University of Alberta

Pivotal role of co-inhibitory molecules in immune tolerance

by

Govindarajan Thangavelu

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Experimental Surgery

Department of Surgery

Fall 2011
Edmonton, Alberta

Dedication

To my parents without them I would not exist in this world

&

To my family for their invincible love and encouragement

Abstract

The main function of co-inhibitory molecules is to regulate T cell immune responses by providing negative signals to those cells. Homeostatic activation of T cells occurs in both natural and artificially induced states of lymphopenia. Although lymphopenia leads to homeostatic proliferation of T cells, it does not always lead to autoimmunity, suggesting that control mechanisms may exist. Controlling these mechanisms may be particularly important during the period when the first T cells are exported from the thymus, as many recent thymic emigrants (RTE) have not yet had the opportunity to undergo peripheral tolerance. In chapters 2 and 3, I tested whether the major function of the co-inhibitory receptors such as programmed death-1 (PD-1) and B and T cell attenuator (BTLA) is to control autoimmunity induced by homeostatic activation, and also to assess their importance in RTE vs. established peripheral T cells. Interestingly, I found that their function is more critical only in newly generated T cells but not in mature T cells as PD-$1^{-/-}$ or BTLA $^{-/-}$ RTE induced a lethal multi-organ inflammatory disease in lymphopenic recipients. The disease induced by lymphopenia induced proliferation (LIP) was inhibited by reducing lymphoid space and also by providing polyclonal T cells as competitors for the pathogenic T cells. In chapter 4, I examined whether co-inhibitory molecules play a critical role in the "spontaneous" allograft tolerance of male islets. Among those tested, only PD-1 has a role in spontaneous acceptance of male islet allografts. While T cells have been observed to induce bystander killing of uninfected cells at the

vicinity of the infected cells during viral infections *in vitro*, very little is known about the existence of bystander killing *in vivo* and also the mechanisms that limit this type of killing. In chapter 5, I investigated the capacity for bystander killing of islet cells *in vivo* and observed that PD-1, but not BTLA, can limit bystander killing. Overall, my studies have divulged the critical roles of co-inhibitory molecules in the maintenance of self-tolerance and these research findings will provide critical insights in the development of novel therapies to combat autoimmunity and rejection of transplants.

ACKNOWLEDGEMENTS

First and foremost thanks to Almighty God for what I am now in my life!

I am very much indebted to Dr. Colin Anderson for his support, patience, guidance and encouragement through out my doctoral program. I could have not completed my projects without his help. I was blessed to have a mentor like Dr. Anderson, who is open minded, friendly and also has a caring attitude towards graduate students. I really enjoyed the scientific discussions I had with him, which not only helped me to understand the immunological concepts but also instigated to expand my depth of thinking. I have many favorite teachers since my schooldays and would say now Dr. Anderson is my most inspirational teacher I had so far. I want to thank his wife for her congeniality and wish his kids all the very best for their future endeavors.

I would like to thank Dr. Gina Rayat for her advice on my projects. She always encouraged me by telling her experiences and I have had wonderful discussions with her. I am grateful to Dr. Lori West for her advice on my research projects. Moreover, I appreciate her patience during my committee meetings.

I would like to thank Dr. Ciriaco Piccirillo for being my external examiner.

I am extremely grateful to Dr. Ronald Gill for his support and insightful discussions on my projects. In the midst of his busy schedule, he was always willing to discuss my projects. I would like to sincerely thank Dr. Thomas

Churchill for his support and advices during my studies. Also, I want to thank Dr. Harissios Vliagoftis who agreed to be one of my examiners.

I thank William Chan, who taught me research techniques and badminton as well when I joined this laboratory. Also, I thank Christa Smolarchuk and Dr. David Al-Adra for their help and input.

I would like to specially thank Dr. Catherine Ewen and Kristofer Ellestad for their inputs, encouragements and help in my projects. Although Kris joined our laboratory last year, he made me to feel that we have been friends for many years. We have had wonderful scientific discussions and I strongly believe that Kris will publish good scientific manuscripts in the future.

I am thankful to all the technicians in Dr. Anderson's laboratory especially Andrea Lo and Perveen Anwar for their help in my PhD projects. Thanks to Dr. Shaipro's laboratory members for their support and friendship. I also want to thank Rosemarie Henley and Christina Smith for their administrative help.

I would like to specially mention my thanks to Deb Dixon and Dr. Baoyou Xu for their contribution in islet transplantation experiments.

I would like to thank the Alberta Diabetes Institute, Muttart Diabetes Training Centre, Blanch Family, Department of Surgery, Canadian Institute for Health

Research, Juvenile Diabetes Research Foundation and Faculty of Graduate Studies and Research for scholarships and financial assistances.

I would like to thank my mother, brother, sister-in law and parents-in-law for their encouragement and support. Affectionate thanks goes to my daughter, Sanjana, for keeping me happy. Finally, I want to thank my wife, Santhi, for her unconditional love, endless support, encouragement and patience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 6

APPENDIX

List of symbols/abbreviations

α	Alpha
ANOVA	Analysis of Variance
β	Beta
γ	Gamma
m	Milli (10^{-3})
μ	Micro (10^{-6})
BTLA	B and T lymphocyte attenuator
B6	C57BL/6
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CCAC	Canadian Council on Animal care
CTLA-4	Cytotoxic T lymphocyte antigen-4
DAPI	4',6'-diamidino-2-phenylindole
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GVH	Graft versus host
HLA	Human leukocyte antigen(s)
HSC	Hematopoietic stem cells
HY	Male antigen
iTregs	Induced Tregs

i.v.	Intravenous
LIP	Lymphopenia induced proliferation
LTα $^{-/-}$	B6.129S2-Lta^{tm1Dch}
mAb	Monoclonal antibody
MHC	Major histompatability complex
NCI-Frederick	National Cancer Institute at Frederick
NIAID	National Institute of Allergy and Infectious Diseases
NK	Natural killer
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PE	Phycoerythrin
Rag	Recombination activation gene
Rag$^{-/-}$	B6.129S7-Rag1^{tm1mom}
Rag$^{-/-}$ γc$^{-/-}$	C57BL/6J × C57BL/10SgSnAi)-[KO]γc-[KO]Rag2
RPM	Revolution (s) per minute
RTE	Recent thymic emigrants
SEM	Standard error of the mean
SP	Splenocytes
STZ	Streptozotocin
TCR	T cell receptor

Treg	T Regulatory cells
Tg	Transgenic
U	Unit (measure of enzyme activity)
WT	Wild Type

Chapter 1: A version of this chapter has been published.

The citation is given below.

Thangavelu et al., 2010. Self Nonself. 1(2): 77-88.

Co-inhibitory molecules: Controlling the effectors or controlling the controllers?

1.1. Introduction

The question of how the adaptive immune system prevents self-reactivity continues to be at or near the top of the hierarchy of important questions in immunology, with the favored solution changing from one decade to the next. Recently regulatory T cells (T_{reg}) have been the focus (again) of research on this question. Increasingly, however, negative regulation by receptors that work together with lymphocyte antigen-receptors to deliver 'co-inhibitory' signals have also taken centre stage. While the rapidly increasing detailed description of co-inhibitory receptors and their intracellular signaling pathways has been reviewed elsewhere[1-7], we focus here on the relationship between co-inhibitory receptors and their functions in terms of minimal models of immune regulation (solutions to self/nonself discrimination), with a particular focus on recent studies that suggest a convergence between co-inhibitory signals and the cells that regulate the immune response (regulatory T cells and innate immune system cells).

1.2. A brief history of efforts to tackle self/nonself discrimination

A unique feature of the immune system is the ability to discriminate self from nonself antigens, with strong responses against many foreign antigens and tolerance to self -antigens. Many theories have been proposed to solve the problem of self/nonself discrimination.

1.2.1. Timing of antigen exposure

Burnet and Fenner proposed that there is a tolerogenic window early in the

ontogeny of organisms[8]. Despite the elegant studies conducted by Billingham, Brent and Medawar[9] that supported Burnet's theory, numerous studies also provided evidence against this view (discussed in refs.[10, 11]). In addition, the fact that lymphocytes are generated throughout life also indicated the Burnet-Fenner theory was either incorrect or incomplete. If tolerance occurs only early in life, how do lymphocytes newly generated in an adult animal become self-tolerant? Lederberg formulated a one-signal model of lymphocyte activation[12] that resolved this problem in the Burnet-Fenner theory on tolerance. He proposed that there is a tolerogenic window early in the ontogeny of each lymphocyte rather than in the organism as a whole, allowing each lymphocyte to go through self-tolerance education whether the lymphocyte was born in a neonatal or adult animal.

Lederberg's 1959 model proposing that antigen exposure in immature lymphocytes is tolerogenic, is not, as recently described[13], an extension of Burnet and Fenner's idea, but instead overturned their incorrect theory that postulated tolerance was a property uniquely of the fetal or neonatal period. The emergence of the central tolerance mechanisms, primarily deletion of autoreactive T cells in the thymus[14, 15], supported the Lederberg explanation that was proposed half a century ago. However, the one-signal model did not consider a need for tolerance in mature lymphocytes, that is a peripheral tolerance, a tolerance that would seem to be demanded by the presence of particular self antigens only outside the central lymphoid organs and by the capacity of lymphocytes to mutate leading to self-reactivity (e.g.: somatic hypermutation). To counter the problem of continuous

lymphocyte generation and mutation in the life of lymphocytes and consequently rescue the Burnet-Fenner tolerogenic window in ontogeny, Bretscher and Cohn proposed the two-signal model of lymphocyte activation[16]. According to this model, the optimal activation of T or B lymphocytes requires two signals in which the first signal arises from the engagement of the antigen with specific receptors of lymphocytes and the second signal is from the antigen specific T-helper cells (T_h), which are required to complete activation of the immune response. Based on this model, absence of self-reactive helpers can enforce tolerance throughout life due to a lack of help for newly generated helpers. This latter concept opened the chicken-egg dilemma by raising the question of which cell helped the first T_h cells? It also suffered from the same problem as Burnet's hypothesis, the experiments showing that there is no tolerance window defined uniquely in the fetal/neonatal period. Thus, while Lederberg explained much of self/nonself discrimination through a central tolerance mechanism, tolerance of the 'peripheral self' remained unresolved.

1.2.2. Co-stimulation, PAMPS and DAMPS

An effort by Lafferty and Cunningham to solve the puzzle of T cell allo vs. xeno reactivity was a major step towards resolving peripheral tolerance, as it led to a revised two-signal model for lymphocyte activation[17]. In this revised model, signal 2 (positive signal) or "co-stimulation" originates from antigen presenting cells (APCs) instead of T_h. In both of the two-signal theories, absence of signal 2 in lymphocytes will lead to tolerance (deletion or inactivation). However, it

remained unclear how co-stimulation could help discriminate self from nonself; how co-stimulation could be present with foreign but not self-antigens. Charles Janeway suggested a solution to the deficiency in Lafferty and Cunningham's theory by proposing that pattern recognition receptors of the innate immune cells influenced the expression of co-stimulation. Janeway translated from lymphocytes to the APC, the Coutinho and Moller concept of mitogen receptors binding microbial products[18], as the primary stimulus for immune responses. According to Janeway's model, the interaction of pattern recognition receptors of APC with their ligands (pathogen associated molecular patterns or PAMPS) of microbes, induced APC activation and expression of co-stimulatory molecules[19]. The identification of Toll like receptors (TLR), a few years later, supported this concept[20]. Presently, more than ten TLRs have been identified in mammals with their respective PAMP ligands. Despite the clear role of TLRs in regulating immune responses, Janeway's theory failed to easily explain transplant rejection and anti-viral immunity or the ability to harbor normal flora. Polly Matzinger introduced a new theory in the "Danger model" to solve the issues in Janeway's proposal and left the idea of a self/nonself discrimination behind in favor of a danger no danger discrimination[21]. This theory allowed for a peaceful co-existence between the immune system and our normal flora[22], unlike the self/nonself models. In the danger model, co-stimulation is induced by endogenous danger signals that arise from host cell damage. The danger model is, in a number of respects, more diverse in offering explanations for tumor immune responses, autoimmunity and transplant rejection and there is an increasing

amount of evidence supporting this model[23-29]. Molecules that signal danger are also now called alarmins or danger associated molecular patterns (DAMPS).

