Shown are percentages of donor cells in the lymphocyte gate in the peripheral blood at designated time points post-BMT (mean ± SEM). The calculation include all mice with 'young' n=4 for all time points; 'old' n=6 for all time points; 'old-diabetic' n=11 for day 4, n=9 for day 9 and 14, n=14 for day 28. Kruskal-Wallis test with Dunn's multiple comparisons test was used for the comparisons shown and denoted * $p<0.05$ and ** $p<0.01$. Data were pooled from more than eight independent experiments.

4.2.2 Bone marrow transplantation significantly prolongs the survival of donor islets

Transient chimerism has been shown to be associated with prolong kidney and lung acceptance, but not for islet nor heart allografts.[216-218,307,308] However, it is unclear whether this difference in allograft tolerance toward different organs/tissues induced *via* transient chimerism depends only on the types of organs/tissues or also the induction protocols. We asked if the transient chimerism induced with our T cell depletion based conditioning protocol can prolong the survival of allogeneic islets in recipients even when chimerism was lost. As shown in Fig 4-2A, the survival of donor islets was significantly prolonged in 'old-diabetic' recipients given the whole conditioning protocol as compared to recipients that were conditioned but did not receive BMT. The one mouse with stable chimerism maintained euglycemia over three months without the need for chronic immunosuppression (Fig 4-2B). In the BMT group, three recipients became hyperglycemic at four or six weeks post BMT when the donor cells could not be detected in the periphery (Fig 4-2B). One BMT recipient rejected donor islets two weeks after the complete loss of donor chimerism at eight weeks post-BMT (Fig 4-2B). To our surprise, the other three mice that lost their chimerism at eight weeks post-BMT maintained normal blood glucose for more than four weeks after the rejection of donor BMC (Fig 4-2B).

To test the stringency of tolerance to the donor islets, we immunized these three transient chimeric recipients with a dose of donor cells in order to boost their anti-alloantigen response. As shown in Fig 4-2B, one of the three mice became hyperglycemic one day after challenge.

The other two mice stayed euglycemic more than two weeks post the infusion of donor cells (Fig 4-2B).

In short, simultaneous bone marrow and islet transplantation prolonged the survival of donor islets. Transient chimerism induced with a T cell depletion based protocol can improve the survival of donor islets even after the donor chimerism is lost, but only in less than half of recipients. In addition, the stringency of such tolerance may be fragile.

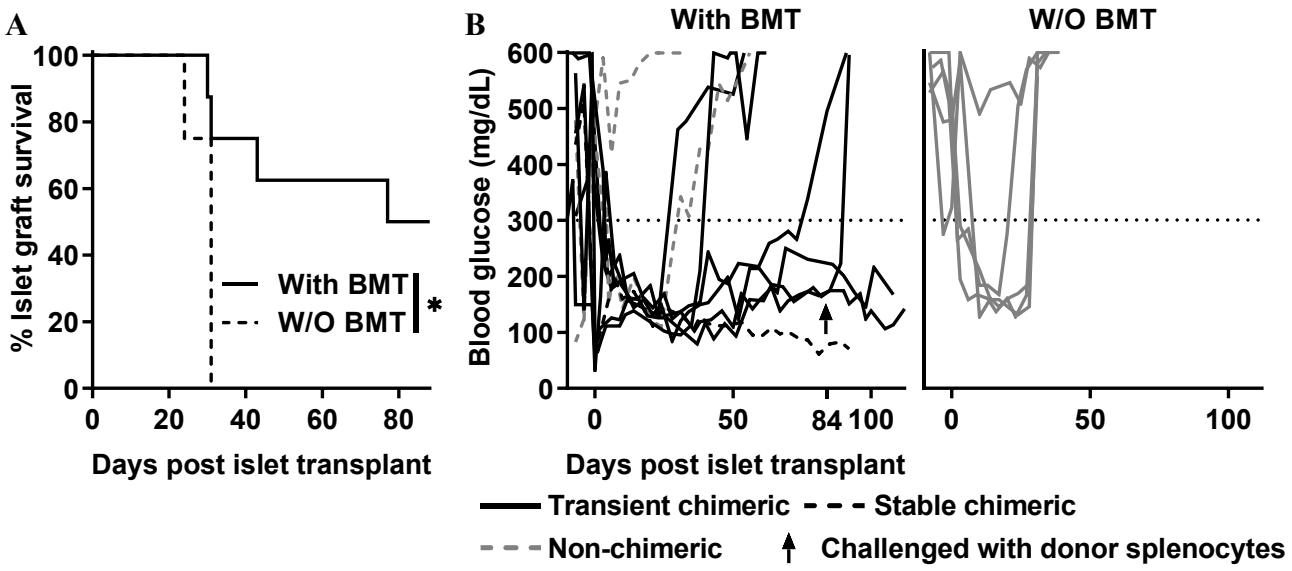

Figure 4-2 Transient chimerism can significantly prolong the survival of donor islets.

(A) Shown are the proportions of recipients that became and maintained euglycemia in 'Old-diabetic' recipients with the conditioning and BMT containing protocol (n=9) or conditioning without BMT protocol (n=5) at the designated time points post islet transplantation. Log-rank (Mantel-Cox) test with * $p<0.05$. (B) Shown are blood glucose levels before and after islet transplant in mice with the conditioning and BMT containing protocol (left panel) and

conditioning without BMT protocol (right panel). Solid black lines represent recipients that became chimeric and lost their donor chimerism at six or eight weeks post-BMT. Dashed gray lines represent recipients that did not become chimeric at four weeks post-BMT. The dashed black line represents one NOD mouse with stable chimerism. The arrow represents an infusion of donor splenocytes i.p. at day 84 post-BMT in three transient chimeric recipients that still maintained normal blood glucose at the time. Solid gray lines are representative curves of blood glucose in mice conditioned without BMT.

4.2.3 Circulating T cells in old and diabetic NOD are depleted similarly to those in young mice but recover quickly after BMT

Recipient T cells are the major barrier towards chimerism induction in young NOD mice that are not yet diabetic.[249,306] We asked if the increased resistance to chimerism induction in 'old' and 'old-diabetic' NOD mice is due to a resistance to T cell depletion as compared to 'young' NOD mice. As shown in Fig 4-3A, the levels of recipient T cells were slightly higher in 'old' and 'old-diabetic' mice at five days post the first dose of T cell depleting mAbs (4 days post-BMT) when compared to those in 'young' mice. After the second dose of T cell depleting mAbs, recipient T cell levels remained similar among the three groups at day 4, 9, and 14 post-BMT.

While T cells were depleted efficiently in all three groups of mice, the recovery of recipient T cells was much faster in 'old' and 'old-diabetic' recipients, in contrast to those in

'young' NOD mice. As shown in Fig 4-3A, recipient T cells repopulated to around 40% in both 'old' and 'old-diabetic' recipients at four weeks post-BMT, whereas recipient T cell levels in 'young' NOD mice remained low. A detailed analysis revealed that the majority of the T cells in these recipients were $CD4^+$ T cells (Fig 4-3B).

We then investigated whether or not the rapid repopulation of recipient T cells was in response to donor BMC. To answer this, a group of 'old-diabetic' NOD mice was conditioned with a standard protocol deprived of BMT. As shown in Fig 4-3C, the levels of recipient $CD4^+$ and $CD8^+$ T cells were similar between 'old-diabetic' mice treated with the whole conditioning protocol or a protocol lacking BMT at both day 14 and 28 post-BMT, suggesting the fast repopulation of recipient T cells was independent of the presence of donor BMC.

Lymphopenia induced proliferation and thymic output are two main sources of recovery after T cell depletion.[309] A functional thymus is critical for a successful reconstitution of the immune system after BMT and tolerance to donor antigens.[310] We next examined if the recipient thymus was involved in the recovery of recipient T cells. To answer this, we generated NOD.Rag2pGFP mice and used them as BMT recipients once they were spontaneously diabetic. In these mice, GFP is expressed under the promotor of the Rag2 gene during T cell development and GFP is lost over time upon cell division, such that only recent thymic emigrants (RTEs) are GFP positive.[255] As shown in Fig 4-3D, the proportion of GFP^+ $CD4^+$ T cells at day 14 post-BMT increased significantly as compared to those before conditioning. More important, the brightness of the GFP signal also increased at day 14 post-BMT compared to 10 days before BMT. This increase of GFP MFI suggested that GFP^+ T

cells detected at day 14 were truly RTE newly exported from the thymus but not RTE that survived T cell depletion.

Briefly, recipient T cells in ‘old’ and ‘old-diabetic’ NOD were depleted efficiently, similar to young mice but recovered quickly after BMT and this recovery included a substantial contribution from newly generated T cells.

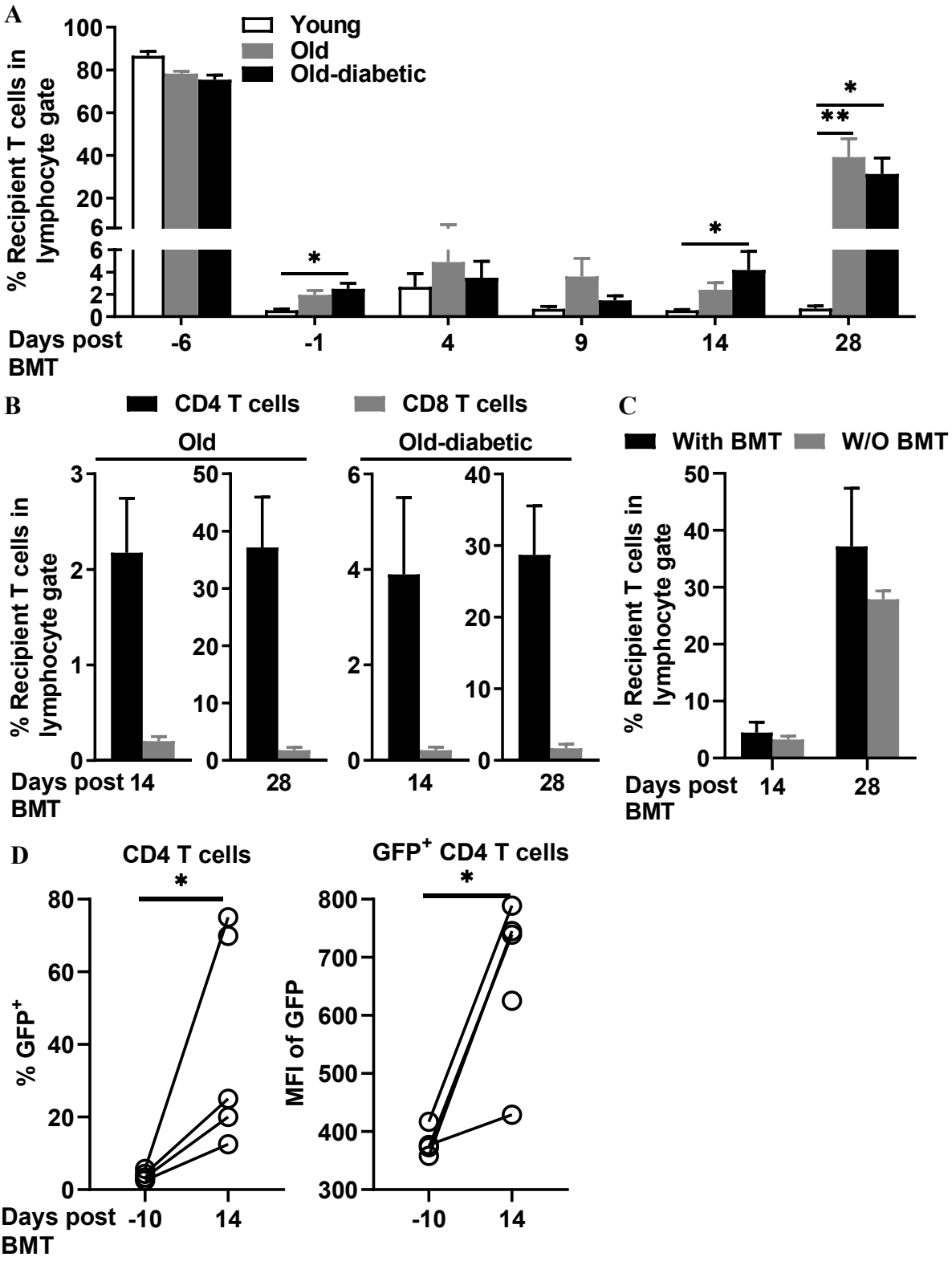
A
Young
Old
Old-diabetic
% Recipient T cells in lymphocyte gate
Days post BMT
-6
-1
4
9
14
28
B
CD4 T cells
CD8 T cells
Old
Old-diabetic
% Recipient T cells in lymphocyte gate
Days post BMT
14
28
C
With BMT
W/O BMT
% Recipient T cells in lymphocyte gate
Days post BMT
14
28
D
CD4 T cells
GFP+ CD4 T cells
% GFP+
MFI of GFP
Days post BMT
-10
14

Figure 4-3 Recipient T cells in old and diabetic NOD are depleted but recover quickly after BMT with a substantial contribution by thymic output.

(A) Shown are the proportions of recipient T cells in peripheral blood at designated time points post-BMT in three groups of recipients. Shown are mean ± SEM. 'young' n=4 for all time points; 'old' n=6 for all time points; 'old-diabetic' n=11 for day 4, n=9 for day 9 and 14, n=14 for day 28. Kruskal-Wallis test with Dunn's multiple comparison test with denoted * $p<0.05$ and ** $p<0.01$ for each time point. (B) The proportion of recipient $CD4^+$ and $CD8^+$ T cells at day 14 and 28 post-BMT in 'old' and 'old-diabetic' recipients treated with our standard protocol. (C) The proportion of recipient T cells at day 14 and 28 post-BMT in 'old-diabetic' recipients treated with the standard BMT containing protocol (n=9) or non-BMT containing protocol (n=5). (D) Shown are 'old-diabetic' NOD.Rag2p-GFP mice (n=5) treated with the standard protocol. The proportion of recipient $CD4^+$ T cells that were GFP positive at designated time points post-BMT were shown on the left. Mean fluorescence intensity (MFI) of GFP from these GFP positive $CD4^+$ T cells are shown on the right. Day 14 data were obtained before mAb injection. Ratio paired *t* test with denoted * $p<0.05$. (C-D) Mice in these Figures received donor islet transplant at day 0 post-BMT.

4.2.4 Delayed infusion of donor CD8α$^+$ cells facilitates the establishment of chimerism and the acceptance of donor islets without GVHD

We previously found that the presence of donor T cells early after BMT was associated with the long term stability of donor chimerism in 'young' NOD mice.[306] We asked whether the failure to generate stable chimerism in 'old' and 'old-diabetic' recipients was associated with lower levels of donor T cells after BMT. Consistant with our previous findings, donor CD4$^+$ or CD8$^+$ T cells were readily detected starting from day 9 post-BMT in 'young' NOD mice. However, donor T cells were not detectable in either 'old' or 'old-diabetic' recipients until day 28 post-BMT. The levels of donor CD8$^+$ T cells were significantly higher in 'young' NOD mice as compared to 'old-diabetic' recipients even as early as day 9 and 14 (Fig 4-4A). Moreover, the levels of donor CD8$^+$ T cells was correlated with donor chimerism levels at day 9 post BMT in all mice. (Fig 4-4B)

Preclinical studies have unveiled subsets of resident cells in the bone marrow that are able to facilitate engraftment of allogeneic HSC, namely facilitating cells, such as CD8$^+$ cells.[311] Similarly, donor splenic CD8$^+$ T cells have also been shown to promote the bone marrow engraftment in young NOD mice, that were also given with a mega-dose of BMC.[250] We next investigated if supplementation of donor peripheral CD8$^+$ cells was able to convert transient chimerism into stable chimerism in 'old-diabetic' NOD mice. 'Old-diabetic' NOD mice were conditioned with the T cell depletion based conditioning protocol as well as a dose of donor CD8α$^+$ splenocytes at day 19 post-BMT. As shown in Fig 4-5A, all of the five mice became stably fully chimeric with donor cells. Multiple lineages of donor cells were detected

in the periphery. Four diabetic recipients given donor islet grafts were able to maintain normal glycemia for more than 240 days (Fig 4-5B). To examine if the islet graft was responsible for reversal of diabetes, three of four islet recipients were subjected to recovery nephrectomy. As expected, all three mice returned to a hyperglycemic state within two days after donor islet grafts were removed (Fig 4-5B). Thus, a conditioning protocol that included a delayed infusion of donor $CD8\alpha^+$ cells generated stable chimerism and the long term survival of donor islet grafts in 100% of recipients without chronic immunosuppressants.

No signs of GVHD were found in these chimeric recipients given donor $CD8\alpha^+$ cells. As shown in Fig 4-5C, no chronic decline of body weight was observed in recipients of $CD8\alpha^+$ cells. Deletion of self-reactive T cell clones is one of the mechanisms for self-tolerance.[312] We examined if donor anti-host T cell clones were deleted in chimeras. In FVB→NOD chimeras, the donor $CD4^+$ T cells expressing Vβ17a bind to NOD-derived superantigen in the presence of the NOD MHC-II I-A^{g7}.[282] This binding leads to a decrease of donor $V\beta17a^+$ $CD4^+$ T cells, which 'mimics' the process of central deletion to conventional peptide antigens in the thymus. As shown in Fig 4-5D, the frequencies of donor $V\beta17a^+$ $CD4^+$ T cells were significantly lower in both young FVB→NOD chimeras treated with our standard protocol and diabetic FVB→NOD chimeras given the standard conditioning protocol plus donor $CD8\alpha^+$ splenocyte infusion, as compared to the $V\beta17a^+$ $CD4^+$ T cell frequencies in untreated FVB mice. The reduction of donor $V\beta17a^+$ $CD4^+$ T cells suggests there was central deletion of recipient-reactive T cells in the chimeras.

Taken together, addition of a delayed infusion of donor CD8α$^+$ cells to the T cell depletion conditioning allowed generation of stable chimerism and the acceptance of donor islets in autoimmune diabetic NOD mice with the involvement of deletional tolerance mechanisms.

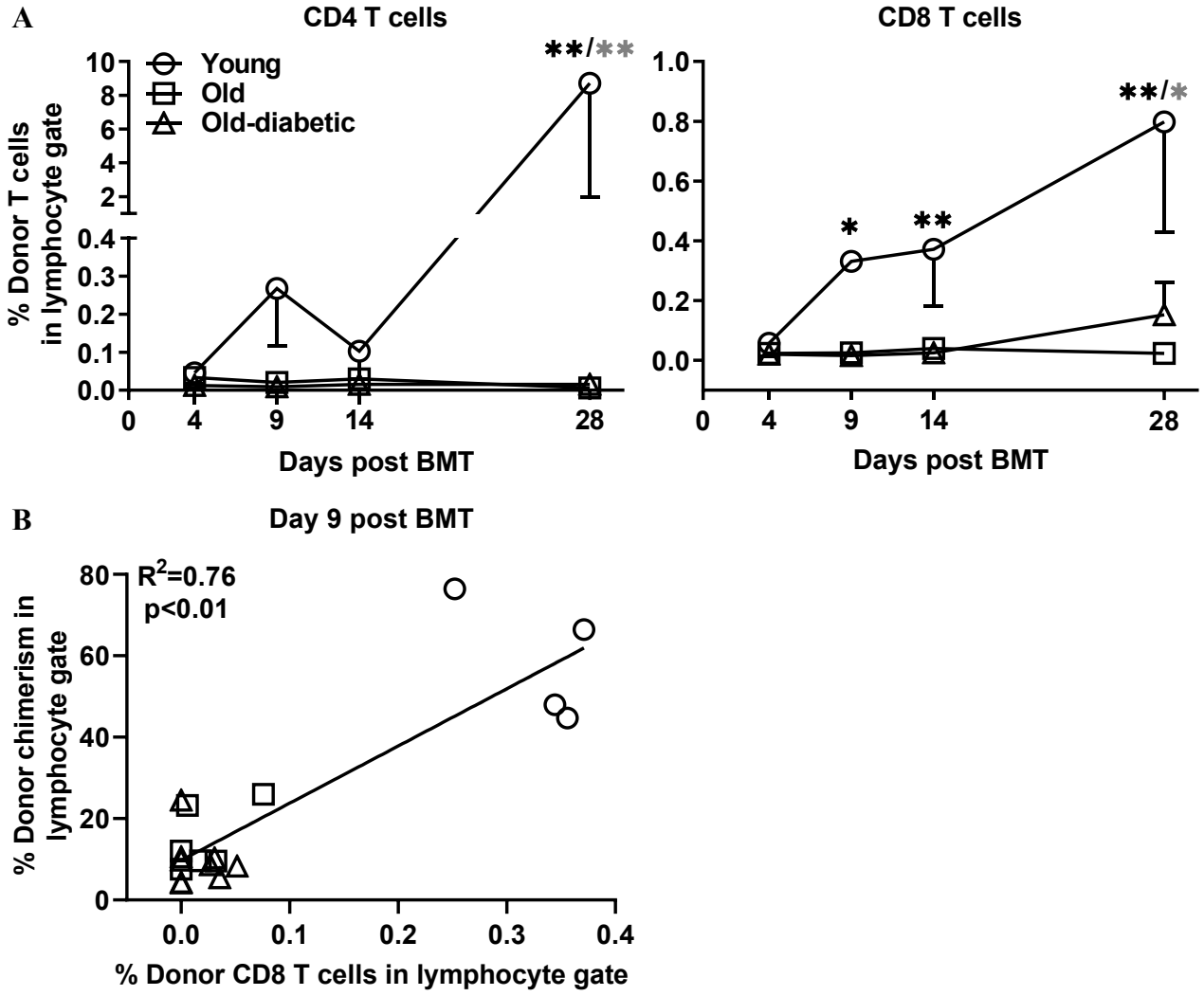

Figure 4-4 Levels of donor T cells early after BMT are lower in autoimmune diabetic and aged recipients

NOD recipients were given the standard conditioning BMT (FVB donor) protocol (see Fig 4-1). (A) Shown are the proportions of donor T cells in the lymphocyte gate in peripheral

blood lymphocytes with $CD4^+$ T cells on the left and $CD8^+$ T cells on the right. Kruskal-Wallis test with Dunn's multiple comparisons test was used to compare data from three groups at each time point with denoted * $p<0.05$ and ** $p<0.01$. Black asterisks denote comparisons between 'young' and 'old-diabetic'; gray asterisks denote comparisons between 'young' and 'old'. (B) Shown is the correlation between donor $CD8^+$ T cell and donor chimerism levels at day 9 post BMT. Pearson correlation coefficients was computed with $R^2=0.76$, $p<0.01$.