1.2.3. Tolerance mediated by co-inhibition

All of these minimal models of immune discrimination, be they self vs. nonself or danger vs. non-danger, give the job of tolerance inducing signals to the antigen receptor of lymphocytes. However, there is increasing evidence that tolerogenic signals are not derived from antigen receptor signals alone, and even before the concept of co-stimulation was proposed, the idea that there are co-receptors that provide inhibitory signals had been put forward. While examining the mechanisms of feedback suppression by antibody, Sinclair and Chan developed a model that explained the importance of the Fc portion of the antibody in suppression of the B cell response. **Figure 1-1** shows the 'Tripartite Inactivation model' from their 1971 publication[30]. Tripartite referred to the three components, antigen, antibody (a co-inhibitory ligand) and the immunologically competent cell. This, the first proposal of a receptor (in this case an Fc receptor) that works together with an activating receptor (when co-aggregated) to mediate inactivation/tolerance, was further substantiated by the identification of Fc receptors on B cells and their negative co-signaling capacity, including the identification of a critical immuno-tyrosine based inhibitory motif (ITIM) in its intracytoplasmic domain. Sinclair later proposed that these negative signals are required for tolerance in T cells as well as B cells[31], and coined the term co-inhibition for this process in further postulating that the fundamental control of

self/nonself discrimination in the periphery is determined by the balance between multiple co-stimulators and co-inhibitors[32]. While co-stimulation contributes to immune discrimination because it is present only with DAMPS or PAMPS (and not in 'healthy' self tissues), we proposed that co-inhibition contributes, at least in part, by being upregulated during prolonged antigen exposure (chronic antigen receptor signaling)[32]. Numerous lines of evidence now support the role of co-inhibitory molecules in self-tolerance[33-35] and in control of responses during chronic antigen exposure[36-43].

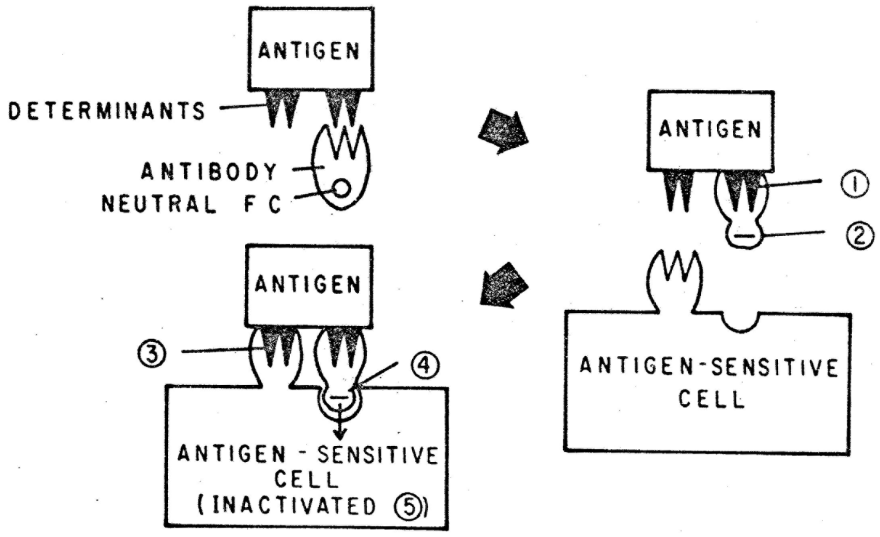


Figure 1-1. The origins of the concept of co-inhibition. The tripartite inactivation model[30] proposed that B cells are inactivated by antibody bound to antigen via the co-aggregation of the B cell antigen receptors with a receptor for the Fc portion of antibody. The model predicted the presence of negative signaling Fc receptors on B cells and that B cells are tolerized not by antigen receptor signals but instead by the co-operative signaling of antigen and Fc receptors. Reprinted with kind permission of Springer Science and Business Media. From page 611 in: K. Lindahl-Kiessling. G. Aim. and M. G. Hanna (eds.). Morphological and Fundamental Aspects of Immunity, pp. 609- 615. New York: Plenum Press, 1971.

1.2.4. Tolerance and regulatory T cells (Tregs)

The presence of autoreactive T cells in the periphery from healthy individuals[44, 45] underscored the importance of peripheral tolerance, especially to control the low affinity autoreactive T cells that escape from the thymus[46]. The potential outcomes of peripheral tolerance are diverse, and include clonal anergy or unresponsiveness[47-51], clonal deletion[52-54], ignorance[55-57], downregulation of T cell receptors or co-receptors[58, 59] and suppression by T_{reg} cells[60]. Among peripheral tolerance mechanisms, T_{reg} cells have become of great interest due to their potential therapeutic applications in controlling autoimmunity and transplant rejection[61-64]. T_{reg} exhibit dominant peripheral tolerance mechanisms by suppressing self-reactive T cells[60]. The suppressive function of T_{reg} is mediated by negative signals to other T cells and APCs through cell contact or cytokines such as TGF-β and IL-10. The concept of T_{reg} or suppressor T cells originated in 1970 from studies of Gershon and Konda[65]. Research on these cells flourished until the discovery that there was no I-J region in major histocompatability gene complex (MHC), which had been expected to be the locus controlling T_{reg}. Moreover, other studies[14, 15, 48] suggested that deletion or inactivation/anergy of lymphocytes were the relevant mechanisms of immunological tolerance, which further dwindled enthusiasm for the T_{reg} field. However, studies by Sakaguchi[66] demonstrated the importance of $CD4^+$ $CD25^+$ suppressor cells in controlling autoimmunity, which rejuvenated enthusiasm for the potential importance of T_{reg} in immunological tolerance. Despite the popularity of T_{reg} studies, there have been very few efforts to incorporate them into a model of self/nonself discrimination[67-

[69]. What are the rules that allow T_{reg} to suppress self but not appropriate foreign antigen specific responses? The rules are far from clear at this point despite the immense amount of data exploring these cells. While T_{reg} mediate a dominant form of tolerance where effectors cells are regulated in a cell extrinsic fashion, co-inhibition has mostly been considered a cell intrinsic recessive form of tolerance. However, recent data that we will discuss is challenging this mutually exclusive viewpoint, and suggesting that many co-inhibitory receptors are involved in both recessive and dominant tolerance.

1.2.4.1. Central tolerance

Self-reactivity of the lymphocytes is controlled by central and peripheral tolerance. Central tolerance or negative selection occurs during the development of lymphocytes, in which lymphocytes that have strong affinity to self peptide-MHC (selfpMHC) will be deleted in primary lymphoid organs. In the case of T cells, central tolerance occurs in the thymus. A classical study by Marrack's group reported the first evidence of clonal deletion by showing that T cells that were specific to MHC class II IE were deleted in the thymus of mice expressing IE[14]. Moreover, Von Boehmer's group also demonstrated central tolerance in developing anti-HY (male antigen) TCR transgenic thymocytes[15]. In male mice, anti-HY CD8+ T cells were deleted in the thymus due to the expression of male antigen in the thymus. On the other hand, in female mice, anti-HY CD8+ T cells were positively selected in the thymus. Recently, discovery of AIRE

(Autoimmune regulator protein) expression in medullary thymic epithelial cells (mTEC) re-emphasized the importance of central tolerance.

AIRE is required for the efficient negative selection in the thymus by up regulating tissue specific antigens in mTEC and so absence of AIRE leads to defective negative selection with the release of autoreactive T cells in the periphery[70]. Mutations in AIRE cause autoimmune disease in humans, namely, APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) or APS-1 (autoimmune polyglandular syndrome type 1)[71]. In the case of mice, AIRE deficiency leads to the development of multi-organ inflammatory infiltrates and auto-antibodies[72].

1.2.4.2. Peripheral tolerance

Although central tolerance is an efficient process in controlling self-reactivity, it does not eliminate completely all the self-reactive T cells. Previous studies also supported this notion by showing the presence of autoreactive T cells in the peripheral blood of healthy patients[73, 74]. However, the autoreactivity of those escaped T cells that are in the periphery is controlled by putative peripheral tolerance mechanisms namely ignorance, anergy, deletion and suppression. In theory, ignorance is mediated through the sequestration of tissue-restricted antigen in tissues that are inaccessible to T cells. Anergy is a condition of functional unresponsiveness of T cells to self-antigens[75]. Anergy in T cells can be classified into two categories namely, clonal anergy and adaptive tolerance or *in*

vivo anergy. Clonal anergy occurs due to lack of second signal in spite of the presence of TCR signaling. However, the induction of clonal anergy can be rescued directly by costimulation of CD28/B7 pathway[76] or indirectly by stimulation of growth factors like IL-2 on cell cycle progression[77]. Adaptive tolerance of a T cell occurs due to the generalized inhibition of proliferation and effector functions. The requirement of co-inhibitory pathways such as, CTLA-4, PD-1 for the induction of T cell anergy has been well-documented[78, 79]. Deletion of autoreactive T cells can occur in the periphery[80] due to Fas-Fas-L, Bim-dependent pathways[81, 82] and also, possibly, due to PD-1-PDL-1 or BTLA-HVEM pathways[83, 84]. T regulatory cells (Tregs) are suppressor cells and have been shown to play an important role in the maintenance of peripheral tolerance by suppressing autoreactive T cells[66].

1.3. Major co-inhibitory molecules

1.3.1. Cytotoxic lymphocyte antigen-4 (CTLA-4; CD152)

CTLA-4 is expressed by activated T cells and Tregs[60, 85]. It exhibits its inhibitory function by binding with B71 (CD80) and B72 (CD86) molecules of antigen presenting cells. It has been reported that the CTLA-4 has 10-20 times higher affinity to bind with B7 molecules than that of its activating counter part, CD28[86, 87].

CTLA-4 plays a critical role in the regulation of T cell tolerance and homeostasis. For example, CTLA-4$^{-/-}$ mice developed rapid lethal inflammatory disease and

died by 3-4 weeks of age[35]. The disease is characterized by increased effector memory T cells with lymphocytic infiltration in major organs such as heart and pancreas. Furthermore, a previous study[88] reported that thymocyte development is normal in CTLA-$4^{-/-}$ mice, which suggests the importance of CTLA-4 in the maintenance of peripheral tolerance. One of the questions in CTLA-$4^{-/-}$ mice that was unanswered was the antigenic specificity of the T cells undergoing rapid proliferation and infiltration of the tissues. It was recently answered by Murphy and colleagues, who demonstrated that the infiltrating T cells in CTLA-$4^{-/-}$ mice were specific to tissue-specific antigens and also autoreactive[89]. The experiment involved CTLA-$4^{-/-}$ mice with fixed TCR-β chain and the antigen specificity was demonstrated by transferring the pathogenic CD4+ T cells from organs like liver or pancreas into secondary $Rag^{-/-}$ recipients. Interestingly, it was found that the T cells from liver tend to home in the liver of $Rag^{-/-}$ recipients and induced pathology only in the liver. Later, it was identified that the pancreatitis in CTLA-$4^{-/-}$ mice is due to specific autoantigen namely acinar cell–specific enzyme PDIA2 (protein disulfide isomerase–associated 2).

1.3.2. Programmed death-1 (PD-1; CD279)

PD-1, a co-inhibitory molecule, was first isolated by Honjo and colleagues in 1992[90] by using a subtractive hybridization technique in two different cell lines namely, LyD9 and 2B4.11. The amino acid sequence of PD-1 is 21-33% identical to CTLA-4/CD28/ICOS. Unlike CTLA-4, PD-1 is a monomer due to the lack of membrane proximal cysteine. The cytoplasmic domain of PD-1 has two different

tyrosine residues, an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM)[91]. It has been reported that the mutation of ITSM abrogated the PD-1 inhibitory function[91].

C57BL/6 PD-1$^{-/-}$ mice developed a narrow spectrum of autoimmunity in the form of destructive arthritis and lupus at an older age[33]. At 6 months of age 30% of mice were affected, where as the incidence increased to 50% by 14 months of age. In the case of BALB/c PD-1 $^{-/-}$ mice, dilated cardiomyopathy was observed with autoantibody production to troponin-1. The majority of the mice (60%) died by 30 weeks of age. Disease was absent in BALB/c PD-1 $^{-/-}$ Rag$^{-/-}$ mice, which suggested that the disease is dependent on T and/or B cells. Also, spleen or bone marrow cells from sick BALB/c PD-1 $^{-/-}$ mice can transfer disease into Rag$^{-/-}$ recipients[34]. Neonatal thymectomy in BALB/c PD-1 $^{-/-}$ induced fatal autoimmune hepatitis with the production of anti-nuclear antibody[92].

Deficiency of PD-1 on autoimmune backgrounds such as NOD[93] and *lpr* mice[33] accelerated the onset of disease. Furthermore, PD-1$^{-/-}$ deficiency can act synergistically with LAG-3 deficiency (lymphocyte activation gene 3) to induce lethal myocarditis in BALB/c mice[94]. In humans, single nucleotide polymorphism in the PD-1 gene has been associated with multiple sclerosis[95], lupus, arthritis[96, 97] and type-1 diabetes[98].

There are two known ligands of PD-1 namely PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L1 is expressed in activated T cells, B cells, Tregs, myeloid and also in dendritic cells. In addition, PD-L1 is also expressed in non-lymphoid tissues such as heart, liver, placenta and pancreatic islets. Cytokines such as IFN-γ can upregulate PD-L1 expression in the tissues[99]. The broader expression of PD-L1 at peripheral tissues emphasized its role in the maintenance of peripheral tolerance by controlling autoreactive T cells. In contrast to PD-L1, PD-L2 expression is restricted to macrophages, dendritic cells and bone marrow derived mast cells. The PD-1/PD-L1 pathway can control the priming of self-reactive T cells in the lymph node and also the effector T cell responses at the target sites[100].

Blocking either PD-1 or PD-L1 but not PD-L2 accelerated the diabetes development in NOD mice, a mouse model for type-1 diabetes[101]. In addition, loss of PD-L1 can induce autoimmune enteritis[102], and myocarditis[99]. Although blockade of PD-L2 did not accelerate diabetes[101], the treatment augmented experimental autoimmune encephalomyelitis (EAE) in wild type mice[103]. Furthermore, lack of PD-L2 abrogated the induction of oral tolerance[104].

1.3.3. B and T lymphocyte attenuator (BTLA; CD272)

B and T lymphocyte attenuator (BTLA) is one of the members of Ig superfamily recently added to the list of co-inhibitory molecules[105]. It is expressed by activated T cells, B cells, NK cells, NK Tcells, macrophages and dendritic cells[1, 105]. BTLA is preferentially expressed by Th1 cells, which suggests its role in

regulating Th1 responses[105]. Previous studies reported that T cell receptor (TCR) engagement, together with BTLA signaling inhibited IL-2 production and decreased T cell proliferation[105, 106]. In contrast to other Ig superfamily members that bind to B7 molecules, BTLA binds with a tumor necrosis factor receptor member namely herpes virus entry mediator (HVEM)[107]. Engagement of BTLA with HVEM exerts inhibitory function on T cell activation[107]. In addition to BTLA, HVEM can also bind to lymphotoxin-α, LIGHT[108] and a co-inhibitory molecule CD160[109] that revealed the complexity of this pathway. The expression pattern of HVEM in T cells is in stark contrast to the BTLA expression. HVEM expression is higher in naïve T cells and becomes lower once the T cells are activated[110].

Various studies have highlighted the importance of BTLA in controlling autoimmunity and the maintenance of self tolerance[83, 105, 111, 112]. Aged $BTLA^{-/-}$ mice on 129SvEv background were susceptible to autoimmune hepatitis with the production of anti-nuclear antibodies. Also, earlier studies reported that $BTLA^{-/-}$ mice were more prone to EAE[105] and allergic airway inflammation[113]. Absence of BTLA impaired the induction and maintenance of oral tolerance and peptide induced tolerance[83]. The expression of BTLA is critical in CD8 + T cells to prevent autoimmune diabetes[83]. BTLA deficiency in MRL-*lpr* mice augmented the autoimmunity by increasing the severity of the lymphocytic infiltration in major organs[111].

1.4. Co-inhibitors in recessive and dominant tolerance mechanisms

For the purposes of this discussion we will consider that dominant tolerance is an antigen specific tolerance that is dominant when lymphocytes from the tolerant animal are mixed with lymphocytes from naïve animals (the mixture acts like the tolerant cells). Conversely, recessive tolerance is manifested by a lack of tolerance when the lymphocytes are mixed. However, it should be noted that there is at least the potential for an additional dominant tolerance mechanism that would not pass the 'mixing' test: The upregulation of co-inhibitory ligands within tissues leading to a local dominant tolerance that is not transferable with 'tolerant' lymphocytes to naïve recipients.

Summarized in **Figure 1-2** and **Table 1-1**, is a minimal model of the currently described cellular interactions, either cell intrinsic (recessive) or extrinsic (dominant/regulatory), in which co-inhibitory pathways are known or thought to be involved. Multiple co-inhibitory receptor ligand pairs are likely to be involved in each of the five pathways illustrated, each serving substantially or slightly different roles in the problem of self/nonself discrimination. Mechanism number 1 in **Figure 1-2** and **Table 1-1** represents the most well documented co-inhibition scenario, where co-inhibitory ligands, which can be soluble (e.g. antibody) or expressed on the surface of cells (non-T cells; APC or other tissue cells), interact with co-inhibitory receptors and generate tolerance. This tolerance is generally considered to be a recessive form of tolerance and involves recruitment of phosphatases to ITIM motifs. However, it cannot be excluded that in some cases

these interactions may turn the T cell into a T_{reg}, in which case dominant tolerance would ensue. In fact recent studies indicate that expression of PD-L1 on APC promotes generation of iT_{reg} in a population of naïve T cells[114, 115]. It remains unclear how such a mechanism could function *in vivo* without causing a state of generalized immunosuppression. Another example of co-inhibition via mechanism 1 is the ability of HVEM on radioresistant cells to prevent T cell activation by its interaction with BTLA and/or CD160 on the responding T cells[116]. PD-L1 is also expressed in non-hematopoeitic cells[117-121] and may bind with PD-1 on conventional T cells (T_{con}) to maintain recessive tolerance within tissues and tumors[122]. Although mechanism 1 is a recessive tolerance (not mediated by T_{reg}) acting directly on responding T cells, the inhibition of proliferation/activation of the responding T cell population could be expected to alter the ratio of effector to T_{reg} cells, favoring the T_{reg}. The concept that mechanism 1 is recessive is contingent upon the determining factor in responsiveness being regulation of co-inhibitor expression on the responding lymphocyte. That is, co-inhibitor levels change while co-inhibitory ligands are a constant (not inducible) and thus do not 'decide' the outcome. However, the picture may become even more complex for mechanism 1 if co-inhibitory ligands are themselves also inducibly expressed in tissues, as has been seen in the setting of inflammatory cytokines and autoimmunity[120, 123]. In this latter case, mechanism 1 would itself seem to be an effort to establish a form of dominant tolerance locally within the tissue, a dominant tolerance that is not mediated by T_{reg} but may nevertheless be useful.

The role of co-inhibitors in dominant tolerance mediated by T_{reg} has only recently emerged. T_{reg} play a key role in immunological tolerance to self-antigens as well as foreign antigens. T_{reg} can be divided into natural T_{reg} (nT_{reg}) and induced Tregs (iT_{reg}). The development of nT_{reg} is different from induced T_{reg} as the former develop in the thymus whereas iT_{reg} are induced in the periphery[124]. The mechanism of suppression by T_{reg} can be contact dependent or through cytokine dependent mechanisms[125, 126]. It has recently emerged that the suppressive function of T_{reg} is mediated by co-inhibitory receptors. For example, T_{reg} lacking PD-L1 or CTLA-4 are not good suppressors[127, 128]. It has been shown recently that the CTLA-4 in T_{reg} down regulates co-stimulatory molecules CD80 and CD86[128, 129] on APCs to maintain tolerance (mechanism 3 in **Figure 1-2** and **Table 1-1**). In contrast to CTLA-4, ligands of PD-1[119] and BTLA[131] are more highly expressed by T_{reg} than T_{con} such that co-inhibitory ligands of T_{reg} bind with their receptors on T_{con} (mechanism 2 in **Figure 1-2** and **Table 1**). However, some studies have suggested an alternate possibility, that PD-1 on T_{reg} could negatively regulate immune responses by binding with its ligand, PD-L1, on other cells[130]. The mechanisms involved in this latter possibility are not clear. It was recently shown that PD-L1 was not only required for T_{reg} functions, but also required for the development and maintenance of iT_{reg}[115]. CTLA-4 and PD-1 are not the only co-inhibitory pathways key to T_{reg} function. T_{reg} that lack HVEM have reduced capacity to suppress naïve wild type (WT) T cells[131]. Conversely, WT T_{reg} could not efficiently suppress $BTLA^{-/-}$ T_{con}, which implied that T_{reg} utilized HVEM to inhibit the effectors through BTLA[131] and possibly CD160. Increasing the

complexity even further, receptors involved in co-inhibition apparently can also have a positive impact on immune responses. BTLA expression and function in T cells is associated with increased T cell survival in both graft versus host disease and colitis models[116, 132-134]. How BTLA functions to increase survival is not yet clear. However, a recent study indicates that BTLA and HVEM can interact in cis on T cells (see mechanism 4 in **Figure 1-2** and **Table 1.1**) and that the cis interaction promotes survival[134]. Surprisingly, it promotes survival even though the cis interaction blocks trans interaction of HVEM ligands (BTLA, CD160) with HVEM on adjacent cells, preventing HVEM signals (NF-κB activation).

T cell immunoglobulin (Ig) domain and mucin domain-3 (Tim-3), is a co-inhibitory molecule expressed by terminally differentiated T_h1 T cells. The binding of Tim-3 with its ligand galectin-9 induced apoptosis of T_h1 cells[135]. A recent study reported that galectin-9 was expressed by T_{reg} and proposed that it could inhibit T_h1 cells by binding with Tim-3 on those cells[136]. Consistent with their speculation, blocking antibodies to Tim-3 reduced the suppressive function of T_{reg} *in vitro* and *in vivo*. Although blocking Tim-3 pathway partially restored T_{con} proliferation *in vitro*, there was no evidence that it directly reduced the suppressive function of T_{reg}. A previous study from the same group reported that the ligand of Tim-3 can negatively regulate alloreactive $CD8^+$ T cells[137]. Based on these findings, the interpretation that blocking Tim-3 pathway *in vivo* negated the suppressive function of T_{reg} is complicated by the possibility that the treatment could have directly enhanced alloreactive CD8+ T cell responses subsequently

resulting in allograft rejection.

Given the above evidence that co-inhibitors are critical in T_{reg} function it raises the question of whether co-inhibitors actually have a critical role in recessive tolerance mechanisms. Conditional deletion of CTLA-4 only in T_{reg} showed delayed onset of the rapid lymphoproliferative disorder and autoimmunity that occurs when there is global deletion of CTLA-4, suggesting that CTLA-4 may also regulate the T_{con} intrinsically[128]. More recently, this concept was supported by elegant experiments by Ise et al.[89] and Jain et al.[138] that demonstrated the requirement for CTLA-4 in controlling T_{con} to prevent autoimmunity. Hence, the expression of CTLA-4 in T cells has a dual role. The expression of CTLA-4 in T_{reg} serves to control aberrant activation of T_{con} extrinsically, whereas CTLA-4 has an intrinsic effect on T_{con} to maintain tolerance. Furthermore, numerous lines of evidence showed the involvement of co-inhibitory molecules in recessive tolerance mechanisms such as deletion and anergy of T cells[139-142]. Interestingly, even the well-known ability of B cell antigen presentation to tolerize naïve T cells[143, 144] has been found to be dependent on the co-inhibitors PD-1 and CTLA-4[145]. In another recent study, the adoptive transfer of $CD25^{-}CD4^{+}CD45RB^{high}$ naive T cells into syngeneic $Rag^{-/-}$ recipients that induces colitis was shown to be accelerated in $HVEM^{-/-}$ $Rag^{-/-}$ recipients. HVEM expression on radioresistant cells reduced the disease via interactions with BTLA and/or CD160[116], indicating a non-T_{reg} mediated tolerance through co-inhibition. Interestingly, BTLA was also required in non-T cells to reduce the disease.