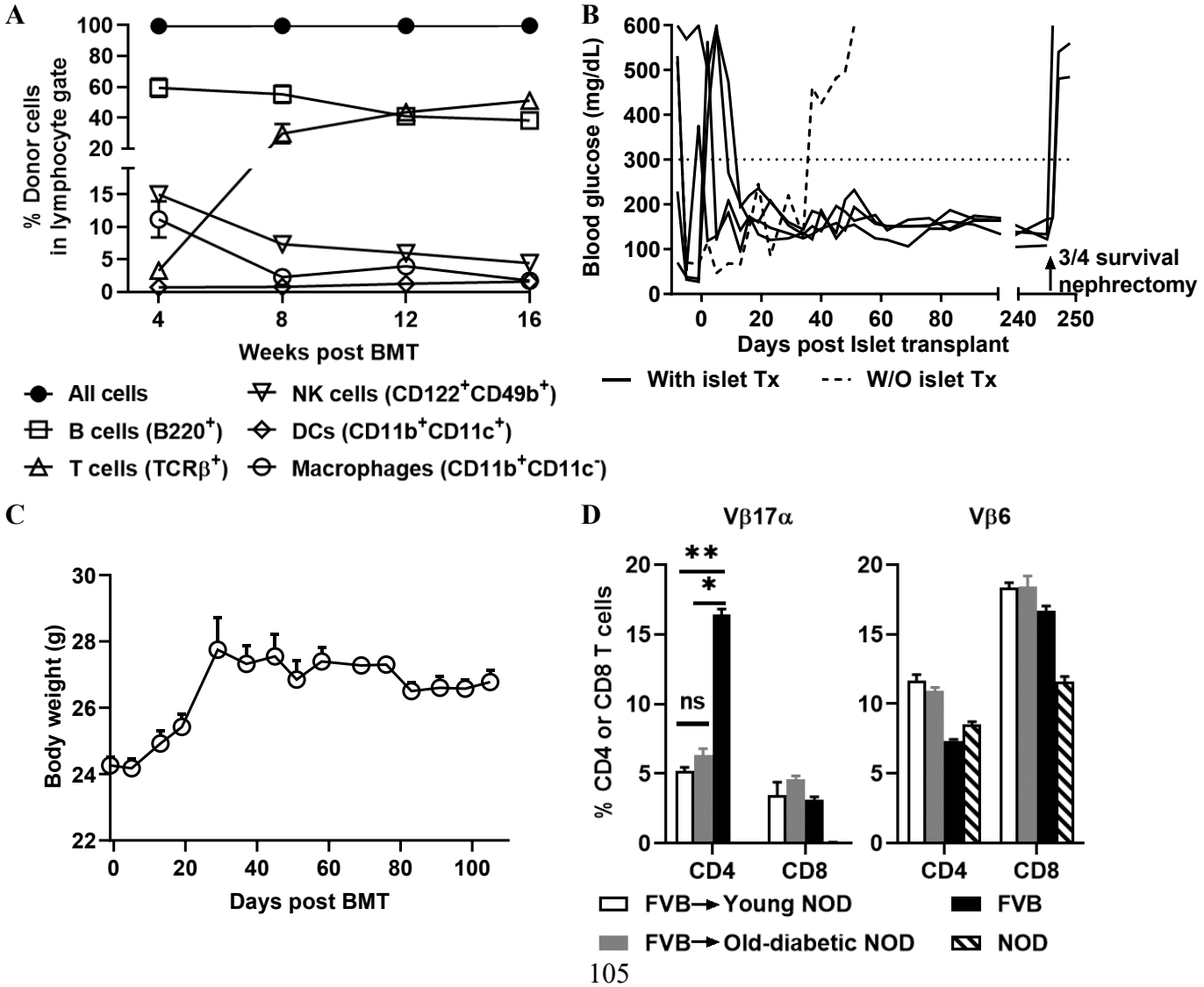

Figure 4-5 T cell depletion conditioning with delayed infusion of donor CD8α$^+$ cells generates stable chimerism and the acceptance of donor islets without GVHD.

Old-diabetic (n=5) NOD recipients were conditioned with our chimerism induction protocol (see Fig. 4-1) and infused with donor CD8α$^+$ cells (day 19, 5×10^6). Four of five mice received a donor islet transplant on day 0. (A) Shown are the percentages of donor-derived MHC-I$^+$ cells, T cells, B cells, NK cells, DCs, and macrophages in the lymphocyte gate in peripheral blood lymphocytes over time (n=5). (B) Blood glucose levels before and after islet transplant. Solid lines represent four mice with an islet transplant, of which three mice received survival nephrectomy; dashed line is for one mouse without an islet transplant showing blood glucose as the s.c. LinBit function declined over time in this mouse. (C) Shown are body weight of four 'old-diabetic' chimeras that also received islet transplantation. (D) Shown are the frequencies of Vβ17a$^+$ and Vβ6$^+$ T cells in peripheral blood lymphocytes at three to four months post BMT. Values are shown as the mean ± SEM. Numbers of animals used for analysis in the left panel: FVB→young NOD n = 3, FVB→old-diabetic NOD n=5, untreated NOD n = 7, and untreated FVB n = 7. Kruskal-Wallis test with Dunn's multiple comparisons test was used for the comparisons shown and denoted * $p<0.05$ and ** $p<0.01$.

4.3 Discussion

Allogeneic islet transplantation has been proved to be a safe method to treat brittle T1D.[184] However, lifelong immunosuppressants are needed to suppress immune reactions

towards these allogeneic islets. Simultaneous allogeneic BMT along with islet engraftment may provide an effective way to induce donor-specific tolerance by creating a state of mixed hematopoietic chimerism. However, the toxicity, demand for mega dose BMC and the risk of GVHD in the current recipient conditioning protocols prevents their application for islet transplant recipients.

Autoimmune diabetes has previously been suspected to be another barrier towards chimerism induction in NOD mice.[143,246,248] A higher dose of costimulation blockade or additional antibody targeting recipient $CD8^+$ T cells was required to overcome this hurdle to chimerism induction.[143,246] How diabetes contributes to the resistance to establishment of chimerism in spontaneously diabetic NOD mice was unclear. From a clinical perspective, it is unknown if diabetes is associated with increased primary graft failure after allogeneic BMT. Previous studies have observed a profound reduction of peripheral T cells and an accumulation of naïve T cells in the BM in NOD mice after diabetes onset.[313,314] However, these age and/or diabetes dependent changes have not yet been associated with difficulty in chimerism induction.

Here, we found that age rather than diabetes was associated with the increased resistance to chimerism induction in autoimmune diabetic NOD mice. A T cell depletion based protocol that generates full chimerism in young NOD mice only led to transient chimerism in a fraction of autoimmune diabetic NOD mice and their aged non-diabetic littermates. While it has been shown that immune senescence did not impair the engraftment of BM in B6 mice at an advanced age, our findings suggest that a factor coinciding with increased age, and

occurring prior to hypergylcemia, contributes to the enhanced resistance to chimerism induction in autoimmune diabetic NOD mice.[315] It remains unknown why such a moderate increase of age strongly precludes a successful BM engraftment in NOD mice.

Despite the eventual loss of chimerism, BMT significantly prolonged the reversal of hyperglycemia after islet transplantation. However, this transient chimerism failed to prevent the rejection of donor islets in most of the diabetic NOD recipients. In an attempt to achieve stable chimerism in autoimmune diabetic NOD mice, we found the enhanced resistance to chimerism induction in these aged recipients, regardless of the diabetes status, was associated with the absence of donor T cells at early time points post-BMT. Future studies should examine whether altered NK cell activity with age contributes to the lack of donor T cells, as NK cells in NOD mice have potent capacity to kill allogeneic cells despite a reduced ability to kill other targets.[274,316] The addition of donor CD8α$^+$ cells from a donor spleen improved the engraftment of donor BMC. CD8α$^+$ cells contain multiple subsets of cells, including CD8$^+$ T cells, CD8α$^+$ pre-plasmacytoid and plasmacytoid dendritic cells. All of these cells have the potential to facilitate BM engraftment.[250,311] However, the mechanisms of graft promotion by donor splenic CD8$^+$ cells require further investigation. The transfer of donor CD8$^+$ T cells might be considered risky in terms of the potential to cause GVHD. However, in our study and several others, this approach appears to be safe.[250] The safety is likely due to the need for T cell help for CD8$^+$ T cells to generate a robust immune response.[317] Consistent with this conclusion, Zeng and colleagues found that if donor CD4$^+$ T cells were given along with the CD8$^+$ T cells, GVHD ensued; similarly, we found if purification of CD8$^+$ cells was not

sufficient, with many contaminating $CD4^+$ T cells, recipients suffered from GVHD (our unpublished data).[318]

In conclusion, donor $CD8\alpha^+$ cells overcame age-dependent resistance to chimerism allowing tolerance to fully allogeneic islets in autoimmune diabetic NOD mice without the need of irradiation, a mega-dose of BM, nor chronic immunosuppressants.

Chapter 5

Desensitization using imlifidase and EndoS enables chimerism induction in allo-sensitized recipient mice

5.1 Introduction

Hematopoietic chimerism *via* allogeneic bone marrow transplantation is a robust method for inducing tolerance to organs from the same donor.[319] However, sensitization to donor antigens prior to transplantation is a significant barrier to both successful bone marrow and solid organ transplantation.[320,321] While challenging in these sensitized recipients, if successful, achieving hematopoietic chimerism could reverse such allosensitization in theory, through the generation of specific tolerance in T and B cells resulting in a reduction of donor antigen specific antibodies (DSA).[322]

Humoral immunity against donor antigens is believed to be the dominant barrier for primary BM engraftment in sensitized hosts.[323,324] Various combinations of DSA desensitization methods, including plasmapheresis that removes DSA, mismatched platelet transfusion that adsorb DSA, as well as rituximab and bortezomib, which inhibit antibody production, have the capacity to improve BM engraftment. Human intravenous immune globulins (IVIG) have also been shown to have some beneficial immune modulatory effects in DSA-positive recipients who underwent transplantation.[320,325] However, these empirical treatments are not always effective in reducing the titer of DSA to the cut-off levels that permit successful engraftment.[320] Furthermore, a rapid rebound of DSA may occur even after successful desensitization and thus additional interventions are required.[321] Novel approaches that can either further reduce the titer or inhibit the effector functions of residual DSA would therefore be helpful in improving the BM engraftment.

Imlifidase (previously called IdeS) enables kidney transplantation in HLA-incompatible highly sensitized recipients through cleavage of donor-specific IgG into Fc- and $F(ab')_2$ fragments, abrogating complement activation and Fc-gamma receptor-mediated mechanisms. Imlifidase is a highly interesting candidate for desensitization to enable bone marrow engraftment and chimerism induction in sensitized hosts.[326] EndoS, which de-glycosylates the Fc portion of all subclasses of human IgG and thus reduces the affinity of IgG to Fcγ receptors (FcγRs), is also an interesting candidate.[327] The inhibition of complement- and FcγR-mediated effector functions has been shown to inhibit RBC lysis and to alleviate antibody-mediated arthritis in animal models.[328,329] We, therefore, hypothesized that imlifidase and/or EndoS could be used for allogeneic BMT in sensitized recipients. In mice, imlifidase only cleaves IgG2c and IgG3 and reduced effects on IgG2c have been demonstrated for EndoS.[328,330,331] We therefore studied imlifidase and EndoS in combination to achieve the greatest effect on DSA-inactivation in mice to establish a model of enzymatic desensitization prior to bone marrow transplantation.

Whereas extensive information exists for imlifidase, no data for EndoS on DSA-inactivation have previously been reported.[326,332] We show here that EndoS inhibits the DSA-mediated killing of donor bone marrow cells in a DSA titer-dependent manner. The combination of imlifidase and EndoS improved the survival of donor hematopoietic cells in allo-sensitized mice. Using a stringent model of primed NOD recipients that are resistant to irradiation and tolerance induction, we show that a combined approach that includes both imlifidase and EndoS permits the generation of mixed hematopoietic chimerism in mice.

5.2 Results

5.2.1 EndoS inhibits the monoclonal DSA mediated killing of donor BMC

To evaluate the effect of EndoS on inhibiting the antibody-mediated killing of donor BMC, DSA passive transfer experiments were performed. Of all DSA, anti-donor MHC or HLA antibodies are of significant importance in the clinic.[320] Therefore, naïve NOD mice expressing MHC-I K^d/D^d were injected with mouse IgG_{2b} antibodies targeting MHC-I K^b expressing cells, treated with EndoS or left untreated, and thereafter subjected to bone marrow transfer from B6 mice.

As shown in Fig 5-1A, in NOD recipients given a single dose of 10μg anti-K^b mAb, the ratios of B6 to NOD cells in blood at one hour post BMT were significantly increased in mice treated with EndoS as compared to those that did not receive enzyme treatment. This difference in ratio of B6 to NOD cells in blood between the two groups remained stable at two and three hours post BMT. Similarly, pre-treatment with 100μg anti-K^b mAb with EndoS led to an increased ratio of B6 to NOD cells in the blood at 1 and 2 hours compared with treatment with 100μg anti-K^b mAb alone but to a lower ratio of B6 to NOD cells at three hours compared with the group that was treated with 10μg anti-K^b mAb with EndoS, possibly due to residual effector function only reaching biological significance at high anti-K^b mAb levels.

At four hours post BMT, a significant increase in the ratio of B6 to NOD cells in both BM and spleen was also observed in mice treated with EndoS and 10μg anti-K^b mAb as compared to those that received 10μg anti-K^b mAb only.

Of note, NOD mice lack hemolytic complement C5, which is essential for complement dependent cytotoxicity and is not genetically linked with MHC genes.[333] Thus, the effect of DSA in NOD mice may be decreased compared with complement sufficient hosts. We, therefore, also examined the role of EndoS on DSA in complement sufficient hosts. NOD MHC congenic B6.H-2^{g7} mice were used as recipients. EndoS improved the ratios of donor to recipient cells to a similar extent in B6.H-2^{g7} mice as compared to NOD hosts, which validates the model (Fig 5-1 B).

In brief, EndoS improved survival of donor cells in the presence of anti-MHC antibodies whether or not the recipients were complement-sufficient.

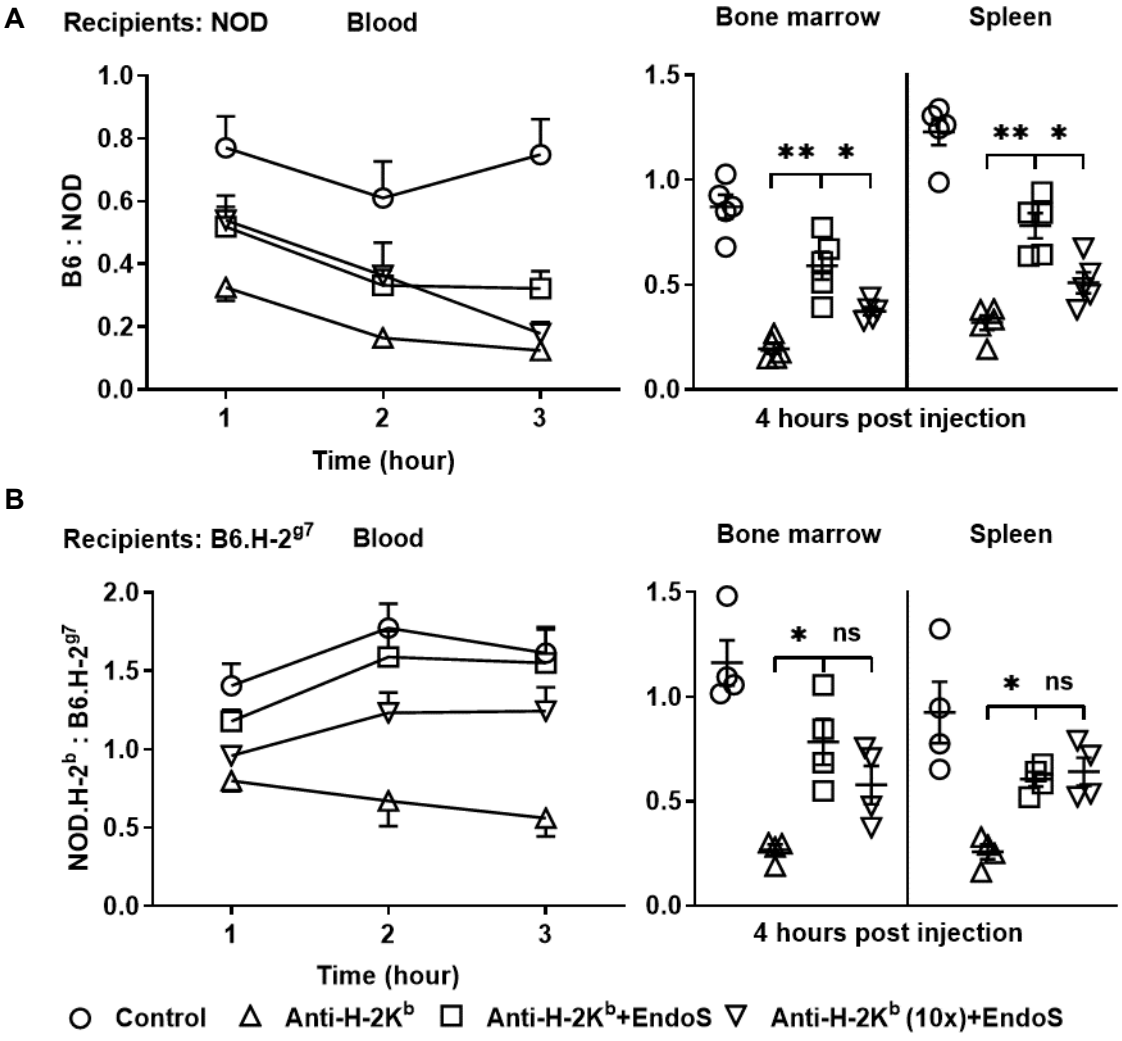

Figure 5-1 EndoS inhibits monoclonal DSA mediated killing of donor bone marrow cells.