Table 1-1

Distinct mechanisms by which co-inhibitory receptors/ligands block conventional T cell (T_{con}) responses depends on the cells expressing co-inhibitors vs. co-inhibitor ligands, and may even switch their function from inhibition to stimulation

	Co-inhibitor	[a]Co-inhibitor ligand	Outcome	Examples; [c]Binding
1	T_{con}	APC or tissue	Inhibitory signals to T_{con}; recessive tolerance	PD-1/PD-L1, Fas; trans
2	T_{con}	T_{reg}	Inhibitory signals to T_{con}; dominant tolerance	PD-1/PD-L1, BTLA/HVEM; trans
3	T_{reg}	APC	Reduced co-stimulation to T_{con}; dominant tolerance	CTLA-4/CD80-CD86; trans
4	T_{con}	[b]T_{con}	Survival signals to T_{con}; prolonged responses	BTLA/CD160/HVEM; cis
5	T_{con}	APC	Reduced co-stimulation to T_{con}; recessive tolerance	CTLA-4/CD80-CD86; trans

[a]In some cases, specifically #3 and 5, ligand for the co-inhibitor is also a co-stimulatory ligand.
[b]The co-inhibitory ligand (HVEM) is also expressed on T_{reg}, and at high levels, although low BTLA levels on these cells likely preclude significant cis interactions.
[c]Interactions between receptors and ligands on the same cell (cis) versus different cells (trans). (Reproduced from Thangavelu et al. (2010) with permission from SelfNonself, Landes Bioscience)

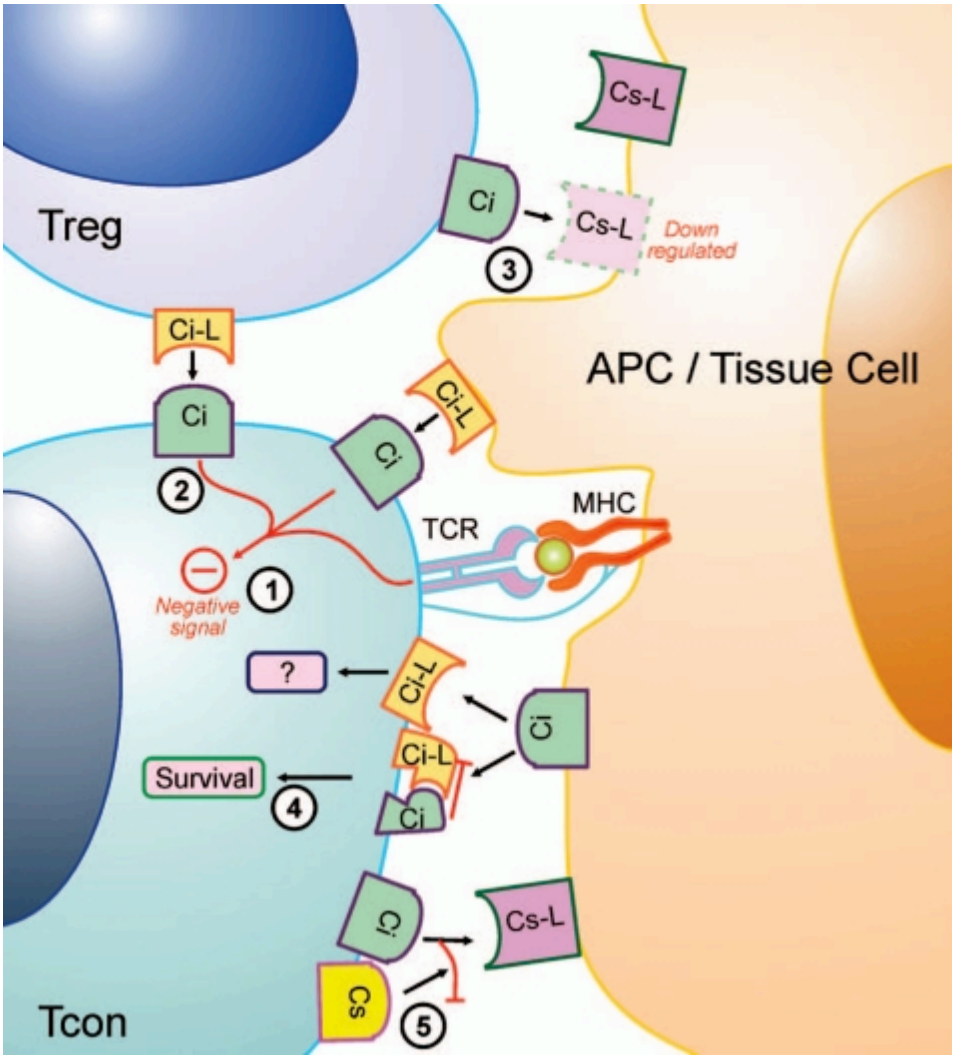


Figure 1-2. Mechanisms of co-inhibitory signaling involving dominant (T_{reg}) versus recessive mechanisms. Abbreviations, include Ci (co-inhibitor), Ci-L (co-inhibitory ligand), Cs (co-stimulator), Cs-L (co-stimulatory ligand), T_{con} (conventional T cell), T_{reg} (regulatory T cell). See also Table 1 for a description of each type of mechanism shown in 1-5 in the figure. (Reproduced from Thangavelu et al. (2010) with permission from SelfNonself, Landes Bioscience)

1.4.1. Feto-maternal tolerance

The mechanisms of feto-maternal tolerance in humans and mice have been discussed in detail elsewhere[146]. Here we will focus on the role of co-inhibitory

molecules in the maintenance of maternal tolerance. Aluvihare et al.[147] reported the expansion of T_{reg} in allogeneic pregnancy in mice when compared to syngeneic pregnancy. Consistent with the mouse studies, it has been demonstrated that there is also expansion of T_{reg} in human pregnancies[148]. Furthermore, adoptive transfer of T_{reg} in an abortion prone mouse model[149] prevented fetal resorption, which suggested the importance of T_{reg} in allogeneic mating. PD-L1 is expressed by mouse[119] and human placenta[150], which may serve to inhibit paternal antigen reactive T cells. Consistent with this possibility, paternal antigen specific T cells up regulated PD-1 upon encounter of cognate fetal antigen in pregnancy[151] and blockade of PD-L1 pathway induced fetal resorption and reduced litter sizes[119]. In contrast to this recessive tolerance action of PD-1 in pregnancy, adoptive transfer of purified T_{reg} from WT mice but not from PD-$L1^{-/-}$ mice was shown to reduce semi-allogeneic fetal resorption in PD-$L1^{-/-}$ mice[127]. However, litter sizes were small when compared to WT females, suggesting the requirement of PD-L1 in other immune cells or tissues. Another study showed that PD-$L1^{-/-}$ mice had an increased percentage of antigen presenting cells, which expressed a higher level of co-stimulatory molecules[152], raising the possibility that this mechanism might have enhanced the alloimmune responses against semi-allogeneic fetuses. It therefore remains an open question as to whether co-inhibition contributes to fetal tolerance primarily via recessive or dominant[127] tolerance mechanisms. The role of co-inhibitory molecules in the maintenance of maternal tolerance may involve protective suppressive functions of T_{reg}[146, 153], induction of apoptosis in paternal antigen specific T cells[151], and a

balancing of Th1/Th2 responses[119, 146].

1.4.2. Exhaustion

T cell adaptation or 'exhaustion' is a property that occurs in T cells due to persistent systemic antigen exposure[43, 58, 154-157] and chronic viral infections, respectively[36, 158]. Previous studies reported that exhausted anti-viral T cells expressed high levels of multiple co-inhibitory receptors[38], including CTLA-4, PD-1, and LAG-3, which leads to T cells dysfunction[36, 159, 160] and persistent viremia. Furthermore, blocking co-inhibitory molecules induced strong immune responses by reversing the state of adaptation or exhaustion of T cells[43, 160-162]. Reversal of exhausted T cells by blocking co-inhibitory pathways has become an important area due to its therapeutic applications in chronic viral infections such as HIV, and blocking multiple co-inhibitors is synergistic in reversing exhaustion[38, 41]. While a number of studies implicate T_{reg} in the reduced responses in chronic viral infection, there are not yet many studies addressing the question of whether co-inhibition's contribution to 'exhaustion' is a recessive tolerance or via T_{reg}. Current data favor a non-T_{reg} contribution of co-inhibition[41].

While the T_{reg} literature may have to some degree promoted a descriptive biology approach to immunology, the exhaustion literature may also have suffered this inertia. Exhaustion studies seem to be an example where evaluation of concepts is lost in the rush to generate descriptions of mechanisms of an immunologic phenomenon. In providing the description of what molecules are involved in

controlling exhaustion, the fact that these descriptions actually overturn (disprove) the concept of exhaustion seems to have been overlooked. The word and concept of exhaustion means to consume or tire completely, that is, the entities or resources used for positive action have been depleted. The literature showing a key role for co-inhibitors in putative "exhaustion" show the phenomenon is in fact not exhaustion, as all the resources for positive action are present; it is instead an upregulation of negative regulatory pathways. As shown in **Figure 1-3**, relieving the cells of these co-inhibitory signals reveals that the cells are not exhausted and have all the resources to respond. Like the term "negative-costimulation", an oxy-moron often used to describe what is really co-inhibition, use of the term exhaustion when discussing tolerance through chronic antigen exposure misconstrues the essence of the phenomenon. While it is exciting to discover molecules that underlie tolerance during chronic antigen exposure, as this provides new avenues for clinical treatments[163], it is not clear why there would not also be excitement in (or even recognition of) the advance that it provides for a fundamental understanding of how the immune system works; that such tolerance works not through exhausting T cells (i.e. too many positive signals exhaust resources) but through a decision to shut down T cells by employing co-inhibitory (negative) signals when positive signals become chronic. As we have argued previously[164], if chronic antigen/virus were truly exhausting T cells, then additional positive signals to T cells should have no effect or deepen the exhaustion if the exhaustion was not already complete. Instead, "exhaustion" can be rescued by providing to T cells what can only be considered additional positive

(exhausting) signals[165]. Given the ability of co-inhibition blockade to restore responses, the only way to maintain the concept that chronic antigen exposure leads to exhaustion would be to postulate that co-inhibition is itself acting as additional positive signals responsible for depletion of resources. Based on existing data we favor the model that co-inhibitory signals inhibit the elaboration of effector functions but do not deplete the resources needed for effector