Naive NOD (panel A) or B6.H-2^{g7} (panel B) were given 30μg EndoS and/or anti-H-$2K^b$ mAb (10μg or 100μg, the latter shown as 10×) intravenously four hours prior to the infusion of a mixture of CFSE labeled NOD/CTV labeled B6 bone marrow cells (BMC; panel A) or CFSE labeled B6.H-2^{g7}/CTV labeled NOD.H-2^b BMC (panel B). Shown are the ratios of dye labeled B6 to NOD cells (panel A) or NOD.H-2^b to B6.H-2^{g7} cells (panel B) in the blood (left panels) collected one to three hours after bone marrow transplant (BMT), in host spleens

(middle panels) and bone marrow (BM, right panels) collected at four hours after BMT. Mean±SEM are shown. Data were pooled from five (panel A) and four (panel B) independent experiments. Mann–Whitney U test (right panel) was used for the comparisons shown; *$p<0.05$ and **$p<0.01$.

5.2.2 EndoS improves survival of donor BMC in presensitized recipients

Next, we investigated if EndoS could improve donor BMC survival in allosensitized recipients that had a diversified antibody repertoire against donor antigens. In order to test this, we combined EndoS with imlifidase. Imlifidase cleaves murine IgG_{2c} and IgG_3 but is not able to cut murine IgG1 and IgG2b. Therefore, EndoS was co-administered to attenuate the effector function of the murine IgG isotypes that are not cleaved by imlifidase.[328,330,331] As shown in Fig 5-2A, imlifidase and EndoS together led to a significant reduction by four hours of DSA-IgG in NOD mice that had been sensitized to FVB splenocytes. This decline of total donor cell-targeting IgG was likely due to imlifidase and not EndoS since deglycosylation still allows the Fc-specific detection antibody to bind. The different sensitivity for murine IgG isotypes is also illustrated by the approximately 80% reduction of DSA-IgG_3, a subclass that is cleaved by imlifidase, whereas no change in the level of DSA-IgG_1 (Fig 5-2B and 5-2C) was seen. Similar results are shown in Fig 5-2C and D where the use of two different secondary antibodies targeting either mouse IgG_3 Fc or IgG_3 heavy chain suggests that the majority of donor cells were not bound by F(ab')2 of the cleaved DSA-IgG_3

from enzyme-treated serum. While the degradation of IgG3 by imlifidase only caused a moderate reduction of intact IgG in the mouse, EndoS could further contribute to the reduction of DSA-IgG effector functions through the deglycosylation of imlifidase resistant IgG molecules. The combination of both enzymes allowed us to analyse donor cell survival in sensitized recipients with polyclonal DSA.

In addition to DSA, primed donor antigen-specific cytotoxic T cells may contribute to the rapid killing of donor BMC. Therefore, sensitized recipients were T cell-depleted two days before imlifidase and EndoS treatment in order to avoid the acute cytotoxic effect mediated by sensitized T cells. Over 95% of T cells in the peripheral blood were depleted in the recipients at two days after giving T cell-depleting mAbs (data not shown). As shown in Fig 5-2E and 5-2F, donor cells were almost completely eliminated at four hours post BMT in sensitized NOD mice when given vehicle control (BM 0.22% and spleen 0.27%) or only imlifidase (BM 0.15% and spleen 0.46%). In contrast, close to 0.5% of BMC and around 1.5% of splenocytes in primed NOD mice treated with EndoS and imlifidase were from the B6 (CD45.2) donor. Thus, administration of imlifidase and EndoS four hours prior to BMT rescued approximately 15-20% donor BMC in allosensitized recipients as compared to naïve recipients, while sensitized recipients or sensitized recipients treated with imlifidase only retained less than 1% (Fig 5-2F). Interestingly, the majority of residual donor cells in recipients treated with imlifidase and EndoS demonstrated low MHC-I K^b staining, suggesting donor MHC epitopes were blocked by either de-glycosylated DSA or $F(ab')_2$ of

DSA (Fig 5-2E). Alternatively, the surviving donor cells may have been those that expressed less MHC class I from the start.

Taken together, these data indicated that imlifidase and EndoS together improved the donor BMC survival in allosensitized recipients.

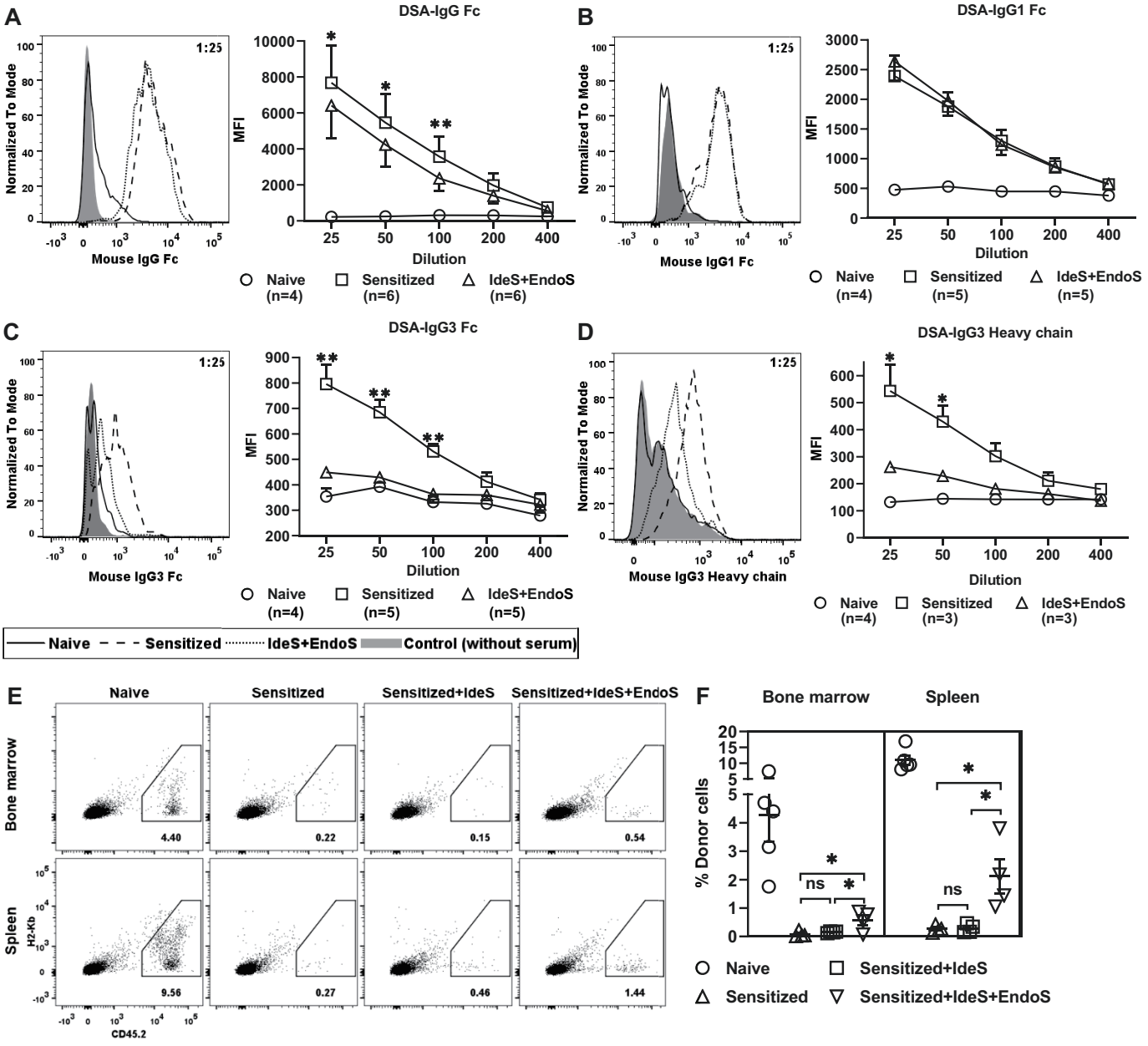

Figure 5-2 EndoS-imlifidase reduces DSA-mediated killing of donor BMC in sensitized recipients.

(A-D) Naive NOD mice were immunized with FVB splenocytes four weeks prior to the administration of IdeS-EndoS. Sera was harvested prior to immunization, prior to and four hours after enzyme treatment. Representative histograms on the left are for DSA-IgG Fc

(panel A), DSA-IgG_1 Fc (panel B), DSA-IgG_3 Fc (panel C) and DSA-IgG_3 heavy chain (panel D) with sera at a 1:25 dilution. Mean fluorescence intensity (MFI) of DSA in the titrated sera is shown on the right. Mean±SEM are shown. Ratio paired *t* test was used to compare MFI of DSA before and after enzyme treatment at each serum dilution with *p<0.05, **p<0.01. (E-F) Naive NOD mice were immunized with B6.CD45.1 splenocytes four weeks prior to injection of T cell depleting mAbs. EndoS-imlifidase was administrated two days post T cells depletion. Four hours after enzyme treatment, sensitized NOD mice were injected with 80 million B6.CD45.2 bone marrow cells intravenously. Splenocytes and bone marrow cells were analyzed for the expression of MHC-I H-$2K^b$ and CD45.2. Shown on the left are representative dot plots of the four different treatment groups and the percentage of donor cells (mean±SEM). One-way ANOVA with Holm-Sidak's multiple comparisons was used to compare values between the three sensitized groups with *p<0.05.

5.2.3 Bortezomib and cyclophosphamide treatment prior to BMT reduced B cells in BM

In order to use imlifidase and/or EndoS for BMT in sensitized recipients, methods that reduce DSA-producing cells are necessary for providing a longer window of the low DSA environment for the continuous survival and further development of donor cells post BMT. In an attempt to reduce existing plasma cells and B cells that can differentiate into plasma cells after BMT, we employed proteasome inhibitior bortezomib to deplete antibody-producing cells and cyclophosphamide to reduce B cells.[334,335] The combination of

bortezomib and cyclophosphamide (CyBor) has been used in patients with non-transplant eligible multiple myeloma and for prevention of GVHD post allogeneic BMT, but rarely used for the purpose of DSA desensitization.[336-338]

We asked if CyBor administration prior to BMT could reduce plasma cells and B cells and if the effect of CyBor could last several days after BMT; note, the BMT can be considered as a second immunization with donor cells in sensitized recipients. At five days after BMT, the cellularity of BMC in the BM did not differ between groups. Interestingly, the overall number of splenocytes increased in the group of mice pretreated with CyBor. However, compared to vehicle group, BM $CD19^+$ B cells, $CD19^-CD138^+B220^+$ plasma blasts and $CD19^-CD138^+B220^-$ plasma cells were significantly reduced in mice treated with CyBor (Fig 5-3A). In contrast to the reduction of B cells in the BM, the reduction of splenic $CD19^+$ B cells was not significant at the time examined in the CyBor treated group. Moreover, there were significant increases of $CD19^-CD138^+B220^+$ plasma blasts and $CD19^-CD138^+B220^-$ plasma cells in the spleens from CyBor treated mice (Fig 5-3B).

We then examined whether the CyBor treatment prevented increased DSA formation stimulated by the BMC injection. As shown in Fig 5-3C, DSA levels increased substantially in two of five mice in the control group and two of five mice in the CyBor treated group, suggesting that CyBor was not able to decrease DSA levels. However, when percentile changes of DSA levels five days after BMT (nine days post CyBor) were compared, the increases of DSA tended to be less in mice treated with CyBor, suggesting that CyBor

treatment prior to BMT may inhibit the increase of DSA stimulated by BMC injection (Fig 5-3D).

In summary, these data showed the effects of CyBor in inhibiting B cells was pronounced in BM and CyBor may limit the increase in DSA caused by the BMC injection.

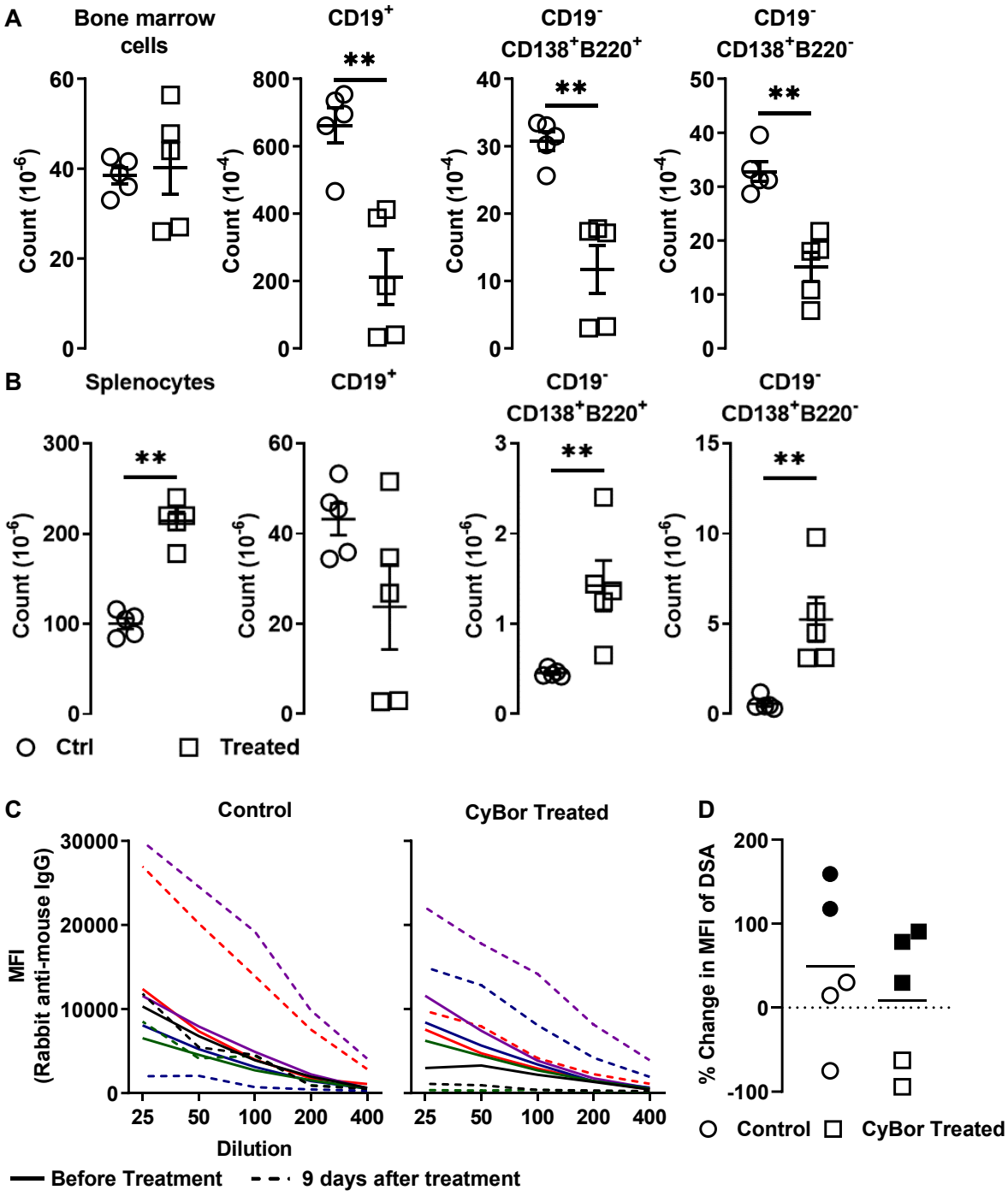

Figure 5-3 Bortezomib/Cyclophosphamide prior to BMT reduces bone marrow B cells in sensitized recipients.

Four weeks after immunization with FVB splenocytes, NOD mice were treated with cyclophosphamide and bortezomib (CyBor) intravenously. Four days after CyBor treatment, bone marrow transplantation with 20 million FVB BMC was done. Splenocytes and bone marrow cells were collected five days after BMT for analysis. Sera were collected before CyBor treatment and five days post BMT. Shown are cell counts of B cells and plasma cells in the bone marrow (panel A) and spleens (panel B) in mice given CyBor or vehicle (Mean±SEM). *$p < 0.05$, Mann–Whitney U test. (C) Sera were collected prior to immunization and five days post BMT, i.e. nine days after CyBor treatment. Shown are MFI of DSA-IgG Fc in the titrated sera from control (on the left) or treated mice (on the right). Shown are mean±SEM. (D) Shown are percentile changes at day 9 in MFI of DSA at the 1:25 dilution compared to pretreatment. Filled and empty symbols represent data collected in two separate experiments.

5.2.4 Engraftment is achievable in presensitized recipients with combination of Imlifidase, EndoS, T cell depletion, and CyBor

With the data above, we hypothesized that imlifidase and EndoS in combination with T- and plasma cell depletion by CyBor together with a non-lethal dose of irradiation and a large dose of BMC would allow engraftment of donor cells in presensitized recipients. We first explored if such protocol induced chimerism in B6.H-2^{g7} mice that are MHC matched with NOD but are not resistant to chimerism induction.[222] B6.H-2^{g7} mice were primed with FVB

cells four weeks prior to the chimerism induction. Naive and primed B6.H-2^{g7} mice were given the same conditioning protocol, as indicated in the methods section. As expected, while naive mice became nearly fully chimeric with FVB cells at four weeks post BMT, donor cells were rejected in primed mice. In contrast to primed mice that were not treated with imlifidase and EndoS, five out of eight mice treated with both enzymes became chimeric (Table 1).

With this preliminary positive result, we next sought to test this protocol on sensitized NOD mice. As shown in Fig 5-4B, donor cells were not detectable even at two days post BMT in sensitized NOD mice that were not treated with enzymes. However, donor cells were more than five percent at day 4 or 9 after BMT in five out of seven sensitized NOD recipients given enzyme treatment. Furthermore, in four enzyme-treated sensitized NOD mice, chimerism levels increased steadily to over 50 percent at day 16 post BMT. Eventually, four of the seven presensitized recipients were chimeric with donor cells at four weeks post BMT, with two mice being transiently chimeric and two being stable mixed chimeras with multiple lineages of donor cells in the periphery (Fig 5-4B). No sign of GVHD was observed in any chimeras. In an attempt to simplify this protocol by eliminating either cyclophosphamide or bortezomib, it appeared that both of them were essential for the success of the current protocol for inducing chimerism in sensitized recipients (Table 5-1).

In summary, imlifidase and EndoS together enable donor BMC engraftment in presensitized recipient mice when combined with CyBor and standard conditioning agents.

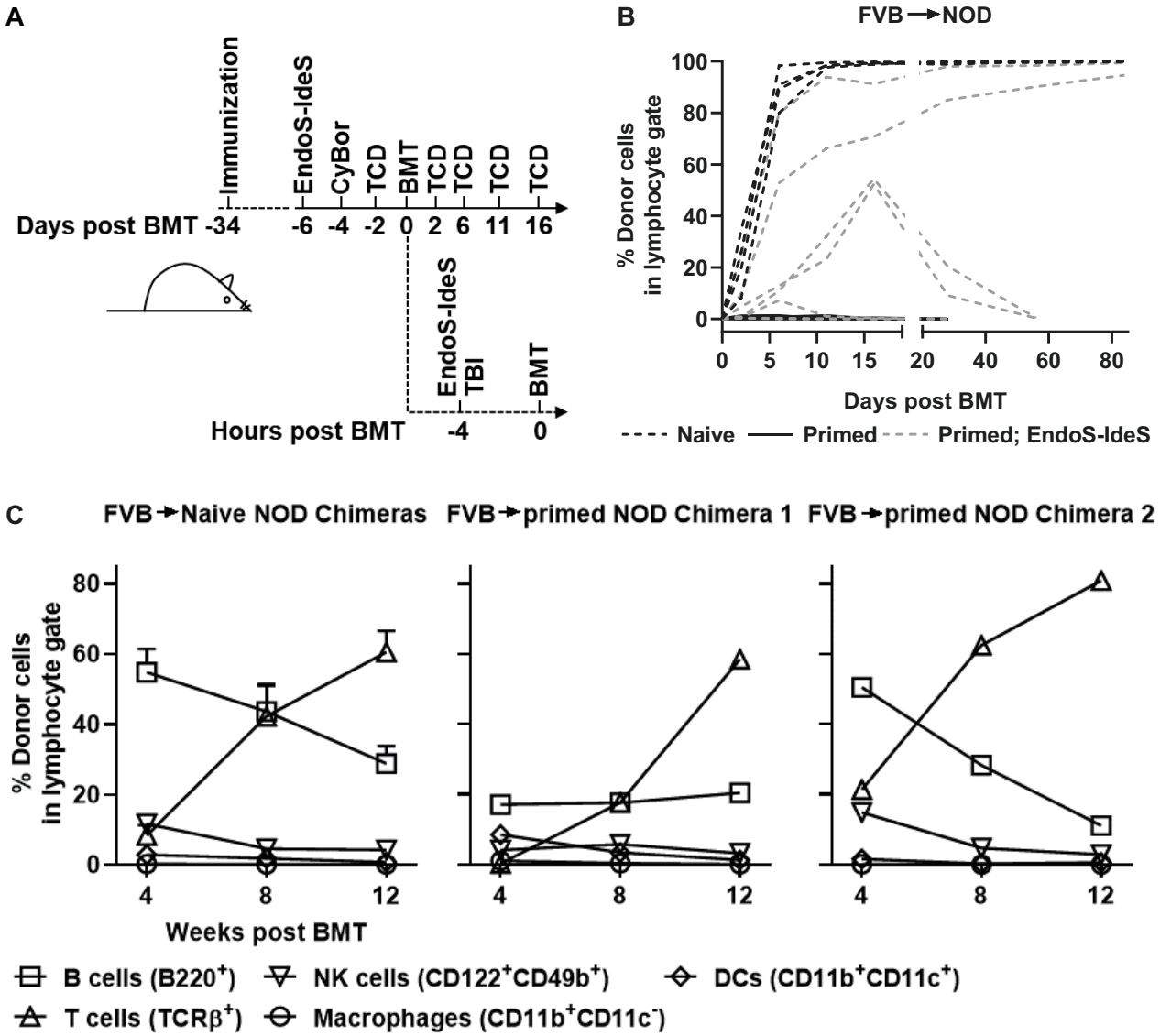

Figure 5-4 EndoS-imlifidase allows hematopoietic chimerism in pre-sensitized recipients

(A) Schematic of the chimerism induction protocol; naive B6.H-2^{g7} or NOD mice were immunized with FVB splenocytes four to six weeks prior to chimerism induction. For chimerism induction, CyBor was given on day -4 with respect to the date of BMT. T cell depleting (TCD) antibodies were administered i.p. on day -2, 2, 6, 11, and 16. Some recipients

that had been sensitized to FVB splenocytes were treated with EndoS-imlifidase i.v. on day -6 and a repeated dose on day 0 at four hours before BMT. Six Gy TBI was given at 4 hours prior to BMT on day 0. FVB BMC (80×10^6) were given on day 0. (B) Shown are the proportions of donor cells in lymphocyte gate in peripheral blood over time. (C) Shown are percentages of different lineages of donor cells in lymphocyte gate in peripheral blood from FVB→naïve NOD chimeras (n=4, on the left, mean±SEM) and FVB→primed NOD chimeras (n=2, on the right). Data were pooled from six independent experiments.

Table 5-1 EndoS-IdeS allows hematopoietic chimerism in pre-sensitized recipients

Treatment group	Engraftment	Chimerism levels[#]
Not primed		
CyBor	6/6[†]	>90%
Primed		
CyBor	0/7[‡]	
CyBor-EndoS-IdeS[*]	5/8[※]	98%, 85%, 57%, 20%, 9%
Cy-EndoS-IdeS	0/2[¤]	
Bor-EndoS-IdeS	0/3[¤]	

See figure legend of Fig 5-4 for details of chimerism induction protocol. † represents two B6.H-2^{g7} and four NOD recipients. ‡ represents two B6.H-2^{g7} and five NOD recipients. ※ represents one B6.H-2^{g7} and seven NOD recipients. ¤ represents NOD recipients. # Shown are chimerism levels at four weeks post BMT. ∗ $p<0.05$ by two-sided Fisher's exact test when compared to "CyBor" primed group.

5.3 Discussion

DSA is a main obstacle for allogeneic BMT in sensitized recipients.[323,324] Previous work showed that both imlifidase and EndoS can be used either for eliminating or inhibiting DSA in different models.[327-329] However, neither of the two enzymes have yet been tested for the purpose of facilitating BMT. With the promising results from recent clinical trials for kidney transplantation in sensitized recipients, together with our pre-clinical BMT results, there is little doubt that imlifidase could be used in BMT for desensitization in humans.[326,332] However, since bone marrow cells have high MHC expression, residual intact antibodies may be more problematic in the setting of bone marrow transplantation.

In the current study, we also addressed the potential use of EndoS in BMT. We found that EndoS alone improved survival of donor cells in the presence of DSA *in vivo*. The fact that EndoS improved the survival of donor cells to a similar extent in B6.H-2^{g7} and NOD suggested that EndoS works even in the presence of an intact complement pathways. The differences between NOD and B6.H-2^{g7} mice given low or high dose of monoclonal DSA and EndoS indicate that the non-MHC genes may have an impact on the efficacy of EndoS in different individuals. This difference between NOD and B6.H-2^{g7} may be attributable to the different binding capacities of IgG_{2b} with various Fc receptors in mice on the NOD and B6 background.[339,340] The FcR polymorphisms may be important as well.[341] Our results also suggest that the effects of EndoS could be more potent if the titer of DSA were reduced.

We found that the combination of imlifidase and EndoS improved the survival of donor BMC and allowed donor chimerism in sensitized mice that had been conditioned with T cell

depletion, CyBor and sublethal irradiation. In our protocol, the effect of T cell depletion in the periphery was not affected by EndoS. This suggests that with appropriately designed timing, EndoS can be used together with antibody-based products like IVIG and B cell depletion antibodies such as rituximab.

With regard to the use of cyclophosphamide and bortezomib, both of them have immune modulatory effects other than targeting B cells or plasma cells.[342] For example, cyclophosphamide can facilitate the chimerism induction in sensitized recipients by reducing memory T cells as shown in our previous study.[306] As for bortezomib, our finding is consistent with the published data showing the compensatory increase of splenic B cells after bortezomib treatment, which in turn resulted in humoral compensation.[343] However, whether or not this increase of splenic B cells after BMT is accompanied with a rebound of DSA in the current study remains unknown. Importantly, T cell depletion employed in our protocol may potentially inhibit the recovery and maturation of both naïve and memory B cells, and the generation of de novo DSA.

Lastly, the findings of this study have to be considered in light of some limitations. Although imlifidase works for all the human IgG subclasses, it only works for two subclasses of mouse IgG. In order to achieve maximum effect on DSA in mice, we had to combine EndoS and imlifidase. It has been shown that imlifidase temporally inhibits the activation of memory B cells by cleavage of membrane bound BCR *in vitro*, but it is unclear if this effect of imlifidase is important in the success of chimerism induction in sensitized recipients.[344] On the other hand, imlifidase only cleaves mouse IgG2c and IgG3 and the effect of imlifidase

on mouse IgG was not complete in this model (Fig 5-2 A). A protocol with imlifidase only as the desensitizing agent is likely to be more efficient in humans where imlifidase completely inactivates the IgG DSA pool.[326,332] The second limitation concerns the toxicity of the chimerism induction protocol. However, the current study is a proof of principle study showing that modulating IgG Fc can be strategically useful for BMT in sensitized recipients. Furthermore, EndoS or imlifidase can be used in combination with other desensitization methods. Currently, it is not known whether the enzyme-mediated blocking of DSA prevents a rebound in antibody. Perhaps maintaining a certain level of DSA while blocking DSA function, i.e. de-glycosylation of IgG Fc, may have less potential to trigger a rebound than complete removal of the DSA.

Finally, we conclude that the combination of imlifidase and EndoS can be used for inducing donor chimerism in allosensitized recipient mice in combination with other desensitization strategies.

Chapter 6

Conclusions and future directions

6.1 Conclusions and limitations

Allogeneic hematopoietic chimerism *via* simultaneous tissue and bone marrow transplantation, in theory, is the most efficient method of inducing tolerance to allo-islet grafts in patients with brittle T1D. The aim of this thesis was to develop a clinically feasible conditioning protocol for generating allogeneic hematopoietic chimerism by using NOD mice as recipients, which are a mouse model for human type 1 diabetes and a stringent mouse model for testing chimerism induction protocols. More specifically, we aimed at overcoming the resistance to chimerism induction in young NOD mice, spontaneously diabetic NOD mice, which were reported to be even more difficult recipients as compared to young NOD mice that were not yet diabetic, and lastly presensitized NOD mice that have an enhanced allo-immune response towards donor antigens.

The work in **Chapter 3** was a continuation of the research done by the previous PhD student in Anderson lab, Dr. Al-adra. He found recipient T cells in NOD mice were the major barrier to generating multi-lineage chimerism. Based on his findings, we developed a T cell depletion based chimerism induction conditioning protocol in NOD mice with hematopoietic stem cells from a fully MHC and MiHA mismatched donor. Toxic modalities or reagents, like irradiation, co-stimulation blockade with anti-CD40L mAb (thrombogenic), and rapamycin (diabetogenic) are no longer needed in this protocol. As the essential components of this conditioning protocol, including T cell depletion, cyclophosphamide, low dose busulfan, and a moderate dose of donor HSCs, are clinically feasible, this protocol can be translated for future clinical studies with other methods for inducing donor specific tolerance

for islet transplantation as well as transplantation of other organs. The finding that the levels of donor chimerism at early time points post bone marrow transplantation were associated with the long-term stability of chimerism emphasizes the importance of closely monitoring the chimerism levels very soon after transplantation, enabling the early recognition of important components in the successful conditioning protocols, and accelerating the screening of translatable conditioning protocols.

While successful in young NOD mice that were not yet diabetic, this protocol failed to induce chimerism in NOD mice that were spontaneously diabetic (**Chapter 4**). We found that donor T cells, which have been positively associated with the stability of chimerism, were less in diabetic NOD mice as compared to young NOD mice that were not yet diabetic early after bone marrow transplantation. We then modified the conditioning protocol by adding donor splenic $CD8\alpha^+$ cells. This new conditioning protocol did lead to full chimerism in autoimmune diabetic NOD mice and tolerance to islets from a fully MHC and MiHA mismatched donor. In the study of inducing chimerism in diabetic NOD mice, we also identified aging as a confounding factor for the increasing resistance to chimerism induction in NOD mice. However, additional studies are required to understand the roles of aging and diabetes in the resistance to chimerism induction as well as the role of donor $CD8\alpha^+$ cells in this new conditioning protocol.

In **Chapter 5**, we explored the possibility of utilizing the bacterial enzymes, EndoS and IdeS, which inhibit the function of antibodies, for overcoming the enhanced alloimmunity in presensitized recipients. We designed a protocol that combined EndoS and IdeS, and induced

chimerism in some of the primed recipients. This finding suggests EndoS and IdeS can be used as desensitization methods for allogeneic bone marrow transplantation in presensitized recipients, who need bone marrow transplantation as a cure for either hematopoietic malignancies or tolerance induction for donor organs. However, future research is required to improve the efficacy and reduce the toxicity of this conditioning protocol for the purpose of tolerance induction for organ transplantation.

Finally, while animal studies have contributed broadly to our understanding in diseases and development of new treatments, it is very important to keep in mind that findings in animal studies may not always be translatable to humans. Mouse models have limitations. Mice used in our studies are housed in a clean environment. Their immune system is less experienced compared to the ones in human. Also, bone marrow donor mice were used at a young age.

6.2 Future directions

6.2.1 Determining the role of diabetes in chimerism induction

While the finding in **Chapter 4** pointed out that aging was a confounding factor for enhanced resistance to chimerism induction in autoimmune diabetic NOD mice, whether or not autoimmune diabetes is a barrier towards chimerism induction is a question that is still unsolved. This is important because the conditioning protocols that have been tried in the clinic may not be applicable to islet transplantation if autoimmune diabetes is indeed an additional barrier towards chimerism induction. Designing experiments to investigate if there

is additional resistance for inducing chimerism in young autoimmune diabetic NOD mice as compared to young NOD mice that are not yet diabetic would, therefore, be important. Young NOD mice can be induced to become diabetic by injecting streptozotocin that is toxic to beta cells. However, chemically induced diabetes cannot fully mimic autoimmune diabetes. Moreover, it has been shown that streptozotocin treatment led to an increase of $CD4^+CD25^+Foxp3^+$ regulatory cell frequency in the periphery.[345,346] Supplementation of recipient Tregs has been shown to facilitate chimerism induction.[347] Therefore, such changes of immune compositions, especially the frequency or function of Tregs may have an impact on the chimerism induction in streptozotocin-induced diabetic young NOD mice.

The co-inhibitory molecule PD1 and its ligand PD-L1 has been shown to be important in the regulation of autoimmunity in NOD mice. Blockade of PD-L1 led to early onset of autoimmune diabetes in young NOD mice.[348] It would be interesting to know if chimerism can be induced in these PD-L1 blockade induced diabetic young NOD mice as compared to young NOD mice treated with control antibodies. However, as the PD1-PD-L1 pathway has an important role in peripheral tolerance, PD-L1 blockade may affect chimerism induction in these young diabetic NOD mice in a way that is not diabetes relevant.[349]

Alternatively, inducing autoimmune diabetes in young NOD mice by injecting syngenic diabetogenic T cells would be of interest. T cells from NOD.BDC2.5 TCR transgenic mice are autoreactive T cells that respond to islet beta cell antigens and are diabetogenic. Injection of NOD.BDC2.5 T cells into neonatal NOD mice led to rapid onset of diabetes in 6 weeks.[350] Similarly, infusion of NOD.BDC2.5 T cells that have been activated *in vitro* with a peptide

mimicking an islet beta cell antigen induced diabetes in young NOD mice within a week.[140] This model can be used to investigate the role of autoimmune diabetes in chimerism induction in NOD mice.

6.2.2 Investigating the role of IL-2 in chimerism induction in aged NOD mice

In **Chapter 4**, I showed that a protocol that induced stable chimerism in young NOD mice failed to induce chimerism in aged NOD mice that were not yet diabetic. This resistance to chimerism induction in aged NOD mice was associated with lower levels of donor T cells early after bone marrow transplantation, which can be overcome by injecting donor $CD8\alpha^+$ cells. It is unknown if these donor passenger T cells did not survive due to the lack of resources or were rejected quickly. Both of these questions are worth pursuing.

It is known that naïve T cells tend to proliferate spontaneously in a lymphopenic environment, which can be generated by depleting T cells, and acquire an effector-memory phenotype.[351] IL-2 is important for the differentiation and homeostasis of effector-memory T cells.[352,353] Therefore, the presence of IL-2 may be important for the donor passenger T cells to survive in the T cell depleted aged NOD mice. The importance of IL-2 for chimerism induction was shown by Wekerle and colleagues.[268] In this study designed to study the role of Tregs in chimerism induction, neutralizing IL-2 at the time of bone marrow transplantation precluded the generation of chimerism in B6 mice. Of note, the bone marrow cells used in this study was not T cell depleted. The failure of chimerism induction in these IL-2 deprived

mice could be due to the decreased survival of passenger T cells rather than the inhibition of host Tregs.

Consistant with this observation, antibodies neutralizing IL-2 were found in NOD mice. The titers of anti-IL-2 antibodies were close to baseline in young NOD mice, but significantly increased in aged NOD mice and were even higher in diabetic NOD mice.[104] Moreover, anti-IL-2 antibody secreting plasma cells and their memory B cell counterparts were also detected. Importantly, the authors also showed that this presence of anti-IL-2 antibodies was associated with human type 1 diabetes. This increased anti-IL-2 antibodies upon aging and diabetes onset can be used to explain the reduced frequencies of $CD44^{hi}$ and $CD25^{+}$ T cells in the bone marrow of diabetic NOD mice found by another group of researchers.[314] In line with the development of anti-IL-2 antibodies in aged NOD mice, it is found that FoxP3 expression on Tregs but not the Treg frequency in pancreatic lymph node declined when NOD mice became aged.[354] Therefore, it will be important to investigate the role of IL-2 during the induction of chimerism in the aged NOD mice.

One can start investigating the role of IL-2 by neutralizing IL-2 in young NOD mice at the time of bone marrow transplantation and examining if these mice can become chimeric. Alternatively, the supplementation of exogenous IL-2 by injecting murine IL-2 or IL-2-anti-IL-2 antibody complex (IL-2 complex) into aged NOD mice that are not yet diabetic may help in inducing stable chimerism in these hosts. A previous study from Wekerle and colleagues aimed at expanding host Tregs showed that incorporation of IL-2 complex into a costimulation blockade based conditioning protocol promoted bone marrow rejection in B6

recipients.[355] The authors found that this treatment not only increase the numbers of Tregs, but also $CD4^+$ $FoxP3^-$ T cells and $CD8^+$ T cells. Such expansion of recipient conventional $CD4^+$ and $CD8^+$ T cells may be prevented by using a T cell depletion based conditioning protocol. Presumably, if the supplementary of IL-2 alone cannot induce chimerism in aged NOD mice, depleting memory B cells with antibodies targeting CD20 and a reagent targeting plasma cells can be considered in combination with IL-2 treatment.

6.2.3 Investigating if the thymus is required for chimerism and tolerance induction in NOD mice

A functional thymus is believed to be important for T cell repopulation after a successful allogeneic bone marrow transplant.[310] In the setting of islet transplantation for late stage diabetes, recipients were likely at their 40s.[356] It was shown that the thymic functional capacity was only 5% at the age of 40 years in human.[357] At the time of islet transplantation and chimerism induction, the thymus in these patients would have shrunk. It was shown that chimerism can be induced in thymectomized SJL/J mice and B6 mice.[358,359] More importantly, thymectomized chimeric B6 accepted donor skin and rejected skin from a third party, suggesting a state of donor specific tolerance. However, with the defects in peripheral tolerance, chimerism and tolerance induction in thymectomized NOD mice is doubtful. Therefore, it is important to know if the reduced thymic functional capacity or the absence of thymus would diminish chimerism and tolerance induction.

To answer this question, one can try to induce chimerism in NOD mice that were hemi-thymectomized or fully thymectomized at the date of bone marrow transplantation by using the protocol presented **in Chapter 3**. If these thymectomized mice can become chimeric to donor, the follow-up question is if these chimeras develop donor specific tolerance, i.e. accepting donor skin but rejecting skin from a third party.

Of note, the rapid thymic involution was associated with aging in NOD mice.[360] This was also supported by an early study showing NOD mice were lymphopenic.[313] The rapid atrophy of NOD thymus may also contribute to the enhanced resistance to chimerism induction in NOD mice, besides the emergence of anti-IL-2 antibodies. In this case, it would be interesting to know if transplantation of thymus from neonatal or young NOD under the kidney capsule of aged NOD mice could enable chimerism induction in these recipients.

Alternatively, one can also rejuvenate aged thymus with IL-7, IL-21, IL-22 or Keratinocyte growth factor (KGF, FGF7).[361-363] It would be interesting to validate the efficacy of these thymopoiesis-stimulators in aged NOD.Rag2pGFP mice and evaluate the absolute counts of DN, DN1, DP, SP thymocytes in the thymus and the frequencies GFP^+ cells in the periphery. Should thymic involution be reversed after these treatments, it would be intriguing to test if chimerism can be induced in these rejuvenated recipients by using the protocol provided in **Chapter 3**.

6.2.4 Investigating the important subset of CD8α$^+$ cells and their functions in chimerism induction

In **Chapter 4**, I showed a new conditioning protocol that included the delay infusion of donor CD8α$^+$ splenocytes overcame the enhanced resistance to chimerism induction in aged spontaneously diabetic NOD mice. However, CD8α$^+$ splenocytes contain mainly CD8α$^+$ T cells, and other CD8α$^+$ cells such as pre-plasmacytoid and plasmacytoid dendritic cells. The 'facilitating cells', which enhance the engraftment of donor hematopoietic cells, used in the clinical trial on chimerism induction led by Leventhal and Ildstad were also CD8α$^+$ cells.[364] CD8α$^+$ T cells have also been shown to improve bone marrow cell engraftment.[254,276,365] It is important to know which subset of donor CD8α$^+$ cells is critical for the generation of chimerism in diabetic NOD mice.