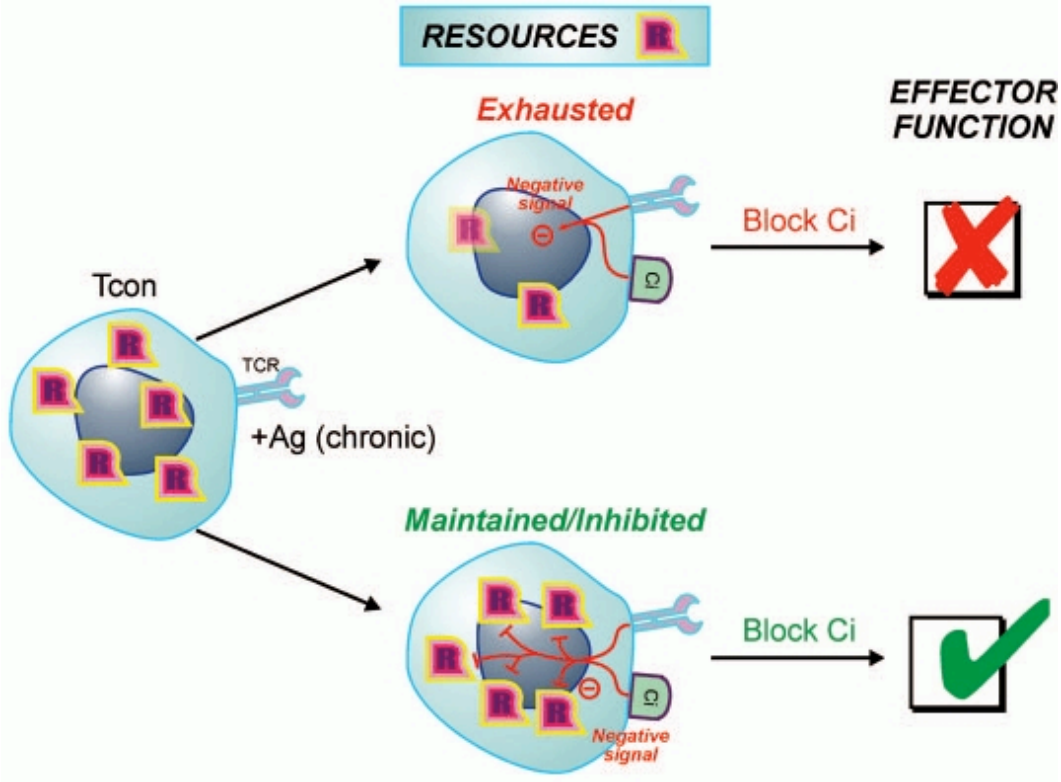


Figure 1-3. Relief from co-inhibition reveals that chronic antigen exposure does not lead to exhaustion of T cells. Chronic antigen exposure leads to long-term expression of co-inhibitory receptors in conventional T cells. Two alternative outcomes of chronic antigen exposure are depicted. The conventional view is shown on top, where chronic antigen exposure (e.g. chronic LCMV infection) leads to exhaustion of T cells. Exhaustion is a loss of resources needed for differentiation to effector function. The resources (R) that are putatively depleted have not been defined but could include signaling elements, transcription factors, cytokines, ATP etc. The second possible outcome is shown at the bottom, where the T cell is not exhausted, resources within the cell are maintained but not deployed because they are held in check by co-inhibitory signals. Blocking co-

inhibitory signals differentiates between these two possibilities, as co-inhibitory blockade is predicted to restore effector function in the second model (bottom) but not if chronic antigen leads to exhaustion (top). Abbreviations are as described in Figure 2. (Reproduced from Thangavelu et al. (2010) with permission from SelfNonself from Landes Bioscience)

function. Chronic antigen exposure can also lead to deletion of some of the responding repertoire of T cells, and co-inhibition is also likely to be central to this process. There is no evidence that this deletion is a result of exhausting resources.

1.4.3. Tumor evasion mechanisms

Tumor cells, as a mechanism of immune evasion, have exploited the property of co-inhibitory molecules that regulates immune responses against self-antigens. The anti-tumor T cell responses are limited due to the expression of co-inhibitory molecule by T cells, and co-inhibitory ligands by antigen presenting cells as well as by the tumor microenvironment. For example PD-L1 expressed by tumor cells induced T cell dysfunction, by binding with PD-1 expressed by tumor specific cytotoxic T cells[122, 166]. A good prognosis for cancer patients was inversely proportional to the expression of PD-L1 in tumor cells[167]. Interestingly, a recent study suggested that PD-L1 sends signals directly to the tumor cells to trigger their resistance to killing, rather than PD-L1 sending co-inhibitory signals to T cells[168]. Another recent study in humans demonstrated the relationship of BTLA expression in anti-tumor effector T cells and inhibition of their function[169]. T_{reg} and APCs can also block the anti-tumor T cells by direct effects and also by the production of the indoleamine 2,3- dioxygenase (IDO) enzyme. The negative

association of T_{reg} with tumor immune responses has been shown in various studies[170]. Depletion of T_{reg} using anti-CD25[171] induced tumor immune responses and other strategies that were meant to attenuate T_{reg} function by anti-CTLA-4; anti-GITR treatment also induced strong tumor immune responses and rejection of tumors[172, 173]. Although the latter studies demonstrated the induction of tumor immune responses, they did not demonstrate that the treatments affected T_{reg} directly. Indirect effects could occur through expression of the targeted molecules on other cells of the immune system. The role of CTLA-4 in T_{reg} mediated tumor immune suppression was demonstrated by the development of a T_{reg} -specific CTLA-4 knockout, lacking CTLA-4 only in Tregs[128]. In this mouse model the tumor immune responses were enhanced. The involvement of multiple co-inhibitory pathways opens up a possibility to develop an innovative tumor immunotherapy.

1.4.4. Role of co-inhibitory molecules in transplantation

Induction of transplantation tolerance to foreign antigens remains the Holy Grail for transplantation immunology. The involvement of co-inhibitory molecules in the mechanisms of peripheral tolerance has allowed immunologists to develop new strategies that promote tolerance to allogeneic tissues. Long-term acceptance of allografts was achieved in various allograft models by using CTLA-4-Ig[174, 175] (although this works by blocking CD28 co-stimulation), PD-L1-Ig[176, 177], anti-BTLA treatments[175, 178] alone or in combination with other therapies. Conversely, blocking co-inhibitory pathways accelerated allograft rejection[179-181]. It has been

demonstrated that intratracheal delivery of alloantigen prolonged the survival of cardiac allografts by allowing the development of donor specific T_{reg}[182]. Blockade of the PD-1/PD-L1 pathway during the administration of alloantigen, by using either anti-PD-1 or anti-PD-L1, accelerated rejection[183]. The conclusion was that PD-L1 blockade prevented the induction of T_{reg}. However, there was no direct evidence that PD-1 or PD-L1 blockade prevented the induction of T_{reg} in this setting, as the adoptive transfer studies employed whole splenocytes rather than purified T_{reg}. Tolerance to various allografts achieved by treating animals with several regimens could be prevented by using blocking antibodies to Tim-3[184] or PD-L1[174] or CTLA-4[185]. The effects could be due to enhanced proliferation and cytokine responses. Blockade of co-inhibitory molecules induced strong immune responses by favoring T_h1 responses and expansion of cytotoxic T cells that lead to accelerated rejection. In terms of strategies to induce transplantation tolerance, there are at least two major approaches that could prove useful. One is the generation of biologics that act as agonists for co-inhibitory signals, with few such agents having been developed at this point. A second approach is to overexpress ligands within tissues to create an immune privileged environment for the transplant[186-189].

1.4.5. Homeostatic proliferation

Homeostatic proliferation of T cells is a physiological mechanism that exists to prevent lymphopenia and also to maintain immunocompetence[190-192]. This phenomenon is characterized by antigen independent conversion of naïve T cells

into a memory like phenotype and occurs in the T cell compartment of the secondary lymph nodes[193]. Lymphopenia can occur due to viral infections, lympho-ablation employed in cancer therapy, and also in primary immunodeficiency disorders.

Although lymphopenia induced proliferation (LIP) is generally beneficial, previous studies reported that there might be a possibility of the expansion of disease causing autoreactive T cells in the periphery[194-196]. The association of lymphopenia with autoimmunity has been well documented in humans[197, 198] and mice[199]. However, other studies have reported that lymphopenia alone does not have the capacity to induce autoimmunity[200, 201]. Moreover, studies in mice [202, 203] and humans[204] showed that LIP can impair the induction of tolerance to allografts. Naïve T cells can proliferate in a lymphopenic host due to the availability of more resources such as self-pMHC ligands and IL-7[191]. Previous studies have demonstrated the critical importance of positively selected self-pMHC ligands in driving homeostatic proliferation of both polyclonal and monoclonal naïve T cells[205, 206]. Higher concentrations of IL-7 can enhance the LIP of T cells[207, 208]. Also, Tak Mak and colleagues reported that the combination of either exogenous or endogenous IL-7 and lymphopenia can induce autoimmune diabetes[209]. However, studies identified that IL-7 independent homeostatic proliferation of CD4+ T cells can occur in chronic immunodeficient mice such as $Rag^{-/-}$ and $TCR^{-/-}$[195, 210]. Apart from IL-7, other cytokines namely, IL-2 and IL-5 also have been shown to play a role in the homeostatic proliferation of CD8+ T cells[211, 212].

1.4.6. Co-inhibition, a controller of homeostasis, antigen specific responses or both?

The engagement of a co-inhibitory receptor with its ligand could influence the homeostasis of T cells. Blockade or absence of co-inhibitory molecules induced expansion of antigen-specific reactive T cells[179, 213]. CTLA-4-/- mice die by 3 weeks of age due to a lymphoproliferative disorder, which implied the importance of CTLA-4 in T cell homeostasis. However, in an important recent study the hyperproliferative response in CTLA-4-/- T cells appears to be autoantigen driven to a large extent, and for the first time it was shown that CTLA-4 is critical in controlling T cells specific to natural autoantigens[89]. It has been demonstrated that antigen independent homeostatic expansion of T cells could be negatively regulated by BTLA[214]. In addition, the loss of BTLA in naïve T cells enhanced the generation of CD8+ memory T cells. Using an elegant model, Welsh and colleagues showed that PD-1 also plays a key role in controlling lymphopenia induced homeostatic proliferation of established anti-viral T cells[215]. Recent thymic emigrants are a T cell population with distinct properties and are particularly important early in immune system generation and during immune reconstitution post lymphocyte depletion that occurs in some viral infections and in conditioning used for bone marrow transplantation. Interestingly, syngeneic bone marrow transplantation induced autoimmunity in sub-lethally irradiated immunodeficient animals, but not in lethally irradiated immunocompetent mice[216].

1.4.7. Interpreting experiments using antibodies targeting co-inhibitors

The blockade of co-inhibitory pathways with monoclonal antibodies (mAb) has been an important strategy in various experimental models to test co-inhibitor function and generally has been found to increase immune responses, although in some cases the antibodies appear to be agonistic. The induction of strong immune responses could occur through releasing effector cells from co-inhibitory signals, by altering the ratio of T_{reg}/T_{con}, reducing T_{reg} function, or favoring a particular class of response ($T_h1/T_h2/T_h17$). Conversely, putative agonistic antibodies are assumed to inhibit responses by providing negative co-inhibitory signals to the cells they bind. However, in most cases there is very limited data to support the contention that the antibodies simply act by blocking or stimulating the co-inhibitory receptor. Often the evidence that a particular mAb blocks or activates a co-inhibitor is derived solely *in vitro* and then assumed to function similarly *in vivo*. However, mAb have the potential to do things *in vivo* that do not readily occur *in vitro*, such as opsonize cells leading to their destruction via various mechanisms. A recent example of this is an interesting study showing the importance of HVEM on radioresistant cells interacting with BTLA on T cells in the prevention of colitis[116]. The mAb 6F7 specific to BTLA was used to show that an agonist mAb (conclusion derived from *in vitro* data) inhibits colitis. However, when we studied the effects of 6F7 *in vivo*, we found that this antibody physically depletes BTLA expressing cells[178]. Although it is possible that depletion is due to induction of apoptosis triggered by BTLA signaling, this seems unlikely given that loss of BTLA expressing cells occurs in a large fraction of these cells, while

only a small fraction are likely to be engaging cognate antigen (a requirement for a co-inhibitory signal). Whether an antibody is blocking, depleting or agonistic when bound to a co-inhibitory receptor (or ligand) has important implications for its use therapeutically. An agonistic anti-co-inhibitor mAb may only temporarily inactivate the relevant antigen specific cells, while depletion would be a permanent elimination of relevant clones that could only be countered by recruitment of new thymic emigrants and newly generated B cells into the peripheral repertoire.

1.4.8. Thymic selection

The role of BTLA and PD-1 in thymic selection is not clear due to differing results and insufficient data. Sharpe's group reported that PD-1 can inhibit positive selection using a mouse that transgenically expressed PD-1 on developing thymocytes[217]. However, there was no effect on negative selection. Experiments in 2C $Rag^{-/-}$ mice suggested that there was an increase in negative selection of double positive thymocytes in the absence of PD-1[218]. By using the OT-1 mouse model, it was reported that there is an association between PD-1 and positive selection of thymocytes[219]. An earlier study reported that BTLA is an early marker for positive selection[106]. But the exact role of BTLA in thymic selection has not yet been determined.

1.5. Islet transplantation

Diabetes is one of the fatal diseases in humans with high morbidity and secondary complications. It has been estimated that close to 400 million people will be affected with diabetes worldwide by 2030[220]. Despite the higher occurrence of type-2 disease in diabetic patients, type-1 remains as the severe form due to loss of insulin producing beta cells as a result of autoimmunity.