Another important question is how these cells overcome the barrier to chimerism induction in diabetic NOD mice. Mechanisms of mouse 'facilitating cells' were extensively studied by Ildstad's group.[364] Early studies have also investigated the requirement of perforin, Granzyme A and Fas ligand on CD8α$^+$ cells for preventing graft rejection.[254,366,367] However, the mechanisms underlying how donor CD8α$^+$ T cells help donor bone marrow cell engraftment is not fully clear. It was shown that successful bone marrow transplantation in recipients infused with CD8α$^+$ TCR$^+$ cells was associated with an elimination of residual host T cells, but not in recipients infused with CD8α$^+$ TCR$^-$ cells.[254] However, how the residual T cells are removed from the host is not clear. It is possible that recipient T cells are killed by donor T cells directly or indirectly. In **Chapter 4**, I showed that the majority of repopulated

host T cells in aged NOD mice were $CD4^+$ T cells. As the presence of MHC-II expression is required for the homeostatic proliferation of $CD4^+$ T cells in a lymphopenic environment, donor $CD8^+$ T cells may limit the survival of host $CD4^+$ T cells by killing host antigen presenting cells.[368]

As donor $CD8^+$ T cells may cause GVHD, especially with the presence of donor $CD4^+$ T cells, it is also important to investigate if anti-recipient immunity from donor $CD8\alpha^+$ T cells is required for them to enhance bone marrow engraftment.[318] To answer this question, donor derived $CD8\alpha^+$ T cells from stable FVB→ NOD chimeric mice or $CD8\alpha^+$ T cells from (FVB x NOD) F1 mice can be used as cell sources for delayed infusion of donor cells. Should the anti-recipient alloreactivity be required for the donor $CD8^+$ T cells to enhance donor bone marrow engraftment, replacing donor $CD8^+$ T cells with other donor facilitating cells that are more susceptible to tolerance induction can be considered, such as donor T cell progenitors and donor CD8 single positive thymocytes.

Donor T cell precursors can be generated *in vitro* by culturing donor hematopoietic stem cells with Notch-1 ligand Delta-like-1 expressing mouse stromal cells (OP9-DL1 cells).[369] It has been shown that the infusion of such T cell progenitors increased thymic cellularity and enhanced donor T cell reconstitution in irradiated mice. Importantly, the newly generated donor T cells were functional and did not cause GVHD.[369,370] Therefore, OP9-DL1 induced donor T cell precursors may be used for tackling the resistance of chimerism induction in aged NOD mice. Alternatively, donor CD8 single positive thymocytes have been shown to improve donor bone marrow transplantation.[365]

6.2.5 Reversing late-stage T1D with chimerism and beta cell regeneration

In clinical islet transplantation, T1D recipients usually receive islets from more than one donor, which limits the application of chimerism induction in these patients.[184] Islet transplantation with islets from a single donor is possible but not routinely achieved.[304,305] It has been shown by Zeng and colleagues that inducing chimerism and transplantation of a very low dose of donor islets can reverse hyperglycemia in NOD mice that had been spontaneously diabetic for more than 3 weeks.[251] Replication of beta cells in the islet graft was found in this study. Their finding suggests that successful induction of chimerism may allow islet transplantation from a single donor. However, whether or not similar findings can be achieved by using other successful chimerism induction conditioning protocols is unknown. Therefore, it would be important to test the survival of a low dose islet graft in autoimmune diabetic NOD that is also conditioned with the protocol provided in **Chapter 3**.

Zeng's group also showed that inducing chimerism restored normal glycaemia in new-onset diabetic NOD mice.[143] While this is an interesting finding, inducing chimerism in patients that are recently diagnosed with T1D may not be feasible considering the relatively younger age in these population and the risk of GVHD. The author later found that in combination with gastrin and epidermal growth factor (EGF), a reagent that stimulates beta cells regeneration, the establishment of chimerism can also reverse late-stage diabetes in NOD mice.[155] However, only 60% of recipients became normal glycaemic after treatment and there is not yet a marker to predict in which mice hyperglycemia can be reversed. Presumably, if chimerism can safely restore euglycemia in all late-stage diabetic patients

without islet transplantation, a mega-dose of hematopoietic stem cells collected from living donors would be possible. This increase of stem cell dose can potentially reduce the requirement of some toxic reagents in the conditioning protocols. Therefore, it is worthy to test if our chimerism induction conditioning protocol can reverse hyperglycemia in all late-stage diabetic recipients with various reagents that stimulate beta cell regeneration.

6.2.6 Combination of enzyme treatment with other desensitization strategies

Although we induced chimerism in some of these primed recipients, only two mice became stable chimeras. It is important to know if there is a rapid rebound of donor antigen specific antibodies in the mice that lost their chimerism or that did not become chimeric. It was reported that the DSA was eventually lost in presensitized recipients that became chimeric to donor.[322] Therefore, it is also important to know if the DSA eventually disappeared in stable chimeras induced by the new conditioning protocol.

While we provided evidence that the combination of both enzymes can be used in sensitized recipients for the purposes for chimerism induction, this conditioning protocol is not yet ready to be applied to islet transplantation due to the toxicity of the reagents involved and the demand of high dose bone marrow cells. The toxicity of irradiation can potentially be reduced by decreasing the dose or by replacement with busulfan or anti-c-Kit antibodies. However, irradiation not only creates a niche for donor cells, but also induces immunosuppression. It would not be surprising if extra reagents were required for targeting alloreactive memory T cells when the dose of irradiation is reduced. Bortezomib, which

targets plasma cells, has been associated with pulmonary fibrosis and may be toxic to endogenous and exogenous pancreatic beta cells.[371,372] However, a reagent to deplete plasma cells is presumably required in order to inhibit the rapid recovery of DSA, as both islets and bone marrow cells are sensitive to the DSA mediated toxicity.[323,324,373,374] Lastly, the use of cyclophosphamide can potentially be substituted with rituximab, which targets CD20 on B cells.

References

1. ONT-WHO Global Observatory on Donation and Transplantation. 'Organ donation and transplantation activities 2016', (2018), online: <http://www.transplant-observatory.org/download/2016-activity-data-report/>.

2. The Canadian Organ Replacement Register (CORR) at the Canadian Institute for Health Information (CIHI). 'Annual statistics on organ replacement in Canada: dialysis, transplantation and donation, 2008 to 2017', (2018), online: <https://www.cihi.ca/sites/default/files/document/snapshot-corr-2018-en.pdf>.

3. Bastani B. The present and future of transplant organ shortage: some potential remedies. *J Nephrol* (2019):1-12.

4. Gorer PA, Lyman S, Snell GD. Studies on the genetic and antigenic basis of tumour transplantation. Linkage between a histocompatibility gene and'fused'in mice. *P ROY SOC LOND B BIO* (1948) **135**:499-505.

5. DAUSSET J. Iso-leuko-antibodies. *Acta haematologica* (1958) **20**:156.

6. Opelz G. Correlation of HLA matching with kidney graft survival in patients with or without cyclosporine treatment. *Transplantation* (1985) **40**:240-3.

7. Zachary AA, Leffell MS. HLA mismatching strategies for solid organ transplantation - a balancing act. *Front Immunol* (2016) **7**:575.

8. Yacoub R, Nadkarni GN, Cravedi P, He JC, Delaney VB, Kent R, et al. Analysis of OPTN/UNOS registry suggests the number of HLA matches and not mismatches is a stronger independent predictor of kidney transplant survival. *Kidney Int* (2018) **93**:482-90.

9. Loveland B, Simpson E. The non-MHC transplantation antigens: neither weak nor minor. *Immunol Today* (1986) **7**:223-9.

10. Perreault C, Decary F, Brochu S, Gyger M, Belanger R, Roy D. Minor histocompatibility antigens. *Blood* (1990) **76**:1269-80.

11. Spierings E. Minor histocompatibility antigens: past, present, and future. *Tissue Antigens* (2014) **84**:374-60.

12. Lindahl KF, Wilson DB. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J Exp Med* (1977) **145**:508-22.

13. Sherman LA, Chattopadhyay S. The molecular basis of allorecognition. *Annu Rev Immunol* (1993) **11**:385-402.

14. Obst R, Netuschil N, Klopfer K, Stevanovic S, Rammensee HG. The role of peptides in T cell alloreactivity is determined by self-major histocompatibility complex molecules. *J Exp Med* (2000) **191**:805-12.

15. Siu JHY, Surendrakumar V, Richards JA, Pettigrew GJ. T cell allorecognition pathways in solid organ transplantation. *Front Immunol* (2018) **9**:2548.

16. Talmage DW, Dart G, Radovich J, Lafferty KJ. Activation of transplant immunity: effect of donor leukocytes on thyroid allograft rejection. *Science* (1976) **191**:385-8.

17. Marino J, Babiker-Mohamed MH, Crosby-Bertorini P, Paster JT, LeGuern C, Germana S, et al. Donor exosomes rather than passenger leukocytes initiate alloreactive T cell responses after transplantation. *Sci Immunol* (2016) **1**:aaf8759.

18. Liu Q, Rojas-Canales DM, Divito SJ, Shufesky WJ, Stolz DB, Erdos G, et al. Donor dendritic cell-derived exosomes promote allograft-targeting immune response. *J Clin Invest* (2016) **126**:2805-20.

19. Burke GW, 3rd, Vendrame F, Pileggi A, Ciancio G, Reijonen H, Pugliese A. Recurrence of autoimmunity following pancreas transplantation. *Curr Diab Rep* (2011) **11**:413-9.

20. Weber DJ, Wilkes DS. The role of autoimmunity in obliterative bronchiolitis after lung transplantation. *Am J Physiol Lung Cell Mol Physiol* (2013) **304**:L307-11.

21. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* (2004) **351**:2715-29.

22. Van Arendonk KJ, Boyarsky BJ, Orandi BJ, James NT, Smith JM, Colombani PM, et al. National trends over 25 years in pediatric kidney transplant outcomes. *Pediatrics* (2014) **133**:594-601.

23. Chapman JR, Webster AC, Wong G. Cancer in the transplant recipient. *Cold Spring Harb Perspect Med* (2013) **3**:a015677.

24. Fishman JA. Infection in solid-organ transplant recipients. *N Engl J Med* (2007) **357**:2601-14.

25. Roussey-Kesler G, Giral M, Moreau A, Subra JF, Legendre C, Noel C, et al. Clinical operational tolerance after kidney transplantation. *Am J Transplant* (2006) **6**:736-46.

26. Feng S, Bucuvalas J. Tolerance after liver transplantation: Where are we? *Liver Transpl* (2017) **23**:1601-14.

27. Levitsky J, Feng S. Tolerance in clinical liver transplantation. *Hum Immunol* (2018) **79**:283-7.

28. Brouard S, Pallier A, Renaudin K, Foucher Y, Danger R, Devys A, et al. The natural history of clinical operational tolerance after kidney transplantation through twenty-seven cases. *Am J Transplant* (2012) **12**:3296-307.

29. Ohe H, Uchida Y, Yoshizawa A, Hirao H, Taniguchi M, Maruya E, et al. Association of anti-human leukocyte antigen and anti-angiotensin II type 1 receptor antibodies with liver allograft fibrosis after immunosuppression withdrawal. *Transplantation* (2014) **98**:1105-11.

30. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* (2005) **436**:709-13.

31. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* (1999) **285**:727-9.

32. van den Berg TK, van der Schoot CE. Innate immune 'self' recognition: a role for CD47-SIRPα interactions in hematopoietic stem cell transplantation. *Trends Immunol* (2008) **29**:203-6.

33. Dai H, Friday AJ, Abou-Daya KI, Williams AL, Mortin-Toth S, Nicotra ML, et al. Donor SIRPα polymorphism modulates the innate immune response to allogeneic grafts. *Sci Immunol* (2017) **2**:eaam6202.

34. Gellert M. V(D)J recombination: RAG proteins, repair factors, and regulation. *Annu Rev Biochem* (2002) **71**:101-32.

35. Motea EA, Berdis AJ. Terminal deoxynucleotidyl transferase: the story of a misguided DNA polymerase. *Biochim Biophys Acta* (2010) **1804**:1151-66.

36. Di Noia JM, Neuberger MS. Molecular mechanisms of antibody somatic hypermutation. *Annu Rev Biochem* (2007) **76**:1-22.

37. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol* (2015) **16**:1114-23.

38. Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* (2005) **5**:772-82.

39. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol* (2014) **14**:377-91.

40. Gascoigne NR, Rybakin V, Acuto O, Brzostek J. TCR signal strength and T cell development. *Annu Rev Cell Dev Biol* (2016) **32**:327-48.

41. Ernst B, Lee DS, Chang JM, Sprent J, Surh CD. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* (1999) **11**:173-81.

42. Myers DR, Zikherman J, Roose JP. Tonic signals: why do lymphocytes bother? *Trends Immunol* (2017) **38**:844-57.

43. Malchow S, Leventhal DS, Lee V, Nishi S, Socci ND, Savage PA. Aire enforces immune tolerance by directing autoreactive T Cells into the regulatory T cell lineage. *Immunity* (2016) **44**:1102-13.

44. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* (2001) **27**:68-73.

45. Walker LS, Abbas AK. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol* (2002) **2**:11.

46. Matzinger P, Kamala T. Tissue-based class control: the other side of tolerance. *Nat Rev Immunol* (2011) **11**:221-30.

47. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med* (1987) **165**:302-19.

48. Bretscher P, Cohn M. A theory of self-nonself discrimination. *Science* (1970) **169**:1042-9.

49. Miller JF, Mitchell GF. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J Exp Med* (1968) **128**:801-20.

50. Mitchell GF, Miller JF. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J Exp Med* (1968) **128**:821-37.

51. Keene J, Forman J. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *J Exp Med* (1982) **155**:768-82.

52. Claman HN, Chaperon EA, Triplett RF. Thymus-marrow cell combinations. Synergism in antibody production. *Proc Soc Exp Biol Med* (1966) **122**:1167-71.

53. Al-Yassin GA, Bretscher PA. Does T cell activation require a quorum of lymphocytes? *J Immunol* (2018) **201**:2855-61.

54. Akkaya B, Oya Y, Akkaya M, Al Souz J, Holstein AH, Kamenyeva O, et al. Regulatory T cells mediate specific suppression by depleting peptide-MHC class II from dendritic cells. *Nat Immunol* (2019) **20**:218-31.

55. Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* (1989) **54 Pt 1**:1-13.

56. Janeway CA, Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* (1992) **13**:11-6.

57. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci* (1975) **53**:27-42.

58. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* (1994) **12**:991-1045.

59. Bingaman AW, Ha J, Waitze SY, Durham MM, Cho HR, Tucker-Burden C, et al. Vigorous allograft rejection in the absence of danger. *J Immunol* (2000) **164**:3065-71.

60. Anderson CC, Carroll JM, Gallucci S, Ridge JP, Cheever AW, Matzinger P. Testing time-, ignorance-, and danger-based models of tolerance. *J Immunol* (2001) **166**:3663-71.

61. Sinclair NS, Chan P. Regulation of the immune response. IV. The role of the Fc-fragment in feedback inhibition by antibody. Morphological and functional aspects of immunity. Springer (1971). p. 609-15.

62. Sinclair NR, Anderson CC. Co-stimulation and co-inhibition: equal partners in regulation. *Scand J Immunol* (1996) **43**:597-603.

63. Sinclair NRS. Commentary I: How many signals are enough? *Cell Immunol* (1990) **130**:204-12.

64. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* (2013) **13**:227-42.

65. Bendelac A, Bonneville M, Kearney JF. Autoreactivity by design: innate B and T lymphocytes. *Nat Rev Immunol* (2001) **1**:177-86.

66. Cornall RJ, Goodnow CC, Cyster JG. The regulation of self-reactive B cells. *Curr Opin Immunol* (1995) **7**:804-11.

67. Brink R, Phan TG. Self-reactive B cells in the germinal center reaction. *Annu Rev Immunol* (2018) **36**:339-57.

68. Nemazee DA, Burki K. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* (1989) **337**:562-6.

69. Halverson R, Torres RM, Pelanda R. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. *Nat Immunol* (2004) **5**:645-50.

70. Goodnow CC, Crosbie J, Adelstein S, Lavoie TB, Smith-Gill SJ, Brink RA, et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* (1988) **334**:676-82.

71. Hartley SB, Crosbie J, Brink R, Kantor AB, Basten A, Goodnow CC. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature* (1991) **353**:765-9.

72. Russell DM, Dembic Z, Morahan G, Miller JF, Burki K, Nemazee D. Peripheral deletion of self-reactive B cells. *Nature* (1991) **354**:308-11.

73. Akkaraju S, Canaan K, Goodnow CC. Self-reactive B cells are not eliminated or inactivated by autoantigen expressed on thyroid epithelial cells. *J Exp Med* (1997) **186**:2005-12.

74. Aplin BD, Keech CL, de Kauwe AL, Gordon TP, Cavill D, McCluskey J. Tolerance through indifference: autoreactive B cells to the nuclear antigen La show no evidence of tolerance in a transgenic model. *J Immunol* (2003) **171**:5890-900.

75. Pelanda R, Torres RM. Central B-cell tolerance: where selection begins. *Cold Spring Harb Perspect Biol* (2012) **4**:a007146.

76. Crotty S. A brief history of T cell help to B cells. *Nat Rev Immunol* (2015) **15**:185-9.

77. Tsubata T. Ligand recognition determines the role of inhibitory B cell co-receptors in the regulation of B cell homeostasis and autoimmunity. *Front Immunol* (2018) **9**:2276.

78. de Ferranti SD, de Boer IH, Fonseca V, Fox CS, Golden SH, Lavie CJ, et al. Type 1 diabetes mellitus and cardiovascular disease: a scientific statement from the American Heart Association and American Diabetes Association. *Diabetes Care* (2014) **37**:2843-63.

79. Huo L, Harding JL, Peeters A, Shaw JE, Magliano DJ. Life expectancy of type 1 diabetic patients during 1997-2010: a national Australian registry-based cohort study. *Diabetologia* (2016) **59**:1177-85.

80. Patterson CC, Harjutsalo V, Rosenbauer J, Neu A, Cinek O, Skrivarhaug T, et al. Trends and cyclical variation in the incidence of childhood type 1 diabetes in 26 European centres in the 25 year period 1989-2013: a multicentre prospective registration study. *Diabetologia* (2019) **62**:408-17.

81. Fox DA, Islam N, Sutherland J, Reimer K, Amed S. Type 1 diabetes incidence and prevalence trends in a cohort of Canadian children and youth. *Pediatr Diabetes* (2018) **19**:501-5.

82. Redondo MJ, Jeffrey J, Fain PR, Eisenbarth GS, Orban T. Concordance for islet autoimmunity among monozygotic twins. *N Engl J Med* (2008) **359**:2849-50.

83. Dorman JS, Steenkiste AR, O'Leary LA, McCarthy BJ, Lorenzen T, Foley TP. Type 1 diabetes in offspring of parents with type 1 diabetes: the tip of an autoimmune iceberg? *Pediatr Diabetes* (2000) **1**:17-22.

84. Mrena S, Virtanen SM, Laippala P, Kulmala P, Hannila ML, Akerblom HK, et al. Models for predicting type 1 diabetes in siblings of affected children. *Diabetes Care* (2006) **29**:662-7.

85. Singal DP, Blajchman MA. Histocompatibility (HL-A) antigens, lymphocytotoxic antibodies and tissue antibodies in patients with diabetes mellitus. *Diabetes* (1973) **22**:429-32.

86. Noble JA, Valdes AM, Cook M, Klitz W, Thomson G, Erlich HA. The role of HLA class II genes in insulin-dependent diabetes mellitus: molecular analysis of 180 Caucasian, multiplex families. *Am J Hum Genet* (1996) **59**:1134-48.

87. Onengut-Gumuscu S, Chen WM, Burren O, Cooper NJ, Quinlan AR, Mychaleckyj JC, et al. Fine mapping of type 1 diabetes susceptibility loci and evidence for colocalization of causal variants with lymphoid gene enhancers. *Nat Genet* (2015) **47**:381-6.

88. Rewers M, Ludvigsson J. Environmental risk factors for type 1 diabetes. *Lancet* (2016) **387**:2340-8.

89. Gundersen E. Is diabetes of infectious origin? *J Infect Dis* (1927) 197-202.

90. Coppieters KT, Wiberg A, von Herrath MG. Viral infections and molecular mimicry in type 1 diabetes. *APMIS* (2012) **120**:941-9.

91. Stene L, Rewers M. Immunology in the clinic review series; focus on type 1 diabetes and viruses: the enterovirus link to type 1 diabetes: critical review of human studies. *Clin Exp Immunol* (2012) **168**:12-23.

92. Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK. Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. *J Clin Invest* (1994) **94**:2125-9.

93. Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia* (2009) **52**:1143-51.

94. Krogvold L, Edwin B, Buanes T, Frisk G, Skog O, Anagandula M, et al. Detection of a low-grade enteroviral infection in the islets of langerhans of living patients newly diagnosed with type 1 diabetes. *Diabetes* (2015) **64**:1682-7.

95. Okada H, Kuhn C, Feillet H, Bach JF. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clin Exp Immunol* (2010) **160**:1-9.

96. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, et al. Toward defining the autoimmune microbiome for type 1 diabetes. *ISME J* (2011) **5**:82-91.

97. de Goffau MC, Luopajärvi K, Knip M, Ilonen J, Ruohtula T, Härkönen T, et al. Fecal microbiota composition differs between children with β-cell autoimmunity and those without. *Diabetes* (2013) **62**:1238-44.

98. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* (1965) **14**:619-33.

99. Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG, Gamble DR. In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. *N Engl J Med* (1985) **313**:353-60.

100. Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Analysis of islet inflammation in human type 1 diabetes. *Clin Exp Immunol* (2009) **155**:173-81.

101. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, et al. Coxsackie B4 virus infection of β cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. *Proc Natl Acad Sci U S A* (2007) **104**:5115-20.

102. Bottazzo GF, Florin-Christensen A, Doniach D. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet* (1974) **2**:1279-83.

103. Visperas A, Vignali DA. Are regulatory T cells defective in type 1 diabetes and can we fix them? *J Immunol* (2016) **197**:3762-70.

104. Perol L, Lindner JM, Caudana P, Nunez NG, Baeyens A, Valle A, et al. Loss of immune tolerance to IL-2 in type 1 diabetes. *Nat Commun* (2016) **7**:13027.

105. Ferreira RC, Simons HZ, Thompson WS, Cutler AJ, Dopico XC, Smyth DJ, et al. IL-21 production by CD4+ effector T cells and frequency of circulating follicular helper T cells are increased in type 1 diabetes patients. *Diabetologia* (2015) **58**:781-90.

106. Mallone R, Brezar V, Boitard C. T cell recognition of autoantigens in human type 1 diabetes: clinical perspectives. *Clin Dev Immunol* (2011) **2011**:513210.

107. Gottlieb PA, Delong T, Baker RL, Fitzgerald-Miller L, Wagner R, Cook G, et al. Chromogranin A is a T cell antigen in human type 1 diabetes. *J Autoimmun* (2014) **50**:38-41.

108. Delong T, Wiles TA, Baker RL, Bradley B, Barbour G, Reisdorph R, et al. Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. *Science* (2016) **351**:711-4.

109. Chen YG, Mathews CE, Driver JP. The role of NOD mice in type 1 diabetes research: lessons from the past and recommendations for the future. *Front Endocrinol (Lausanne)* (2018) **9**:51.

110. Driver JP, Chen YG, Mathews CE. Comparative genetics: synergizing human and NOD mouse studies for identifying genetic causation of type 1 diabetes. *Rev Diabet Stud* (2012) **9**:169-87.

111. Hattori M, Buse JB, Jackson RA, Glimcher L, Dorf ME, Minami M, et al. The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. *Science* (1986) **231**:733-5.

112. Pearson JA, Wong FS, Wen L. The importance of the Non Obese Diabetic (NOD) mouse model in autoimmune diabetes. *J Autoimmun* (2016) **66**:76-88.

113. Wicker LS, Miller BJ, Mullen Y. Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. *Diabetes* (1986) **35**:855-60.

114. Harada M, Makino S. Suppression of overt diabetes in NOD mice by anti-thymocyte serum or anti-Thy 1, 2 antibody. *Exp Anim* (1986) **35**:501-4.

115. Koike T, Itoh Y, Ishii T, Ito I, Takabayashi K, Maruyama N, et al. Preventive effect of monoclonal anti-L3T4 antibody on development of diabetes in NOD mice. *Diabetes* (1987) **36**:539-41.

116. Charlton B, Bacelj A, Mandel TE. Administration of silica particles or anti-Lyt2 antibody prevents beta-cell destruction in NOD mice given cyclophosphamide. *Diabetes* (1988) **37**:930-5.

117. Christianson SW, Shultz LD, Leiter EH. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice: relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD. NON-Thy-1a donors. *Diabetes* (1993) **42**:44-55.

118. Yang M, Charlton B, Gautam AM. Development of insulitis and diabetes in B cell-deficient NOD mice. *J Autoimmun* (1997) **10**:257-60.

119. Serreze DV, Fleming SA, Chapman HD, Richard SD, Leiter EH, Tisch RM. B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Immunol* (1998) **161**:3912-8.

120. Noorchashm H, Lieu YK, Noorchashm N, Rostami SY, Greeley SAS, Schlachterman A, et al. I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet β cells of nonobese diabetic mice. *J Immunol* (1999) **163**:743-50.

121. Carrero JA, McCarthy DP, Ferris ST, Wan X, Hu H, Zinselmeyer BH, et al. Resident macrophages of pancreatic islets have a seminal role in the initiation of autoimmune diabetes of NOD mice. *Proc Natl Acad Sci U S A* (2017) **114**:E10418-E27.

122. Kishimoto H, Sprent J. A defect in central tolerance in NOD mice. *Nat Immunol* (2001) **2**:1025-31.

123. Lesage S, Hartley SB, Akkaraju S, Wilson J, Townsend M, Goodnow CC. Failure to censor forbidden clones of CD4 T cells in autoimmune diabetes. *J Exp Med* (2002) **196**:1175-88.

124. Liston A, Lesage S, Gray DH, O'Reilly LA, Strasser A, Fahrer AM, et al. Generalized resistance to thymic deletion in the NOD mouse; a polygenic trait characterized by defective induction of Bim. *Immunity* (2004) **21**:817-30.

125. Mellanby RJ, Thomas D, Phillips JM, Cooke A. Diabetes in non-obese diabetic mice is not associated with quantitative changes in CD4+ CD25+ Foxp3+ regulatory T cells. *Immunology* (2007) **121**:15-28.

126. Yamanouchi J, Rainbow D, Serra P, Howlett S, Hunter K, Garner VE, et al. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat Genet* (2007) **39**:329-37.

127. D'Alise AM, Auyeung V, Feuerer M, Nishio J, Fontenot J, Benoist C, et al. The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. *Proc Natl Acad Sci U S A* (2008) **105**:19857-62.

128. Juvet SC, Zhang L. Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions. *J Mol Cell Biol* (2012) **4**:48-58.

129. Duncan B, Nazarov-Stoica C, Surls J, Kehl M, Bona C, Casares S, et al. Double negative (CD3+ 4- 8-) TCR αβ splenic cells from young NOD mice provide long-lasting protection against type 1 diabetes. *PLoS One* (2010) **5**:e11427.

130. Dugas V, Beauchamp C, Chabot-Roy G, Hillhouse EE, Lesage S. Implication of the CD47 pathway in autoimmune diabetes. *J Autoimmun* (2010) **35**:23-32.

131. Dugas V, Liston A, Hillhouse EE, Collin R, Chabot-Roy G, Pelletier AN, et al. Idd13 is involved in determining immunoregulatory DN T-cell number in NOD mice. *Genes Immun* (2014) **15**:82-7.

132. Collin R, Dugas V, Pelletier AN, Chabot-Roy G, Lesage S. The mouse Idd2 locus is linked to the proportion of immunoregulatory double-negative T cells, a trait associated with autoimmune diabetes resistance. *J Immunol* (2014) **193**:3503-12.

133. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic extracts in the treatment of diabetes mellitus. *Can Med Assoc J* (1922) **12**:141-6.

134. Fullerton B, Jeitler K, Seitz M, Horvath K, Berghold A, Siebenhofer A. Intensive glucose control versus conventional glucose control for type 1 diabetes mellitus. *Cochrane Database Syst Rev* (2014).

135. Chatenoud L, Thervet E, Primo J, Bach J-F. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci U S A* (1994) **91**:123-7.

136. Ryu S, Kodama S, Ryu K, Schoenfeld DA, Faustman DL. Reversal of established autoimmune diabetes by restoration of endogenous beta cell function. *J Clin Invest* (2001) **108**:63-72.

137. Ogawa N, List JF, Habener JF, Maki T. Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4. *Diabetes* (2004) **53**:1700-5.

138. Maki T, Gottschalk R, Ogawa N, Monaco AP. Prevention and cure of autoimmune diabetes in nonobese diabetic mice by continuous administration of FTY720. *Transplantation* (2005) **79**:1051-5.

139. Bresson D, Togher L, Rodrigo E, Chen Y, Bluestone JA, Herold KC, et al. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. *J Clin Invest* (2006) **116**:1371-81.

140. Fife BT, Guleria I, Gubbels Bupp M, Eagar TN, Tang Q, Bour-Jordan H, et al. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. *J Exp Med* (2006) **203**:2737-47.

141. Yang Z, Chen M, Carter JD, Nunemaker CS, Garmey JC, Kimble SD, et al. Combined treatment with lisofylline and exendin-4 reverses autoimmune diabetes. *Biochem Biophys Res Commun* (2006) **344**:1017-22.

142. Sherry NA, Chen W, Kushner JA, Glandt M, Tang Q, Tsai S, et al. Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of β-cells. *Endocrinology* (2007) **148**:5136-44.

143. Zhang C, Todorov I, Lin CL, Atkinson M, Kandeel F, Forman S, et al. Elimination of insulitis and augmentation of islet beta cell regeneration via induction of chimerism in overtly diabetic NOD mice. *Proc Natl Acad Sci U S A* (2007) **104**:2337-42.

144. Fiorina P, Vergani A, Dada S, Jurewicz M, Wong M, Law K, et al. Targeting CD22 reprograms B-cells and reverses autoimmune diabetes. *Diabetes* (2008) **57**:3013-24.

145. Louvet C, Szot GL, Lang J, Lee MR, Martinier N, Bollag G, et al. Tyrosine kinase inhibitors reverse type 1 diabetes in nonobese diabetic mice. *Proc Natl Acad Sci U S A* (2008) **105**:18895-900.

146. Perone MJ, Bertera S, Shufesky WJ, Divito SJ, Montecalvo A, Mathers AR, et al. Suppression of autoimmune diabetes by soluble galectin-1. *J Immunol* (2009) **182**:2641-53.

147. Tian B, Hao J, Zhang Y, Tian L, Yi H, O'Brien TD, et al. Upregulating CD4+CD25+FOXP3+ regulatory T cells in pancreatic lymph nodes in diabetic NOD mice by adjuvant immunotherapy. *Transplantation* (2009) **87**:198-206.

148. Zhao Y, Lin B, Darflinger R, Zhang Y, Holterman MJ, Skidgel RA. Human cord blood stem cell-modulated regulatory T lymphocytes reverse the autoimmune-caused type 1 diabetes in nonobese diabetic (NOD) mice. *PLoS One* (2009) **4**:e4226.

149. Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, et al. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med* (2010) **207**:1871-8.

150. Ablamunits V, Henegariu O, Hansen JB, Opare-Addo L, Preston-Hurlburt P, Santamaria P, et al. Synergistic reversal of type 1 diabetes in NOD mice with anti-CD3 and interleukin-1 blockade: evidence of improved immune regulation. *Diabetes* (2012) **61**:145-54.

151. Davoodi-Semiromi A, Wasserfall CH, Xia CQ, Cooper-DeHoff RM, Wabitsch M, Clare-Salzler M, et al. The tyrphostin agent AG490 prevents and reverses type 1 diabetes in NOD mice. *PLoS One* (2012) **7**:e36079.

152. Lee L-F, Logronio K, Tu GH, Zhai W, Ni I, Mei L, et al. Anti-IL-7 receptor-α reverses established type 1 diabetes in nonobese diabetic mice by modulating effector T-cell function. *Proc Natl Acad Sci U S A* (2012) **109**:12674-9.

153. Penaranda C, Kuswanto W, Hofmann J, Kenefeck R, Narendran P, Walker LS, et al. IL-7 receptor blockade reverses autoimmune diabetes by promoting inhibition of effector/memory T cells. *Proc Natl Acad Sci U S A* (2012) **109**:12668-73.

154. Takiishi T, Korf H, Van Belle TL, Robert S, Grieco FA, Caluwaerts S, et al. Reversal of autoimmune diabetes by restoration of antigen-specific tolerance using genetically modified Lactococcus lactis in mice. *J Clin Invest* (2012) **122**:1717-25.

155. Wang M, Racine JJ, Song X, Li X, Nair I, Liu H, et al. Mixed chimerism and growth factors augment beta cell regeneration and reverse late-stage type 1 diabetes. *Sci Transl Med* (2012) **4**:133ra59.

156. Yi Z, Diz R, Martin AJ, Morillon YM, Kline DE, Li L, et al. Long-term remission of diabetes in NOD mice is induced by nondepleting anti-CD4 and anti-CD8 antibodies. *Diabetes* (2012) **61**:2871-80.

157. Grant CW, Moran-Paul CM, Duclos SK, Guberski DL, Arreaza-Rubin G, Spain LM. Testing agents for prevention or reversal of type 1 diabetes in rodents. *PLoS One* (2013) **8**:e72989.

158. Hu C, Ding H, Zhang X, Wong FS, Wen L. Combination treatment with anti-CD20 and oral anti-CD3 prevents and reverses autoimmune diabetes. *Diabetes* (2013) **62**:2849-58.

159. Wang M, Racine J, Zhang M, Wu T, Deng R, Johnston H, et al. MHC-mismatched chimerism is required for induction of transplantation tolerance in autoimmune nonobese diabetic recipients. *J Immunol* (2014) **193**:2005-15.

160. Bednar KJ, Tsukamoto H, Kachapati K, Ohta S, Wu Y, Katz JD, et al. Reversal of new-onset type 1 diabetes with an agonistic TLR4/MD-2 monoclonal antibody. *Diabetes* (2015) **64**:3614-26.

161. Liu T, Cong M, Sun G, Wang P, Tian Y, Shi W, et al. Combination of double negative T cells and anti-thymocyte serum reverses type 1 diabetes in NOD mice. *J Transl Med* (2016) **14**:57.

162. Ben Nasr M, Tezza S, D'Addio F, Mameli C, Usuelli V, Maestroni A, et al. PD-L1 genetic overexpression or pharmacological restoration in hematopoietic stem and progenitor cells reverses autoimmune diabetes. *Sci Transl Med* (2017) **9**:eaam7543.

163. Ryden AK, Perdue NR, Pagni PP, Gibson CB, Ratliff SS, Kirk RK, et al. Anti-IL-21 monoclonal antibody combined with liraglutide effectively reverses established hyperglycemia in mouse models of type 1 diabetes. *J Autoimmun* (2017) **84**:65-74.

164. Besancon A, Goncalves T, Valette F, Dahllof MS, Mandrup-Poulsen T, Chatenoud L, et al. Oral histone deacetylase inhibitor synergises with T cell targeted immunotherapy to preserve beta cell metabolic function and induce stable remission of new-onset autoimmune diabetes in NOD mice. *Diabetologia* (2018) **61**:389-98.

165. Jacobsen LM, Newby BN, Perry DJ, Posgai AL, Haller MJ, Brusko TM. Immune mechanisms and pathways targeted in type 1 diabetes. *Curr Diab Rep* (2018) **18**:90.

166. Herold KC, Bundy BN, Long SA, Bluestone JA, DiMeglio LA, Dufort MJ, et al. An Anti-CD3 antibody, teplizumab, in relatives at risk for type 1 diabetes. *N Engl J Med* (2019) **381**:603-13.

167. Kuhn C, Weiner HL. Therapeutic anti-CD3 monoclonal antibodies: from bench to bedside. *Immunotherapy* (2016) **8**:889-906.

168. Lewis EC, Blaabjerg L, Størling J, Ronn SG, Mascagni P, Dinarello CA, et al. The oral histone deacetylase inhibitor ITF2357 reduces cytokines and protects islet β cells in vivo and in vitro. *Mol Med* (2011) **17**:369-77.

169. Cobbold SP, Castejon R, Adams E, Zelenika D, Graca L, Humm S, et al. Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol* (2004) **172**:6003-10.

170. Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet β-cell destruction and insulin-dependent diabetes mellitus. *Biochem Pharmacol* (1998) **55**:1139-49.

171. Alkanani AK, Rewers M, Dong F, Waugh K, Gottlieb PA, Zipris D. Dysregulated toll-like receptor-induced interleukin-1β and interleukin-6 responses in subjects at risk for the development of Type 1 diabetes. *Diabetes* (2012) **61**:2525-33.

172. Mohammad MK, Morran M, Slotterbeck B, Leaman DW, Sun Y, Grafenstein Hv, et al. Dysregulated Toll-like receptor expression and signaling in bone marrow-derived macrophages at the onset of diabetes in the non-obese diabetic mouse. *Int Immunol* (2006) **18**:1101-13.

173. Sadelain MW, Qin HY, Lauzon J, Singh B. Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. *Diabetes* (1990) **39**:583-9.

174. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* (2010) **236**:219-42.

175. Keymeulen B, Walter M, Mathieu C, Kaufman L, Gorus F, Hilbrands R, et al. Four-year metabolic outcome of a randomised controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass. *Diabetologia* (2010) **53**:614-23.

176. Hagopian W, Ferry RJ, Sherry N, Carlin D, Bonvini E, Johnson S, et al. Teplizumab preserves C-peptide in recent-onset type 1 diabetes: two-year results from the randomized, placebo-controlled Protégé trial. *Diabetes* (2013) **62**:3901-8.

177. Malmegrim KC, de Azevedo JT, Arruda L, Abreu JR, Couri CE, de Oliveira GL, et al. Immunological balance is associated with clinical outcome after autologous hematopoietic stem cell transplantation in type 1 diabetes. *Front Immunol* (2017) **8**:167.

178. Wan X, Guloglu FB, Vanmorlan AM, Rowland LM, Zaghouani S, Cascio JA, et al. Recovery from overt type 1 diabetes ensues when immune tolerance and β-cell formation are coupled with regeneration of endothelial cells in the pancreatic islets. *Diabetes* (2013) **62**:2879-89.

179. Jurczyk A, Bortell R, Alonso LC. Human β-cell regeneration: progress, hurdles, and controversy. *Curr Opin Endocrinol Diabetes Obes* (2014) **21**:102.

180. Jiang Y, Zhong F. Endogenous pancreatic β-cell regeneration: a potential strategy for the recovery of β-cell deficiency in diabetes. *Front Endocrinol (Lausanne)* (2019) **10**:101.

181. Gamble A, Pepper AR, Bruni A, Shapiro AMJ. The journey of islet cell transplantation and future development. *Islets* (2018) **10**:80-94.

182. Wiseman AC. Pancreas transplant options for patients with type 1 diabetes mellitus and chronic kidney disease: simultaneous pancreas kidney or pancreas after kidney? *Curr Opin Organ Transplant* (2012) **17**:80-6.

183. Troppmann C. Complications after pancreas transplantation. *Curr Opin Organ Transplant* (2010) **15**:112-8.

184. Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ. Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges. *Diabetes Metab Syndr Obes* (2014) **7**:211-23.

185. Scalea JR, Tomita Y, Lindholm CR, Burlingham W. Transplantation tolerance induction: cell therapies and their mechanisms. *Front Immunol* (2016) **7**:87.

186. Vaikunthanathan T, Safinia N, Boardman D, Lechler RI, Lombardi G. Regulatory T cells: tolerance induction in solid organ transplantation. *Clin Exp Immunol* (2017) **189**:197-210.

187. Oura T, Cosimi AB, Kawai T. Chimerism-based tolerance in organ transplantation: preclinical and clinical studies. *Clin Exp Immunol* (2017) **189**:190-6.

188. Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* (1995) **92**:9560-4.

189. Markees TG, Serreze DV, Phillips NE, Sorli CH, Gordon EJ, Shultz LD, et al. NOD mice have a generalized defect in their response to transplantation tolerance induction. *Diabetes* (1999) **48**:967-74.

190. Demirci G, Strom TB, Li XC. Islet allograft rejection in nonobese diabetic mice involves the common γ-chain and CD28/CD154-dependent and -independent mechanisms. *J Immunol* (2003) **171**:3878-85.

191. Moore DJ, Huang X, Lee IV MK, Lian MM, Chiaccio M, Chen H, et al. Resistance to anti-CD45RB-induced tolerance in NOD mice: mechanisms involved. *Transpl Int* (2004) **17**:261-9.