Insulin was discovered to treat diabetes by Banting, Best and Collip. The downsides of the insulin therapy are failure of complete glycemic control, development of secondary complications such as retinopathy, neuropathy and renal failure. However, the problem of glycemic control can potentially be prevented by islet or pancreas transplantation. Islet transplantation is considered to be much superior to whole pancreas due to its minimal invasiveness.

The initial attempt for pancreas/islet transplantation was performed in a young patient in 1893 by Watson–Williams and Harsant. Minced pancreases from sheep were transplanted subcutaneously. However, the patient died 3 days later despite of the mild control of glucose levels. In 1915, Charles Pybus transplanted diabetic patients with human cadeveric pancreatic tissues. The result from this study was similar to Watson-William's study. A major breakthrough in islet transplantation was made by Paul Lacy. He developed a novel method for islet isolation by using collagenase[221] and later demonstrated that the transplanted islets can reverse diabetes in animals[222]. Since 1977, various clinical trials, for islet transplantation,

were conducted all over the world but the results from those trials were not promising[223-225]. However, the development of the Edmonton protocol in 2000 reignited the islet transplantation field by demonstrating 100% insulin independence in seven patients by using steroid free immunosuppressive therapy[226]. After the success of the Edmonton protocol, nine centers participated in clinical trials. Interestingly, the centres could reproduce the success, but with varied insulin independence rates (23% to 82%)[227, 228].

The major hurdles for the successful islet transplantation are insufficient donor islets and rejection of the grafts. Islet stem cells or xenogeneic porcine islets could be used to meet the rising demand for donor islets. Induction of tolerance towards donor islet grafts would avert the use of harmful immunosuppressive drugs.

Recent studies have demonstrated the significance of co-inhibitory signals in the attenuation/prevention of autoimmune diabetes or the induction of tolerance to islet allografts. An earlier study showed that transgenic expression of anti-CTLA-4 scFV, agonistic CTLA-4 signaling molecule, on pancreatic β cells of NOD mice decreased the incidence of autoimmune diabetes[229]. Moreover, combination of blocking anti-ICOS mAb and CTLA-4 Ig therapy significantly delayed the islet allograft rejection in a fully MHC mismatched mouse model of islet transplantation[230]. In the case of PD-1, absence[93] or blockade[101] accelerated the onset of diabetes in NOD mice. Interestingly, insulin promoter driven PD-L1 over expression in NOD mice delayed the onset of autoimmune diabetes[231]. HVEM,

the ligand of BTLA, is expressed by β cells of pancreas[232]. Studies from our group demonstrated that depleting anti-BTLA antibody delayed the onset of diabetes in NOD mice[178]. Furthermore, the combination of anti-BTLA and CTLA-4 Ig therapy induced donor specific islet allograft tolerance[175, 233]. Recently, a study reported that BTLA expressing transgenic dendritic cells ameliorated autoimmune diabetes by inducing CD8+ T cell tolerance[234].

1.5.1. Bystander killing or collateral damage

It is not known whether the existence of co-inhibitory molecule is to limit bystander killing. The unique nature of T cell effector function is the selective killing of target cells without affecting bystander cells during infection or allograft rejection. This specificity of the T cell function has been tested by a limited number of *in vitro* and *in vivo* studies. Although *in vitro* studies using CTL clones reported the lysis of bystander cells[235-237], the results from *in vivo* studies[238, 239] were mixed. A previous study[240] reported that influenza infection is always associated with liver pathology despite the absence of the detectable virus in the liver, which suggests the possibility of bystander killing or collateral damage in the liver. In the fifth chapter of this thesis, I tested the occurrence of bystander killing *in vivo* using islet transplantation as a model. Also, the role of co-inhibitory molecules in limiting bystander killing was examined.

1.6. Overview of my thesis

It has been shown that co-inhibitory molecules play an important role in the regulation of immune responses. Although studies have been conducted to understand the role of co-inhibitory molecules in tolerance mechanisms, the role of these molecules in understudied areas such as lymphopenia induced homeostatic proliferation and bystander killing is unclear. Co-inhibitory molecules regulate the tolerance of T cells. This led to the hypothesis of this thesis that co-inhibitory molecules are required to prevent lymphopenia induced autoimmunity and bystander killing. I investigated the role of co-inhibitory molecules in lymphopenia induced homeostatic proliferation and bystander killing by using stem cell transplantation and islet transplantation models, respectively.

In chapters 2 and 3 **(Figure 1-4)** of my thesis, I report studies of the role of co-inhibitory molecules namely PD-1 and BTLA in controlling lymphopenia-induced autoimmunity of T cells by using lymphopenic ($Rag^{-/-}$) recipients. I tested the role of these two co-inhibitory molecules in two kinds of T cells, namely mature T cells and also newly generated T cells or recent thymic emigrants (RTE). Fetal liver cells and adult splenocytes were used as a source of RTE and mature T cells, respectively. Syngeneic $Rag^{-/-}$ recipients were given either fetal liver cells or mature T cells from PD-$1^{-/-}$ or BTLA $^{-/-}$ mice. I used fetal liver cells from wild type (WT) mice as a control. Interestingly, I found that co-inhibitory molecules are required in newly generated T cells to prevent lymphopenia-induced autoimmunity. The disease in lymphopenic recipients was controlled by reducing

lymphoid space or providing competitor T cells.

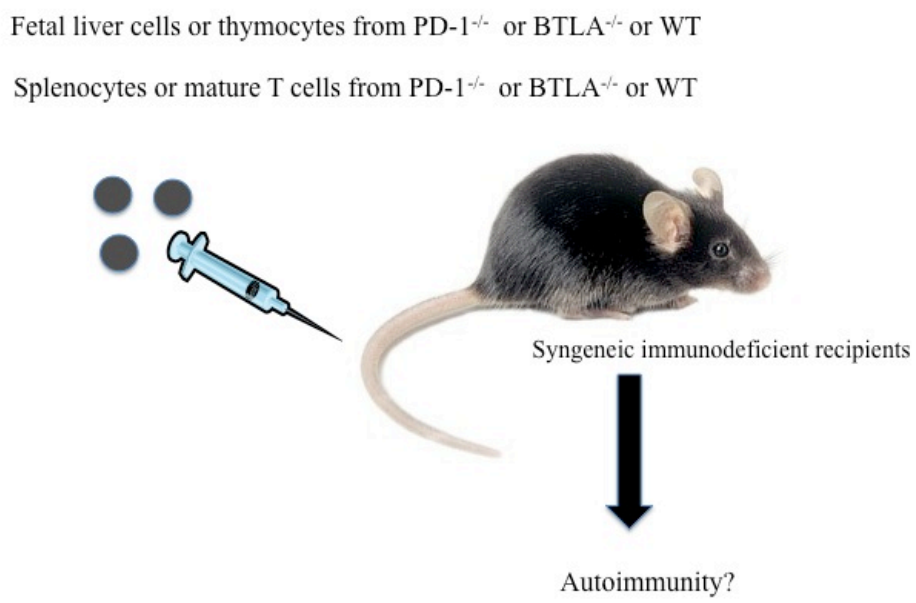


Figure 1-4. Testing the capacity of co-inhibitory molecules to prevent lymphopenia induced autoimmunity. Syngeneic immunodeficient (T and B cell deficient) mice were given fetal liver cells or thymocytes or splenocytes from PD-1$^{-/-}$ or BTLA$^{-/-}$ or Wild type (WT) mice.

In chapter 4 **(Figure 1-5)** of my thesis, I report studies testing the role of co-inhibitory molecules (CTLA-4, PD-1 and BTLA) in the maintenance of spontaneous acceptance of weakly mismatched allografts (male antigen). Among the co-inhibitory molecules, I found that only PD-1 is required for maintenance of spontaneous acceptance and tolerance of male islet grafts in syngeneic female mice.

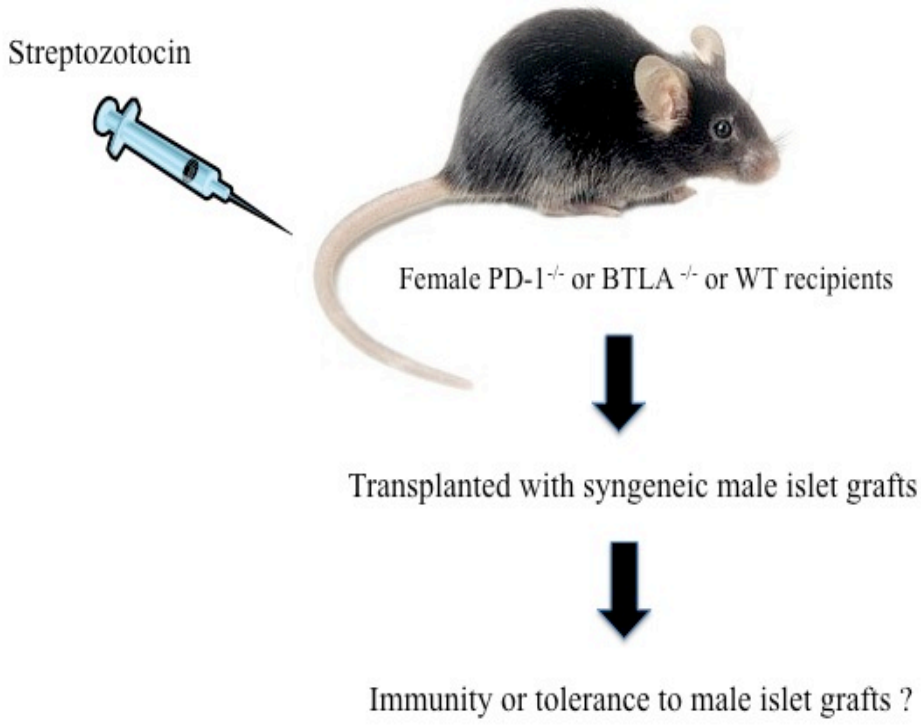


Figure 1-5. Testing the capacity of co-inhibitory molecules in the maintenance of tolerance to male islet allografts. Chemically induced diabetic female PD-1$^{-/-}$ or BTLA$^{-/-}$ or Wild type (WT) mice were transplanted with their syngeneic male islet grafts.

In chapter 5 **(Figure 1-6)** of my thesis, I describe studies that tested whether bystander killing or collateral damage can occur in islet transplantation by using TCR transgenic (Tg) and wild type mice. Results from TCR transgenic experiments demonstrated that bystander killing can occur in islet transplantation. However, bystander killing was not observed in wild type mice, which have a

polyclonal T cell repertoire. Furthermore, I investigated the mechanisms that can limit bystander killing *in vivo*.

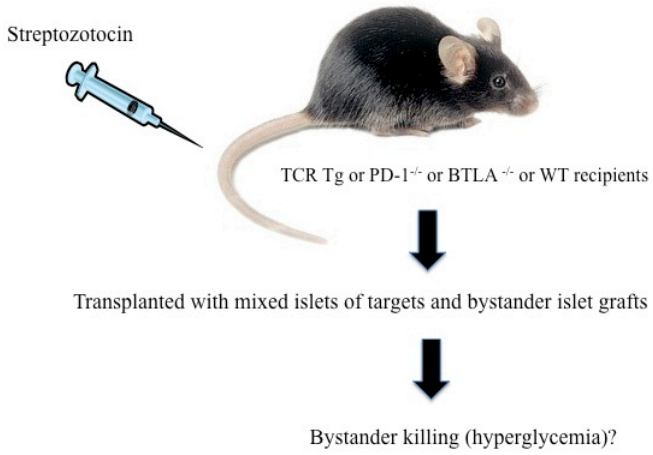


Figure 1-6. Testing the occurrence of bystander killing *in vivo* and mechanisms that limit bystander killing. Chemically induced diabetic TCR Tg or PD-1$^{-/-}$ or BTLA$^{-/-}$ or Wild type (WT) mice were transplanted with mixed islets of targets and bystander islets.

In chapter 6 of my thesis, I described the possible implications of my research findings in clinical settings. Additionally, I discussed the future directions of my research. Taken together, my research findings uncovered the most critical role of the co-inhibitory molecules PD-1 & BTLA in the maintenance of self-tolerance.

1.7. Conclusions

The data we have reviewed here present a strong case that co-inhibitory receptor ligand pathways are central to both recessive and dominant tolerance mechanisms, and that their control of innate immunity is a promising area for

future research. The complexity of their interactions, including the cell types that express the receptors/ligands and other issues related to the context in which these signals are perceived can alter the outcome. Receptors predominantly contributing negative co-inhibitory signals may under some conditions positively regulate responses. It therefore becomes difficult to neatly categorize receptors based simply on their structural relationships or predominant functions. The precise role of co-inhibition in recessive vs. dominant tolerance needs to be more fully defined. Most experiments investigating co-inhibitors in dominant T_{reg} function suffer from the flaws common to experimental systems evaluating T_{reg}. The T_{reg} studied usually do not have a defined antigen specificity[89, 241]. In systems where tolerance depends on a particular receptor/pathway (e.g. a co-inhibitor) the loss of tolerance by T_{reg} does not by itself prove the receptor/pathway works via T_{reg}. In addition, dominant tolerance is tested by studying the response to an antigen of naïve cells that have been mixed with cells that are putatively tolerant via a T_{reg} mechanism. The control for such experiments has almost universally been the addition of non-T_{reg} (e.g. $CD25^-$ cells) to the naïve population to show that the non-T_{reg} do not inhibit the response. However, if one is to demonstrate a true dominant tolerance preventing immune responses specifically to self, the required control is instead the addition of a control population of T cells that is tolerant via a recessive mechanism (e.g. tolerant by deletion of T cells with the appropriate specificity). Without this control, the dominant T_{reg} tolerance demonstrated might simply be a non-specific cellular competition that raises the threshold for naïve T cell activation. Nevertheless, such a non-specific suppression could be important

for inhibiting low affinity anti-self cells, buffering against homeostatic activation, and allowing recessive tolerance mechanisms to take hold[141, 241]. Defining the role of co-inhibitors in these processes should provide important insights into the evolutionary solution for self/nonself discrimination and new avenues of immune intervention in disease.

1.8. References

1. Murphy KM, Nelson CA, Sedy JR. Balancing co-stimulation and inhibition with BTLA and HVEM. Nat Rev Immunol 2006; 6:671-81.
2. Cady CT, Rice JS, Ott VL, Cambier JC. Regulation of hematopoietic cell function by inhibitory immunoglobulin G receptors and their inositol lipid phosphatase effectors. Immunol Rev 2008; 224:44-57.
3. Cai G, Freeman GJ. The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation. Immunol Rev 2009; 229:244-58.
4. Daeron M, Jaeger S, Du Pasquier L, Vivier E. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev 2008; 224:11-43.
5. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunol Rev 2008; 224:166-82.
6. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008; 26:677-704.
7. Riley JL. PD-1 signaling in primary T cells. Immunol Rev 2009; 229:114-25.
8. Burnet FM, Fenner F. The production of Antibodies. Melbourne: Macmillan and Company Limited, 1949.
9. Billingham RE, Brent L, Medawar PB. 'Actively acquired tolerance' of foreign cells. Nature 1953; 172:603-6.
10. Anderson CC. On the sorting of the repertoire: an analysis of Cohn's challenge to integrity (Dembic), Round 2. Scand J Immunol 2009; 70:321-5.
11. Chan WF, Perez-Diez A, Razavy H, Anderson CC. The ability of natural tolerance to be applied to allogeneic tissue: determinants and limits. Biol Direct 2007; 2:10.
12. Lederberg J. Genes and antibodies. Science 1959; 129:1649-53.
13. Guerau-de-Arellano M, Martinic M, Benoist C, Mathis D. Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity. J Exp Med 2009; 206:1245-52.
14. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. Cell 1987; 49:273-80.
15. Kisielow P, Bluthmann H, Staerz UD, Steinmetz M, von Boehmer H. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. Nature 1988; 333:742-6.
16. Bretscher P, Cohn M. A theory of self-nonself discrimination. Science 1970; 169:1042-9.
17. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. Aust J Exp Biol Med Sci 1975; 53:27-42.
18. Coutinho A, Moller G. The self-nonself discrimination: A one-signal mechanism. Scand J Immunol 1975; 4:99-102.

19. Janeway CA, Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today 1992; 13:11-6.
20. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997; 388:394-7.
21. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 1994; 12:991-1045.
22. Anderson CC, Matzinger P. Danger: the view from the bottom of the cliff. Semin Immunol 2000; 12:231-8; discussion 57-344.
23. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 2003; 425:516-21.
24. Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science 2009; 323:1722-5.
25. Hreggvidsdottir HS, Ostberg T, Wahamaa H, Schierbeck H, Aveberger AC, Klevenvall L, et al. The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation. J Leukoc Biol 2009; 86:655-62.
26. Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A. HMGB1: endogenous danger signaling. Mol Med 2008; 14:476-84.
27. Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? PLoS One 2008; 3:e3331.
28. Peter ME. ROS eliminate danger. Immunity 2008; 29:1-2.
29. Johnson GB, Brunn GJ, Kodaira Y, Platt JL. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. J Immunol 2002; 168:5233-9.
30. Sinclair NRS, Chan PL. Regulation of the immune response. IV. The role of the Fc-fragment in feedback inhibition by antibody. Adv Exp Med Biol 1971; 12:609-15.
31. Sinclair NR. How many signals are enough? Cell Immunol 1990; 130:204-12; discussion 13-35.
32. Sinclair NR, Anderson CC. Co-stimulation and co-inhibition: equal partners in regulation. Scand J Immunol 1996; 43:597-603.
33. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999; 11:141-51.
34. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001; 291:319-22.
35. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995; 270:985-8.
36. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 2006; 439:682-7.

37. Blackburn SD, Crawford A, Shin H, Polley A, Freeman GJ, Wherry EJ. Tissue specific differences in PD-1 and PD-L1 expression during chronic viral infection: Implications for CD8 T cell exhaustion. J Virol 2009.
38. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 2009; 10:29-37.
39. Fisicaro P, Valdatta C, Massari M, Loggi E, Biasini E, Sacchelli L, et al. Antiviral Intrahepatic T-Cell Responses Can Be Restored by Blocking Programmed Death-1 Pathway in Chronic Hepatitis B. Gastroenterology 2009.
40. Ha SJ, Mueller SN, Wherry EJ, Barber DL, Aubert RD, Sharpe AH, et al. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. J Exp Med 2008; 205:543-55.
41. Nakamoto N, Cho H, Shaked A, Olthoff K, Valiga ME, Kaminski M, et al. Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. PLoS Pathog 2009; 5:e1000313.
42. Radziewicz H, Dunham RM, Grakoui A. PD-1 tempers Tregs in chronic HCV infection. J Clin Invest 2009; 119:450-3.
43. Rivas MN, Weatherly K, Hazzan M, Vokaer B, Dremier S, Gaudray F, et al. Reviving function in CD4+ T cells adapted to persistent systemic antigen. J Immunol 2009; 183:4284-91.
44. Liblau R, Tournier-Lasserve E, Maciazek J, Dumas G, Siffert O, Hashim G, et al. T cell response to myelin basic protein epitopes in multiple sclerosis patients and healthy subjects. Eur J Immunol 1991; 21:1391-5.
45. Sun JB, Olsson T, Wang WZ, Xiao BG, Kostulas V, Fredrikson S, et al. Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls. Eur J Immunol 1991; 21:1461-8.
46. Liu GY, Fairchild PJ, Smith RM, Prowle JR, Kioussis D, Wraith DC. Low avidity recognition of self-antigen by T cells permits escape from central tolerance. Immunity 1995; 3:407-15.
47. Burkly LC, Lo D, Kanagawa O, Brinster RL, Flavell RA. Clonal anergy of I-E-tolerant T cells in transgenic mice with pancreatic expression of MHC class II I-E. Cold Spring Harb Symp Quant Biol 1989; 54 Pt 2:815-20.
48. 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.
49. Rammensee HG, Kroschewski R, Frangoulis B. Clonal anergy induced in mature V beta 6+ T lymphocytes on immunizing Mls-1b mice with Mls-1a expressing cells. Nature 1989; 339:541-4.
50. Cambier JC, Getahun A. B cell activation versus anergy; the antigen receptor as a molecular switch. Immunol Lett 2009.
51. Getahun A, O'Neill SK, Cambier JC. Establishing anergy as a bona fide in vivo mechanism of B cell tolerance. J Immunol 2009; 183:5439-41.
52. Jones LA, Chin LT, Longo DL, Kruisbeek AM. Peripheral clonal elimination of functional T cells. Science 1990; 250:1726-9.

53. Rocha B, von Boehmer H. Peripheral selection of the T cell repertoire. Science 1991; 251:1225-8.
54. Webb S, Morris C, Sprent J. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. Cell 1990; 63:1249-56.
55. Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell 1991; 65:319-31.
56. Starzl TE, Zinkernagel RM. Transplantation tolerance from a historical perspective. Nat Rev Immunol 2001; 1:233-9.
57. Zinkernagel RM, Ehl S, Aichele P, Oehen S, Kundig T, Hengartner H. Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity. Immunol Rev 1997; 156:199-209.
58. Chan WF, Razavy H, Anderson CC. Differential susceptibility of allogeneic targets to indirect CD4 immunity generates split tolerance. J Immunol 2008; 181:4603-12.
59. Schonrich G, Kalinke U, Momburg F, Malissen M, Schmitt-Verhulst AM, Malissen B, et al. Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. Cell 1991; 65:293-304.
60. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008; 133:775-87.
61. Long E, Wood KJ. Regulatory T cells in transplantation: transferring mouse studies to the clinic. Transplantation 2009; 88:1050-6.
62. Wright GP, Notley CA, Xue SA, Bendle GM, Holler A, Schumacher TN, et al. Adoptive therapy with redirected primary regulatory T cells results in antigen-specific suppression of arthritis. Proc Natl Acad Sci U S A 2009; 106:19078-83.
63. Jankowska-Gan E, Sollinger HW, Pirsch JD, Cai J, Pascual J, Haynes LD, et al. Successful reduction of immunosuppression in older renal transplant recipients who exhibit donor-specific regulation. Transplantation 2009; 88:533-41.
64. Molitor-Dart ML, Andrassy J, Kwun J, Kayaoglu HA, Roenneburg DA, Haynes LD, et al. Developmental exposure to noninherited maternal antigens induces CD4+ T regulatory cells: relevance to mechanism of heart allograft tolerance. J Immunol 2007; 179:6749-61.
65. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology 1970; 18:723-37.
66. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155:1151-64.
67. Cohn M. What roles do regulatory T cells play in the control of the adaptive immune response? Int Immunol 2008; 20:1107-18.

68. Modigliani Y, Bandeira A, Coutinho A. A model for developmentally acquired thymus-dependent tolerance to central and peripheral antigens. Immunol Rev 1996; 149:155-20.
69. Dembic Z. Beginning of the end of (understanding) the immune response. Scand J Immunol 2008; 68:381-2.
70. Liston A, Lesage S, Wilson J, Peltonen L, Goodnow CC. Aire regulates negative selection of organ-specific T cells. Nat Immunol 2003; 4:350-4.
71. Nagamine K, Peterson P, Scott HS, Kudoh J, Minoshima S, Heino M, et al. Positional cloning of the APECED gene. Nat Genet 1997; 17:393-8.
72. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science 2002; 298:1395-401.
73. Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T cells in healthy individuals. J Immunol 2004; 172:5967-72.
74. Jingwu Z, Medaer R, Hashim GA, Chin Y, van den Berg-Loonen E, Raus JC. Myelin basic protein-specific T lymphocytes in multiple sclerosis and controls: precursor frequency, fine specificity, and cytotoxicity. Ann Neurol 1992; 32:330-8.
75. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. Science 1990; 248:1349-56.
76. Becker JC, Brabletz T, Kirchner T, Conrad CT, Brocker EB, Reisfeld RA. Negative transcriptional regulation in anergic T cells. Proc Natl Acad Sci U S A 1995; 92:2375-8.
77. Jenkins MK. The role of cell division in the induction of clonal anergy. Immunol Today 1992; 13:69-73.
78. 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.
79. Kearney ER, Walunas TL, Karr RW, Morton PA, Loh DY, Bluestone JA, et al. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. J Immunol 1995; 155:1032-6.
80. Kurts C, Kosaka H, Carbone FR, Miller JF, Heath WR. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. J Exp Med 1997; 186:239-45.
81. Davey GM, Kurts C, Miller JF, Bouillet P, Strasser A, Brooks AG, et al. Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. J Exp Med 2002; 196:947-55.
82. Kurts C, Heath WR, Kosaka H, Miller JF, Carbone FR. The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). J Exp Med 1998; 188:415-20.
83. Liu X, Alexiou M, Martin-Orozco N, Chung Y, Nurieva RI, Ma L, et al. Cutting edge: A critical role of B and T lymphocyte attenuator in peripheral T cell tolerance induction. J Immunol 2009; 182:4516-20.