192. Ansari MJ, Fiorina P, Dada S, Guleria I, Ueno T, Yuan X, et al. Role of ICOS pathway in autoimmune and alloimmune responses in NOD mice. *Clin Immunol* (2008) **126**:140-7.

193. Suarez-Pinzon W, Korbutt GS, Power R, Hooton J, Rajotte RV, Rabinovitch A. Testicular sertoli cells protect islet beta-cells from autoimmune destruction in NOD mice by a transforming growth factor-beta1-dependent mechanism. *Diabetes* (2000) **49**:1810-8.

194. Shieh SJ, Chou FC, Yu PN, Lin WC, Chang DM, Roffler SR, et al. Transgenic expression of single-chain anti-CTLA-4 Fv on β cells protects nonobese diabetic mice from autoimmune diabetes. *J Immunol* (2009) **183**:2277-85.

195. Graham JG, Zhang X, Goodman A, Pothoven K, Houlihan J, Wang S, et al. PLG scaffold delivered antigen-specific regulatory T cells induce systemic tolerance in autoimmune diabetes. *Tissue Eng Part A* (2013) **19**:1465-75.

196. Molano RD, Berney T, Li H, Cattan P, Pileggi A, Vizzardelli C, et al. Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation. *Diabetes* (2001) **50**:270-6.

197. Molano R, Berney T, Pileggi A, Ricordi C, Burkly L, Rothstein D, et al. Prolonged survival of allogeneic islet grafts in NOD mice treated with a combination of anti-CD45RB and anti-CD154 antibodies. *Transplant Proc* (2001) **33**:248-9.

198. Carvello M, Petrelli A, Vergani A, Lee KM, Tezza S, Chin M, et al. Inotuzumab ozogamicin murine analog-mediated B-cell depletion reduces anti-islet allo-and autoimmune responses. *Diabetes* (2012) **61**:155-65.

199. Vergani A, D'Addio F, Jurewicz M, Petrelli A, Watanabe T, Liu K, et al. A novel clinically relevant strategy to abrogate autoimmunity and regulate alloimmunity in NOD mice. *diabetes* (2010) **59**:2253-64.

200. Deng R, Khattar M, Xie A, Schroder PM, He X, Chen W, et al. Anti-TCR mAb induces peripheral tolerance to alloantigens and delays islet allograft rejection in autoimmune diabetic NOD mice. *Transplantation* (2014) **97**:1216-24.

201. Fousteri G, Jofra T, Di Fonte R, Battaglia M. Combination of an antigen-specific therapy and an immunomodulatory treatment to simultaneous block recurrent autoimmunity and alloreactivity in non-obese diabetic mice. *PLoS One* (2015) **10**:e0127631.

202. Fu F, Hu S, Deleo J, Li S, Hopf C, Hoover J, et al. Long-term islet graft survival in streptozotocin-and autoimmune-induced diabetes models by immunosuppressive and potential insulinotropic agent FTY720. *Transplantation* (2002) **73**:1425-30.

203. Posselt AM, Szot GL, Frassetto LA, Masharani U, Tavakol M, Amin R, et al. Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive protocols based on T-cell adhesion or costimulation blockade. *Transplantation* (2010) **90**:1595.

204. Shapiro AM. State of the art of clinical islet transplantation and novel protocols of immunosuppression. *Curr Diab Rep* (2011) **11**:345-54.

205. Li H, Kaufman CL, Ildstad ST. Allogeneic chimerism induces donor-specific tolerance to simultaneous islet allografts in nonobese diabetic mice. *Surgery* (1995) **118**:192-7; discussion 7-8.

206. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* (1945) **102**:400-1.

207. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* (1953) **172**:603-6.

208. Main JM, Prehn RT. Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow. *J Natl Cancer Inst* (1955) **15**:1023-9.

209. Yolcu ES, Shirwan H, Askenasy N. Mechanisms of tolerance induction by hematopoietic chimerism: the immune perspective. *Stem Cells Transl Med* (2017) **6**:700-12.

210. Scandling JD, Busque S, Dejbakhsh-Jones S, Benike C, Millan MT, Shizuru JA, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* (2008) **358**:362-8.

211. Scandling JD, Busque S, Shizuru JA, Lowsky R, Hoppe R, Dejbakhsh-Jones S, et al. Chimerism, graft survival, and withdrawal of immunosuppressive drugs in HLA matched and mismatched patients after living donor kidney and hematopoietic cell transplantation. *Am J Transplant* (2015) **15**:695-704.

212. Leventhal J, Abecassis M, Miller J, Gallon L, Ravindra K, Tollerud DJ, et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med* (2012) **4**:124ra28-ra28.

213. Leventhal J GJ, Mathew J, Gallon L, Stare D, Sweeney A, Miller J, Abecassis M, Ildstad S. Eight year follow-up of a phase 2 clinical trial to induce tolerance in living donor renal transplant recipients. *Am J Transplant* (2017) **17**:suppl 3.

214. Soderdahl G, Tammik C, Remberger M, Ringden O. Cadaveric bone marrow and spleen cells for transplantation. *Bone Marrow Transplant* (1998) **21**:79-84.

215. Kawai T, Sachs DH, Sykes M, Cosimi AB, Immune Tolerance N. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* (2013) **368**:1850-2.

216. Kawai T, Cosimi AB, Wee SL, Houser S, Andrews D, Sogawa H, et al. Effect of mixed hematopoietic chimerism on cardiac allograft survival in cynomolgus monkeys. *Transplantation* (2002) **73**:1757-64.

217. Tonsho M, Lee S, Aoyama A, Boskovic S, Nadazdin O, Capetta K, et al. Tolerance of lung allografts achieved in nonhuman primates via mixed hematopoietic chimerism. *Am J Transplant* (2015) **15**:2231-9.

218. Oura T, Ko DS, Boskovic S, O'Neil JJ, Chipashvili V, Koulmanda M, et al. Kidney versus islet allograft survival after induction of mixed chimerism with combined donor bone marrow transplantation. *Cell Transplant* (2016) **25**:1331-41.

219. Gordon EJ, Wicker LS, Peterson LB, Serreze DV, Markees TG, Shultz LD, et al. Autoimmune diabetes and resistance to xenograft transplantation tolerance in NOD mice. *Diabetes* (2005) **54**:107-15.

220. Pearson T, Markees TG, Wicker LS, Serreze DV, Peterson LB, Mordes JP, et al. NOD congenic mice genetically protected from autoimmune diabetes remain resistant to transplantation tolerance induction. *Diabetes* (2003) **52**:321-6.

221. Pearson T, Markees TG, Serreze DV, Pierce MA, Marron MP, Wicker LS, et al. Genetic disassociation of autoimmunity and resistance to costimulation blockade-induced transplantation tolerance in nonobese diabetic mice. *J Immunol* (2003) **171**:185-95.

222. Chan WF, Razavy H, Luo B, Shapiro AM, Anderson CC. Development of either split tolerance or robust tolerance along with humoral tolerance to donor and third-party alloantigens in nonmyeloablative mixed chimeras. *J Immunol* (2008) **180**:5177-86.

223. Baron F, Mohty M, Blaise D, Socie G, Labopin M, Esteve J, et al. Anti-thymocyte globulin as graft-versus-host disease prevention in the setting of allogeneic peripheral blood stem cell transplantation: a review from the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Haematologica* (2017) **102**:224-34.

224. Kroger N, Solano C, Wolschke C, Bandini G, Patriarca F, Pini M, et al. Antilymphocyte globulin for prevention of chronic graft-versus-host disease. *N Engl J Med* (2016) **374**:43-53.

225. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* (2000) **6**:114.

226. Pinelli DF, Ford ML. Novel insights into anti-CD40/CD154 immunotherapy in transplant tolerance. *Immunotherapy* (2015) **7**:399-410.

227. Yamada Y, Ochiai T, Boskovic S, Nadazdin O, Oura T, Schoenfeld D, et al. Use of CTLA4Ig for induction of mixed chimerism and renal allograft tolerance in nonhuman primates. *Am J Transplant* (2014) **14**:2704-12.

228. Langford-Smith KJ, Sandiford Z, Langford-Smith A, Wilkinson FL, Jones SA, Wraith JE, et al. Signal one and two blockade are both critical for non-myeloablative murine HSCT across a major histocompatibility complex barrier. *PLoS One* (2013) **8**:e77632.

229. Barlow AD, Nicholson ML, Herbert TP. Evidence for rapamycin toxicity in pancreatic β-cells and a review of the underlying molecular mechanisms. *Diabetes* (2013) **62**:2674-82.

230. Yang SB, Lee HY, Young DM, Tien AC, Rowson-Baldwin A, Shu YY, et al. Rapamycin induces glucose intolerance in mice by reducing islet mass, insulin content, and insulin sensitivity. *J Mol Med (Berl)* (2012) **90**:575-85.

231. Chan CK, Chen CC, Luppen CA, Kim JB, DeBoer AT, Wei K, et al. Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature* (2009) **457**:490-4.

232. Chhabra A, Ring AM, Weiskopf K, Schnorr PJ, Gordon S, Le AC, et al. Hematopoietic stem cell transplantation in immunocompetent hosts without radiation or chemotherapy. *Sci Transl Med* (2016) **8**:351ra105.

233. Ikehara S, Ohtsuki H, Good RA, Asamoto H, Nakamura T, Sekita K, et al. Prevention of type I diabetes in nonobese diabetic mice by allogenic bone marrow transplantation. *Proc Natl Acad Sci U S A* (1985) **82**:7743-7.

234. Li H, Kaufman CL, Boggs SS, Johnson PC, Patrene KD, Ildstad ST. Mixed allogeneic chimerism induced by a sublethal approach prevents autoimmune diabetes and reverses insulitis in nonobese diabetic (NOD) mice. *J Immunol* (1996) **156**:380-8.

235. Sykes M, Szot GL, Swenson KA, Pearson DA. Induction of high levels of allogeneic hematopoietic reconstitution and donor-specific tolerance without myelosuppressive conditioning. *Nat Med* (1997) **3**:783-7.

236. Seung E, Iwakoshi N, Woda BA, Markees TG, Mordes JP, Rossini AA, et al. Allogeneic hematopoietic chimerism in mice treated with sublethal myeloablation and anti-CD154 antibody: absence of graft-versus-host disease, induction of skin allograft tolerance, and prevention of recurrent autoimmunity in islet-allografted NOD/Lt mice. *Blood* (2000) **95**:2175-82.

237. Beilhack GF, Scheffold YC, Weissman IL, Taylor C, Jerabek L, Burge MJ, et al. Purified allogeneic hematopoietic stem cell transplantation blocks diabetes pathogenesis in NOD mice. *Diabetes* (2003) **52**:59-68.

238. Elkin G, Prigozhina TB, Slavin S. Prevention of diabetes in nonobese diabetic mice by nonmyeloablative allogeneic bone marrow transplantation. *Exp Hematol* (2004) **32**:579-84.

239. Nikolic B, Takeuchi Y, Leykin I, Fudaba Y, Smith RN, Sykes M. Mixed hematopoietic chimerism allows cure of autoimmune diabetes through allogeneic tolerance and reversal of autoimmunity. *Diabetes* (2004) **53**:376-83.

240. Prigozhina TB, Elkin G, Gurevitch O, Morecki S, Yakovlev E, Khitrin S, et al. Depletion of alloantigen-primed lymphocytes overcomes resistance to allogeneic bone marrow in mildly conditioned recipients. *Blood Cells Mol Dis* (2004) **33**:238-47.

241. Ildstad ST, Chilton PM, Xu H, Domenick MA, Ray MB. Preconditioning of NOD mice with anti-CD8 mAb and costimulatory blockade enhances chimerism and tolerance and prevents diabetes, while depletion of alpha beta-TCR+ and CD4+ cells negates the effect. *Blood* (2005) **105**:2577-84.

242. Serreze DV, Osborne MA, Chen YG, Chapman HD, Pearson T, Brehm MA, et al. Partial versus full allogeneic hemopoietic chimerization is a preferential means to inhibit type 1 diabetes as the latter induces generalized immunosuppression. *J Immunol* (2006) **177**:6675-84.

243. Asari S, Itakura S, Rawson J, Ito T, Todorov I, Nair I, et al. Mesenchymal stem cells facilitate mixed hematopoietic chimerism induction and prevent onset of diabetes in NOD mice. *Pancreas* (2011) **40**:846.

244. Guo Z, Wu T, Sozen H, Pan Y, Heuss N, Kalscheuer H, et al. A substantial level of donor hematopoietic chimerism is required to protect donor-specific islet grafts in diabetic NOD mice. *Transplantation* (2003) **75**:909-15.

245. Liu B, Hao J, Pan Y, Luo B, Westgard B, Heremans Y, et al. Increasing donor chimerism and inducing tolerance to islet allografts by post-transplant donor lymphocyte infusion. *Am J Transplant* (2006) **6**:933-46.

246. Luo B, Wu T, Pan Y, Sozen H, Hao J, Zhang Y, et al. Resistance to the induction of mixed chimerism in spontaneously diabetic NOD mice depends on the CD40/CD154 pathway and donor MHC disparity. *Ann N Y Acad Sci* (2007) **1103**:94-102.

247. Nikolic B, Onoe T, Takeuchi Y, Khalpey Z, Primo V, Leykin I, et al. Distinct requirements for achievement of allotolerance versus reversal of autoimmunity via nonmyeloablative mixed chimerism induction in NOD mice. *Transplantation* (2010) **89**:23-32.

248. Bozulic LD, Huang Y, Xu H, Wen Y, Ildstad ST. Differential outcomes in prediabetic vs. overtly diabetic NOD mice nonmyeloablatively conditioned with costimulatory blockade. *Exp Hematol* (2011) **39**:977-85.

249. Al-Adra DP, Pawlick R, Shapiro AM, Anderson CC. Targeting cells causing split tolerance allows fully allogeneic islet survival with minimal conditioning in NOD mixed chimeras. *Am J Transplant* (2012) **12**:3235-45.

250. Liang Y, Huang T, Zhang C, Todorov I, Atkinson M, Kandeel F, et al. Donor CD8+ T cells facilitate induction of chimerism and tolerance without GVHD in autoimmune NOD mice conditioned with anti-CD3 mAb. *Blood* (2005) **105**:2180-8.

251. Zhang C, Wang M, Racine JJ, Liu H, Lin CL, Nair I, et al. Induction of chimerism permits low-dose islet grafts in the liver or pancreas to reverse refractory autoimmune diabetes. *Diabetes* (2010) **59**:2228-36.

252. Racine J, Wang M, Zhang C, Lin CL, Liu H, Todorov I, et al. Induction of mixed chimerism with MHC-mismatched but not matched bone marrow transplants results in thymic deletion of host-type autoreactive T-cells in NOD mice. *Diabetes* (2011) **60**:555-64.

253. Al-Adra DP, Anderson CC. Toward minimal conditioning protocols for allogeneic chimerism in tolerance resistant recipients. *Chimerism* (2013) **4**:23-5.

254. Gandy KL, Domen J, Aguila H, Weissman IL. CD8+TCR+ and CD8+TCR- cells in whole bone marrow facilitate the engraftment of hematopoietic stem cells across allogeneic barriers. *Immunity* (1999) **11**:579-90.

255. Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ. Continued maturation of thymic emigrants in the periphery. *Nat Immunol* (2004) **5**:418-25.

256. Yu W, Nagaoka H, Jankovic M, Misulovin Z, Suh H, Rolink A, et al. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature* (1999) **400**:682-7.

257. Ricordi C. Quantitative and qualitative standards for islet isolation assessment in humans and large mammals. *Pancreas* (1991) **6**:242-4.

258. Slavin S, Strober S, Fuks Z, Kaplan HS. Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long-term survival of allogeneic bone marrow and skin grafts. *J Exp Med* (1977) **146**:34-48.

259. Ildstad ST, Wren SM, Bluestone JA, Barbieri SA, Sachs DH. Characterization of mixed allogeneic chimeras. Immunocompetence, in vitro reactivity, and genetic specificity of tolerance. *J Exp Med* (1985) **162**:231-44.

260. Al-Adra DP, Anderson CC. Mixed chimerism and split tolerance: mechanisms and clinical correlations. *Chimerism* (2011) **2**:89-101.

261. Benichou G, Nadazdin O, Cosimi AB, Kawai T. Successful tolerance to fully MHC disparate renal allografts via donor hematopoietic mixed chimerism in non-human primates. *Am J Transplant* (2013) **13**:2500.

262. Kawai T, Sachs DH. Tolerance induction: hematopoietic chimerism. *Curr Opin Organ Transplant* (2013) **18**:402-7.

263. Sachs DH, Kawai T, Sykes M. Induction of tolerance through mixed chimerism. *Cold Spring Harb Perspect Med* (2014) **4**:a015529.

264. Resnick IB, Metodiev K, Lazarova P. Hematopoietic cell transplantation for autoimmune diseases: a review of history, current state, and future issues. Immunotherapy-Myths, Reality, Ideas, Future. InTechOpen (2017)

265. Swart JF, Delemarre EM, van Wijk F, Boelens JJ, Kuball J, van Laar JM, et al. Haematopoietic stem cell transplantation for autoimmune diseases. *Nat Rev Rheumatol* (2017) **13**:244-56.

266. Wekerle T, Kurtz J, Ito H, Ronquillo JV, Dong V, Zhao G, et al. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med* (2000) **6**:464-9.

267. Wekerle T, Nikolic B, Pearson DA, Swenson KG, Sykes M. Minimal conditioning required in a murine model of T cell depletion, thymic irradiation and high-dose bone marrow transplantation for the induction of mixed chimerism and tolerance. *Transpl Int* (2002) **15**:248-53.

268. Bigenzahn S, Blaha P, Koporc Z, Pree I, Selzer E, Bergmeister H, et al. The role of non-deletional tolerance mechanisms in a murine model of mixed chimerism with costimulation blockade. *Am J Transplant* (2005) **5**:1237-47.

269. Westerhuis G, Maas WG, Willemze R, Toes RE, Fibbe WE. Long-term mixed chimerism after immunologic conditioning and MHC-mismatched stem-cell transplantation is dependent on NK-cell tolerance. *Blood* (2005) **106**:2215-20.

270. Oura T, Hotta K, Cosimi AB, Kawai T. Transient mixed chimerism for allograft tolerance. *Chimerism* (2015) **6**:21-6.

271. Maeda T, Eto M, Nishimura Y, Nomoto K, Kong YY, Nomoto K. Direct evidence for clonal destruction of allo-reactive T cells in the mice treated with cyclophosphamide after allo-priming. *Immunology* (1993) **78**:113-21.

272. Lee K, Nguyen V, Lee KM, Kang SM, Tang Q. Attenuation of donor-reactive T cells allows effective control of allograft rejection using regulatory T cell therapy. *Am J Transplant* (2014) **14**:27-38.

273. Sharabi Y, Sachs D, Sykes M. T cell subsets resisting induction of mixed chimerism across various histocompatibility barriers. Progress in Immunology Vol. VIII. Springer (1993). p. 801-5.

274. Al-Adra DP, Chan WF, Anderson CC. Nonobese diabetic natural killer cells: a barrier to allogeneic chimerism that can be reduced by rapamycin. *Transplantation* (2011) **92**:977-84.

275. Herberman RB, Nunn ME, Holden HT. Low density of Thy 1 antigen on mouse effector cells mediating natural cytotoxicity against tumor cells. *J Immunol* (1978) **121**:304-9.

276. Martin PJ. Donor CD8 cells prevent allogeneic marrow graft rejection in mice: potential implications for marrow transplantation in humans. *J Exp Med* (1993) **178**:703-12.

277. Racine JJ, Zeng D. Induction of mixed chimerism for reversal of autoimmunity in type 1 diabetes. *J Clin Cell Immunol* (2013) **S2**:006.

278. Koehn BH, Williams MA, Borom K, Gangappa S, Pearson TC, Ahmed R, et al. Fully MHC-disparate mixed hemopoietic chimeras show specific defects in the control of chronic viral infections. *J Immunol* (2007) **179**:2616-26.

279. Frankel WN, Rudy C. Linkage of Mls genes to endogenous mammary tumour viruses of inbred mice. *Nature* (1991) **349**:526.

280. Bill J, Kanagawa O, Woodland DL, Palmer E. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of V beta 11-bearing T cells. *J Exp Med* (1989) **169**:1405-19.

281. Dyson PJ, Knight AM, Fairchild S, Simpson E, Tomonari K. Genes encoding ligands for deletion of V beta 11 T cells cosegregate with mammary tumour virus genomes. *Nature* (1991) **349**:531-2.

282. McDuffie M, Schweiger D, Reitz B, Ostrowska A, Knight AM, Dyson PJ. I-E-independent deletion of V beta 17a+ T cells by Mtv-3 from the nonobese diabetic mouse. *J Immunol* (1992) **148**:2097-102.

283. Nicosia M, Valujskikh A. Total recall: can we reshape T cell memory by lymphoablation? *Am J Transplant* (2016) **17**:1713-8.

284. Neujahr DC, Chen C, Huang X, Markmann JF, Cobbold S, Waldmann H, et al. Accelerated memory cell homeostasis during T cell depletion and approaches to overcome it. *J Immunol* (2006) **176**:4632-9.

285. Wu Z, Bensinger SJ, Zhang J, Chen C, Yuan X, Huang X, et al. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med* (2004) **10**:87-92.

286. Ross D, Jones M, Komanduri K, Levy RB. Antigen and lymphopenia-driven donor T cells are differentially diminished by post-transplantation administration of cyclophosphamide after hematopoietic cell transplantation. *Biol Blood Marrow Transplant* (2013) **19**:1430-8.

287. MIYAUCHI A, KIRAMINE C, TANAKA S, HOJO K. Differential effects of a single dose of cyclophosphamide on T cell subsets of the thymus and spleen in mice: flow cytofluorometry analysis. *Tohoku J Exp Med* (1990) **162**:147-67.

288. Brode S, Raine T, Zaccone P, Cooke A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+CD25+Foxp3+ regulatory T cells. *J Immunol* (2006) **177**:6603-12.

289. Hotta K, Aoyama A, Oura T, Yamada Y, Tonsho M, Huh KH, et al. Induced regulatory T cells in allograft tolerance via transient mixed chimerism. *JCI Insight* (2016) **1**.

290. Korngold R, Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *J Exp Med* (1978) **148**:1687-98.

291. Luznik L, Jones RJ, Fuchs EJ. High dose cyclophosphamide for GVHD prevention. *Curr Opin Hematol* (2010) **17**:493.

292. Kanakry CG, Tsai H-L, Bolaños-Meade J, Smith BD, Gojo I, Kanakry JA, et al. Single-agent GVHD prophylaxis with posttransplantation cyclophosphamide after myeloablative, HLA-matched BMT for AML, ALL, and MDS. *Blood* (2014) **124**:3817-27.

293. Baron F, Mohty M, Blaise D, Socié G, Labopin M, Esteve J, et al. Anti-thymocyte globulin as graft-versus-host disease prevention in the setting of allogeneic peripheral blood stem cell transplantation: a review from the acute leukemia working party of the European society for blood and marrow transplantation. *Haematologica* (2017) **102**:224-34.

294. Leventhal J, Abecassis M, Miller J, Gallon L, Ravindra K, Tollerud DJ, et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med* (2012) **4**:124ra28.

295. Leventhal JR, Elliott MJ, Yolcu ES, Bozulic LD, Tollerud DJ, Mathew JM, et al. Immune reconstitution/immunocompetence in recipients of kidney plus hematopoietic stem/facilitating cell transplants. *Transplantation* (2015) **99**:288-98.

296. Chhabra A, Ring AM, Weiskopf K, Schnorr PJ, Gordon S, Le AC, et al. Hematopoietic stem cell transplantation in immunocompetent hosts without radiation or chemotherapy. *Sci Transl Med* (2016) **8**:351ra105.

297. Menge T, Stuve O, Kieseier BC, Hartung HP. Alemtuzumab: the advantages and challenges of a novel therapy in MS. *Neurology* (2014) **83**:87-97.

298. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* (2003) **111**:1887-95.

299. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* (2016) **532**:512-6.

300. Mahr B, Granofszky N, Muckenhuber M, Wekerle T. Transplantation tolerance through hematopoietic chimerism: progress and challenges for clinical translation. *Front Immunol* (2017) **8**:1762.

301. Zuber J, Sykes M. Mechanisms of mixed chimerism-based transplant tolerance. *Trends Immunol* (2017) **38**:829-43.

302. Millan MT, Shizuru JA, Hoffmann P, Dejbakhsh-Jones S, Scandling JD, Grumet FC, et al. Mixed chimerism and immunosuppressive drug withdrawal after HLA-mismatched kidney and hematopoietic progenitor transplantation. *Transplantation* (2002) **73**:1386-91.

303. Kawai T, Sachs DH, Sprangers B, Spitzer TR, Saidman SL, Zorn E, et al. Long-term results in recipients of combined HLA-mismatched kidney and bone marrow transplantation without maintenance immunosuppression. *Am J Transplant* (2014) **14**:1599-611.

304. Al-Adra DP, Gill RS, Imes S, O'Gorman D, Kin T, Axford SJ, et al. Single-donor islet transplantation and long-term insulin independence in select patients with type 1 diabetes mellitus. *Transplantation* (2014) **98**:1007-12.

305. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *JAMA* (2005) **293**:830-5.

306. Lin J, Chan WFN, Boon L, Anderson CC. Stability of chimerism in non-obese diabetic mice achieved by rapid T cell depletion is associated with high levels of donor cells very early after transplant. *Front Immunol* (2018) **9**:837.

307. Li H, Inverardi L, Molano RD, Pileggi A, Ricordi C. Nonlethal conditioning for the induction of allogeneic chimerism and tolerance to islet allografts. *Transplantation* (2003) **75**:966-70.

308. Mineo D, Ricordi C, Xu X, Pileggi A, Garcia-Morales R, Khan A, et al. Combined islet and hematopoietic stem cell allotransplantation: a clinical pilot trial to induce chimerism and graft tolerance. *Am J Transplant* (2008) **8**:1262-74.

309. Mackall CL, Hakim FT, Gress RE. Restoration of T-cell homeostasis after T-cell depletion. *Semin Immunol* (1997) **9**:339-46.

310. Roux E, Dumont-Girard F, Starobinski M, Siegrist CA, Helg C, Chapuis B, et al. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. *Blood* (2000) **96**:2299-303.

311. Kaufman CL, Colson YL, Wren SM, Watkins S, Simmons RL, Ildstad ST. Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells. *Blood* (1994) **84**:2436-46.

312. Xing Y, Hogquist KA. T-cell tolerance: central and peripheral. *Cold Spring Harb Perspect Biol* (2012) **4**:a006957.

313. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* (2004) **117**:265-77.

314. Leng Q, Nie Y, Zou Y, Chen J. Elevated CXCL12 expression in the bone marrow of NOD mice is associated with altered T cell and stem cell trafficking and diabetes development. *BMC Immunol* (2008) **9**:51.

315. Hock K, Oberhuber R, Lee YL, Wrba F, Wekerle T, Tullius SG. Immunosenescence does not abrogate engraftment of murine allogeneic bone marrow. *Transplantation* (2013) **95**:1431-8.

316. Kubota A, Kubota S, Lohwasser S, Mager DL, Takei F. Diversity of NK cell receptor repertoire in adult and neonatal mice. *J Immunol* (1999) **163**:212-6.

317. Al-Yassin GA, Bretscher PA. Does T cell activation require a quorum of lymphocytes? *The Journal of Immunology* (2018) **201**:2855-61.

318. Ni X, Song Q, Cassady K, Deng R, Jin H, Zhang M, et al. PD-L1 interacts with CD80 to regulate graft-versus-leukemia activity of donor CD8+ T cells. *J Clin Invest* (2017) **127**:1960-77.

319. Oura T, Cosimi AB, Kawai T. Chimerism-based tolerance in organ transplantation: Preclinical and Clinical Studies. *Clin Exp Immunol* (2017) **189**:190-6.

320. Ciurea SO, Cao K, Fernandez-Vina M, Kongtim P, Malki MA, Fuchs E, et al. The European society for blood and marrow transplantation (EBMT) consensus guidelines for the detection and treatment of donor-specific anti-HLA antibodies (DSA) in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant* (2018) **53**:521-34.

321. Sethi S, Choi J, Toyoda M, Vo A, Peng A, Jordan SC. Desensitization: overcoming the immunologic barriers to transplantation. *J Immunol Res* (2017) **2017**:6804678.

322. Colson YL, Schuchert MJ, Ildstad ST. The abrogation of allosensitization following the induction of mixed allogeneic chimerism. *J Immunol* (2000) **165**:637-44.

323. Taylor PA, Ehrhardt MJ, Roforth MM, Swedin JM, Panoskaltsis-Mortari A, Serody JS, et al. Preformed antibody, not primed T cells, is the initial and major barrier to bone marrow engraftment in allosensitized recipients. *Blood* (2007) **109**:1307-15.

324. Xu H, Chilton PM, Tanner MK, Huang Y, Schanie CL, Dy-Liacco M, et al. Humoral immunity is the dominant barrier for allogeneic bone marrow engraftment in sensitized recipients. *Blood* (2006) **108**:3611-9.

325. Jordan SC, Vo AA, Peng A, Toyoda M, Tyan D. Intravenous gammaglobulin (IVIG): a novel approach to improve transplant rates and outcomes in highly HLA-sensitized patients. *Am J Transplant* (2006) **6**:459-66.

326. Jordan SC, Lorant T, Choi J, Kjellman C, Winstedt L, Bengtsson M, et al. IgG endopeptidase in highly sensitized patients undergoing transplantation. *N Engl J Med* (2017) **377**:442-53.

327. Collin M, Olsen A. EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG. *EMBO J* (2001) **20**:3046-55.

328. Albert H, Collin M, Dudziak D, Ravetch JV, Nimmerjahn F. In vivo enzymatic modulation of IgG glycosylation inhibits autoimmune disease in an IgG subclass-dependent manner. *Proc Natl Acad Sci U S A* (2008) **105**:15005-9.

329. Allhorn M, Briceno JG, Baudino L, Lood C, Olsson ML, Izui S, et al. The IgG-specific endoglycosidase EndoS inhibits both cellular and complement-mediated autoimmune hemolysis. *Blood* (2010) **115**:5080-8.

330. Nandakumar KS, Johansson BP, Bjorck L, Holmdahl R. Blocking of experimental arthritis by cleavage of IgG antibodies in vivo. *Arthritis Rheum* (2007) **56**:3253-60.

331. Kao D, Danzer H, Collin M, Gross A, Eichler J, Stambuk J, et al. A monosaccharide residue is sufficient to maintain mouse and human IgG subclass activity and directs IgG effector functions to cellular Fc receptors. *Cell Rep* (2015) **13**:2376-85.

332. Lorant T, Bengtsson M, Eich T, Eriksson BM, Winstedt L, Jarnum S, et al. Safety, immunogenicity, pharmacokinetics, and efficacy of degradation of anti-HLA antibodies by IdeS (imlifidase) in chronic kidney disease patients. *Am J Transplant* (2018) **18**:2752-62.

333. Baxter AG, Cooke A. Complement lytic activity has no role in the pathogenesis of autoimmune diabetes in NOD mice. *Diabetes* (1993) **42**:1574-8.

334. Neubert K, Meister S, Moser K, Weisel F, Maseda D, Amann K, et al. The proteasome inhibitor bortezomib depletes plasma cells and protects mice with lupus-like disease from nephritis. *Nat Med* (2008) **14**:748-55.

335. Turk JL, Poulter LW. Selective depletion of lymphoid tissue by cyclophosphamide. *Clin Exp Immunol* (1972) **10**:285-96.

336. Al-Homsi AS, Cole K, Bogema M, Duffner U, Williams S, Mageed A. Short course of post-transplantation cyclophosphamide and bortezomib for graft-versus-host disease prevention after allogeneic peripheral blood stem cell transplantation is feasible and yields favorable results: a phase I study. *Biol Blood Marrow Transplant* (2015) **21**:1315-20.

337. Reeder CB, Reece DE, Kukreti V, Chen C, Trudel S, Hentz J, et al. Cyclophosphamide, bortezomib and dexamethasone induction for newly diagnosed multiple myeloma: high response rates in a phase II clinical trial. *Leukemia* (2009) **23**:1337-41.

338. Yoshihara S, Maruya E, Taniguchi K, Kaida K, Kato R, Inoue T, et al. Risk and prevention of graft failure in patients with preexisting donor-specific HLA antibodies undergoing unmanipulated haploidentical SCT. *Bone Marrow Transplant* (2012) **47**:508-15.

339. Gavin AL, Tan PS, Hogarth PM. Gain-of-function mutations in FcγRI of NOD mice: implications for the evolution of the Ig superfamily. *EMBO J* (1998) **17**:3850-7.

340. Luan JJ, Monteiro RC, Sautes C, Fluteau G, Eloy L, Fridman WH, et al. Defective Fc gamma RII gene expression in macrophages of NOD mice: genetic linkage with up-regulation of IgG1 and IgG2b in serum. *J Immunol* (1996) **157**:4707-16.

341. van Sorge NM, van der Pol WL, van de Winkel JG. FcγR polymorphisms: implications for function, disease susceptibility and immunotherapy. *Tissue Antigens* (2003) **61**:189-202.

342. Al-Homsi AS, Feng Y, Duffner U, Al Malki MM, Goodyke A, Cole K, et al. Bortezomib for the prevention and treatment of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Exp Hematol* (2016) **44**:771-7.

343. Kwun J, Burghuber C, Manook M, Iwakoshi N, Gibby A, Hong JJ, et al. Humoral Compensation after Bortezomib Treatment of Allosensitized Recipients. *J Am Soc Nephrol* (2017) **28**:1991-6.

344. Jarnum S, Bockermann R, Runstrom A, Winstedt L, Kjellman C. The bacterial enzyme IdeS cleaves the IgG-type of B cell receptor (BCR), abolishes BCR-mediated cell signaling, and inhibits memory B cell activation. *J Immunol* (2015) **195**:5592-601.

345. Muller YD, Golshayan D, Ehirchiou D, Wyss JC, Giovannoni L, Meier R, et al. Immunosuppressive effects of streptozotocin-induced diabetes result in absolute lymphopenia and a relative increase of T regulatory cells. *Diabetes* (2011) **60**:2331-40.

346. Zhen Y, Sun L, Liu H, Duan K, Zeng C, Zhang L, et al. Alterations of peripheral CD4+CD25+Foxp3+ T regulatory cells in mice with STZ-induced diabetes. *Cell Mol Immunol* (2012) **9**:75-85.

347. Pilat N, Baranyi U, Klaus C, Jaeckel E, Mpofu N, Wrba F, et al. Treg-therapy allows mixed chimerism and transplantation tolerance without cytoreductive conditioning. *Am J Transplant* (2010) **10**:751-62.

348. Ansari MJ, Salama AD, Chitnis T, Smith RN, Yagita H, Akiba H, et al. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med* (2003) **198**:63-9.

349. Sharpe AH, Pauken KE. The diverse functions of the PD1 inhibitory pathway. *Nat Rev Immunol* (2018) **18**:153-67.

350. Katz JD, Benoist C, Mathis D. T helper cell subsets in insulin-dependent diabetes. *Science* (1995) **268**:1185-8.

351. Surh CD, Sprent J. Homeostatic T cell proliferation: how far can T cells be activated to self-ligands? *J Exp Med* (2000) **192**:F9-F14.

352. Dooms H, Wolslegel K, Lin P, Abbas AK. Interleukin-2 enhances CD4+ T cell memory by promoting the generation of IL-7Rα-expressing cells. *J Exp Med* (2007) **204**:547-57.

353. Kalia V, Sarkar S. Regulation of effector and memory CD8 T cell differentiation by IL-2-a balancing act. *Front Immunol* (2018) **9**:2987.

354. Li W, Ji F, Zhang Y, Wang Y, Yang N, Ge H, et al. Cooperation of invariant NKT cells and CD4+CD25+ T regulatory cells in prevention of autoimmune diabetes in non-obese

diabetic mice treated with α-galactosylceramide. *Acta Biochim Biophys Sin (Shanghai)* (2008) **40**:381-90.

355. Mahr B, Unger L, Hock K, Pilat N, Baranyi U, Schwarz C, et al. IL-2/α-IL-2 complex treatment cannot be substituted for the adoptive transfer of regulatory T cells to promote bone marrow engraftment. *PLoS One* (2016) **11**:e0146245.

356. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* (2000) **343**:230-8.

357. Muller-Hermelink HK, Steinmann G, Stein H. Structural and functional alterations of the aging human thymus. *Adv Exp Med Biol* (1982) **149**:303-12.

358. Morison J, Homann J, Hammett M, Lister N, Layton D, Malin M, et al. Establishment of transplantation tolerance via minimal conditioning in aged recipients. *Am J Transplant* (2014) **14**:2478-90.

359. Wu L, Li N, Zhang M, Xue SL, Cassady K, Lin Q, et al. MHC-mismatched mixed chimerism augments thymic regulatory T-cell production and prevents relapse of EAE in mice. *Proc Natl Acad Sci U S A* (2015) **112**:15994-9.

360. Ferreira C, Palmer D, Blake K, Garden OA, Dyson J. Reduced regulatory T cell diversity in NOD mice is linked to early events in the thymus. *J Immunol* (2014) **192**:4145-52.

361. Ventevogel MS, Sempowski GD. Thymic rejuvenation and aging. *Curr Opin Immunol* (2013) **25**:516-22.

362. Al‐Chami E, Tormo A, Pasquin S, Kanjarawi R, Ziouani S, Rafei M. Interleukin-21 administration to aged mice rejuvenates their peripheral T-cell pool by triggering de novo thymopoiesis. *Aging cell* (2016) **15**:349-60.

363. Tormo A, Khodayarian F, Cui Y, Al-Chami E, Kanjarawi R, Noe B, et al. Interleukin-21 promotes thymopoiesis recovery following hematopoietic stem cell transplantation. *J Hematol Oncol* (2017) **10**:120.

364. Merchak A, Chhabra A, Ildstad S. Facilitating cells: a journey from bench to bedside. *J Clin Cell Immunol* (2018) **9**:545.

365. Lapidot T, Faktorowich Y, Lubin I, Reisner Y. Enhancement of T-cell-depleted bone marrow allografts in the absence of graft-versus-host disease is mediated by CD8+ CD4- and not by CD8- CD4+ thymocytes. *Blood* (1992) **80**:2406-11.

366. Martin PJ, Akatsuka Y, Hahne M, Sale G. Involvement of donor T-cell cytotoxic effector mechanisms in preventing allogeneic marrow graft rejection. *Blood* (1998) **92**:2177-81.

367. Jiang Z, Adams GB, Hanash AM, Scadden DT, Levy RB. The contribution of cytotoxic and noncytotoxic function by donor T-cells that support engraftment after allogeneic bone marrow transplantation. *Biol Blood Marrow Transplant* (2002) **8**:588-96.

368. Ellestad KK, Lin J, Boon L, Anderson CC. PD-1 controls tonic signaling and lymphopenia-induced proliferation of T lymphocytes. *Front Immunol* (2017) **8**:1289.

369. Zakrzewski JL, Kochman AA, Lu SX, Terwey TH, Kim TD, Hubbard VM, et al. Adoptive transfer of T-cell precursors enhances T-cell reconstitution after allogeneic hematopoietic stem cell transplantation. *Nat Med* (2006) **12**:1039-47.

370. Smith MJ, Reichenbach DK, Parker SL, Riddle MJ, Mitchell J, Osum KC, et al. T cell progenitor therapy-facilitated thymopoiesis depends upon thymic input and continued thymic microenvironment interaction. *JCI Insight* (2017) **2**.

371. Broca C, Varin E, Armanet M, Tourrel-Cuzin C, Bosco D, Dalle S, et al. Proteasome dysfunction mediates high glucose-induced apoptosis in rodent beta cells and human islets. *PLoS One* (2014) **9**:e92066.

372. Duek A, Feldberg E, Haran M, Berrebi A. Pulmonary fibrosis in a myeloma patient on bortezomib treatment. A new severe adverse effect of a new drug. *Am J Hematol* (2007) **82**:502-3.

373. Campbell PM, Salam A, Ryan EA, Senior P, Paty BW, Bigam D, et al. Pretransplant HLA antibodies are associated with reduced graft survival after clinical islet transplantation. *Am J Transplant* (2007) **7**:1242-8.

374. Chen CC, Pouliquen E, Broisat A, Andreata F, Racape M, Bruneval P, et al. Endothelial chimerism and vascular sequestration protect pancreatic islet grafts from antibody-mediated rejection. *J Clin Invest* (2018) **128**:219-32.