84. Martin-Orozco N, Wang YH, Yagita H, Dong C. Cutting Edge: Programmed death (PD) ligand-1/PD-1 interaction is required for CD8+ T cell tolerance to tissue antigens. J Immunol 2006; 177:8291-5.
85. Chambers CA, Krummel MF, Boitel B, Hurwitz A, Sullivan TJ, Fournier S, et al. The role of CTLA-4 in the regulation and initiation of T-cell responses. Immunol Rev 1996; 153:27-46.
86. Leung HT, Bradshaw J, Cleaveland JS, Linsley PS. Cytotoxic T lymphocyte-associated molecule-4, a high-avidity receptor for CD80 and CD86, contains an intracellular localization motif in its cytoplasmic tail. J Biol Chem 1995; 270:25107-14.
87. Linsley PS, Greene JL, Tan P, Bradshaw J, Ledbetter JA, Anasetti C, et al. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. J Exp Med 1992; 176:1595-604.
88. Chambers CA, Cado D, Truong T, Allison JP. Thymocyte development is normal in CTLA-4-deficient mice. Proc Natl Acad Sci U S A 1997; 94:9296-301.
89. Ise W, Kohyama M, Nutsch KM, Lee HM, Suri A, Unanue ER, et al. CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. Nat Immunol 2010; 11:129-35.
90. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J 1992; 11:3887-95.
91. Shlapatska LM, Mikhalap SV, Berdova AG, Zelensky OM, Yun TJ, Nichols KE, et al. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. J Immunol 2001; 166:5480-7.
92. Kido M, Watanabe N, Okazaki T, Akamatsu T, Tanaka J, Saga K, et al. Fatal autoimmune hepatitis induced by concurrent loss of naturally arising regulatory T cells and PD-1-mediated signaling. Gastroenterology 2008; 135:1333-43.
93. Wang J, Yoshida T, Nakaki F, Hiai H, Okazaki T, Honjo T. Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes. Proc Natl Acad Sci U S A 2005; 102:11823-8.
94. Okazaki T, Okazaki IM, Wang J, Sugiura D, Nakaki F, Yoshida T, et al. PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. J Exp Med 2011; 208:395-407.
95. Kroner A, Mehling M, Hemmer B, Rieckmann P, Toyka KV, Maurer M, et al. A PD-1 polymorphism is associated with disease progression in multiple sclerosis. Ann Neurol 2005; 58:50-7.
96. Prokunina L, Castillejo-Lopez C, Oberg F, Gunnarsson I, Berg L, Magnusson V, et al. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. Nat Genet 2002; 32:666-9.
97. Prokunina L, Padyukov L, Bennet A, de Faire U, Wiman B, Prince J, et al. Association of the PD-1.3A allele of the PDCD1 gene in patients with

rheumatoid arthritis negative for rheumatoid factor and the shared epitope. Arthritis Rheum 2004; 50:1770-3.
98. Nielsen C, Hansen D, Husby S, Jacobsen BB, Lillevang ST. Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes. Tissue Antigens 2003; 62:492-7.
99. Grabie N, Gotsman I, DaCosta R, Pang H, Stavrakis G, Butte MJ, et al. Endothelial programmed death-1 ligand 1 (PD-L1) regulates CD8+ T-cell mediated injury in the heart. Circulation 2007; 116:2062-71.
100. Keir ME, Freeman GJ, Sharpe AH. PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues. J Immunol 2007; 179:5064-70.
101. 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.
102. Reynoso ED, Elpek KG, Francisco L, Bronson R, Bellemare-Pelletier A, Sharpe AH, et al. Intestinal tolerance is converted to autoimmune enteritis upon PD-1 ligand blockade. J Immunol 2009; 182:2102-12.
103. Salama AD, Chitnis T, Imitola J, Ansari MJ, Akiba H, Tushima F, et al. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. J Exp Med 2003; 198:71-8.
104. Zhang Y, Chung Y, Bishop C, Daugherty B, Chute H, Holst P, et al. Regulation of T cell activation and tolerance by PDL2. Proc Natl Acad Sci U S A 2006; 103:11695-700.
105. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 2003; 4:670-9.
106. Han P, Goularte OD, Rufner K, Wilkinson B, Kaye J. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. J Immunol 2004; 172:5931-9.
107. Sedy JR, Gavrieli M, Potter KG, Hurchla MA, Lindsley RC, Hildner K, et al. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. Nat Immunol 2005; 6:90-8.
108. Murphy TL, Murphy KM. Slow down and survive: Enigmatic immunoregulation by BTLA and HVEM. Annu Rev Immunol 2010; 28:389-411.
109. Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. Nat Immunol 2008; 9:176-85.
110. Morel Y, Schiano de Colella JM, Harrop J, Deen KC, Holmes SD, Wattam TA, et al. Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. J Immunol 2000; 165:4397-404.
111. Oya Y, Watanabe N, Kobayashi Y, Owada T, Oki M, Ikeda K, et al. Lack of B and T lymphocyte attenuator exacerbates autoimmune disorders and induces Fas-independent liver injury in MRL-lpr/lpr mice. Int Immunol 2011; 23:335-44.

112. Oya Y, Watanabe N, Owada T, Oki M, Hirose K, Suto A, et al. Development of autoimmune hepatitis-like disease and production of autoantibodies to nuclear antigens in mice lacking B and T lymphocyte attenuator. Arthritis Rheum 2008; 58:2498-510.
113. Deppong C, Juehne TI, Hurchla M, Friend LD, Shah DD, Rose CM, et al. Cutting edge: B and T lymphocyte attenuator and programmed death receptor-1 inhibitory receptors are required for termination of acute allergic airway inflammation. J Immunol 2006; 176:3909-13.
114. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. Proc Natl Acad Sci U S A 2008; 105:9331-6.
115. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 2009; 206:3015-29.
116. Steinberg MW, Turovskaya O, Shaikh RB, Kim G, McCole DF, Pfeffer K, et al. A crucial role for HVEM and BTLA in preventing intestinal inflammation. J Exp Med 2008; 205:1463-76.
117. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med 1999; 5:1365-9.
118. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000; 192:1027-34.
119. Guleria I, Khosroshahi A, Ansari MJ, Habicht A, Azuma M, Yagita H, et al. A critical role for the programmed death ligand 1 in fetomaternal tolerance. J Exp Med 2005; 202:231-7.
120. Liang SC, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, et al. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. Eur J Immunol 2003; 33:2706-16.
121. Rodig N, Ryan T, Allen JA, Pang H, Grabie N, Chernova T, et al. Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. Eur J Immunol 2003; 33:3117-26.
122. Blank C, Brown I, Peterson AC, Spiotto M, Iwai Y, Honjo T, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. Cancer Res 2004; 64:1140-5.
123. Mazanet MM, Hughes CC. B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis. J Immunol 2002; 169:3581-8.
124. Rubtsov YP, Rudensky AY. TGFbeta signalling in control of T-cell-mediated self-reactivity. Nat Rev Immunol 2007; 7:443-53.
125. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol 2000; 164:183-90.
126. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 2001; 194:629-44.

127. Habicht A, Dada S, Jurewicz M, Fife BT, Yagita H, Azuma M, et al. A link between PDL1 and T regulatory cells in fetomaternal tolerance. J Immunol 2007; 179:5211-9.
128. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008; 322:271-5.
129. Schmidt EM, Wang CJ, Ryan GA, Clough LE, Qureshi OS, Goodall M, et al. Ctla-4 controls regulatory T cell peripheral homeostasis and is required for suppression of pancreatic islet autoimmunity. J Immunol 2009; 182:274-82.
130. Kitazawa Y, Fujino M, Wang Q, Kimura H, Azuma M, Kubo M, et al. Involvement of the programmed death-1/programmed death-1 ligand pathway in CD4+CD25+ regulatory T-cell activity to suppress alloimmune responses. Transplantation 2007; 83:774-82.
131. Tao R, Wang L, Murphy KM, Fraser CC, Hancock WW. Regulatory T cell expression of herpesvirus entry mediator suppresses the function of B and T lymphocyte attenuator-positive effector T cells. J Immunol 2008; 180:6649-55.
132. Hurchla MA, Sedy JR, Murphy KM. Unexpected role of B and T lymphocyte attenuator in sustaining cell survival during chronic allostimulation. J Immunol 2007; 178:6073-82.
133. Cheung TC, Steinberg MW, Oborne LM, Macauley MG, Fukuyama S, Sanjo H, et al. Unconventional ligand activation of herpesvirus entry mediator signals cell survival. Proc Natl Acad Sci U S A 2009; 106:6244-9.
134. Cheung TC, Oborne LM, Steinberg MW, Macauley MG, Fukuyama S, Sanjo H, et al. T cell intrinsic heterodimeric complexes between HVEM and BTLA determine receptivity to the surrounding microenvironment. J Immunol 2009; 183:7286-96.
135. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol 2005; 6:1245-52.
136. Wang F, Wan L, Zhang C, Zheng X, Li J, Chen ZK. Tim-3-Galectin-9 pathway involves the suppression induced by CD4+CD25+ regulatory T cells. Immunobiology 2009; 214:342-9.
137. Wang F, He W, Zhou H, Yuan J, Wu K, Xu L, et al. The Tim-3 ligand galectin-9 negatively regulates CD8+ alloreactive T cell and prolongs survival of skin graft. Cell Immunol 2007; 250:68-74.
138. Jain N, Nguyen H, Chambers C, Kang J. Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity. Proc Natl Acad Sci U S A 2010.
139. Chikuma S, Terawaki S, Hayashi T, Nabeshima R, Yoshida T, Shibayama S, et al. PD-1-mediated suppression of IL-2 production induces CD8+ T cell anergy in vivo. J Immunol 2009; 182:6682-9.
140. Dong H, Zhu G, Tamada K, Flies DB, van Deursen JM, Chen L. B7-H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes. Immunity 2004; 20:327-36.

141. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. Nat Immunol 2005; 6:280-6.
142. Tsushima F, Yao S, Shin T, Flies A, Flies S, Xu H, et al. Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy. Blood 2007; 110:180-5.
143. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells. Science 1992; 258:1156-9.
144. Lassila O, Vainio O, Matzinger P. Can B cells turn on virgin T cells? Nature 1988; 334:253-5.
145. Frommer F, Heinen TJ, Wunderlich FT, Yogev N, Buch T, Roers A, et al. Tolerance without clonal expansion: self-antigen-expressing B cells program self-reactive T cells for future deletion. J Immunol 2008; 181:5748-59.
146. Guleria I, Sayegh MH. Maternal acceptance of the fetus: true human tolerance. J Immunol 2007; 178:3345-51.
147. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol 2004; 5:266-71.
148. Heikkinen J, Mottonen M, Alanen A, Lassila O. Phenotypic characterization of regulatory T cells in the human decidua. Clin Exp Immunol 2004; 136:373-8.
149. Zenclussen AC. CD4(+)CD25+ T regulatory cells in murine pregnancy. J Reprod Immunol 2005; 65:101-10.
150. Petroff MG. Immune interactions at the maternal-fetal interface. J Reprod Immunol 2005; 68:1-13.
151. Taglauer ES, Yankee TM, Petroff MG. Maternal PD-1 regulates accumulation of fetal antigen-specific CD8+ T cells in pregnancy. J Reprod Immunol 2009; 80:12-21.
152. Wang W, Carper K, Malone F, Latchman Y, Perkins J, Fu Y, et al. PD-L1/PD-1 signal deficiency promotes allogeneic immune responses and accelerates heart allograft rejection. Transplantation 2008; 86:836-44.
153. Wafula PO, Teles A, Schumacher A, Pohl K, Yagita H, Volk HD, et al. PD-1 but not CTLA-4 blockage abrogates the protective effect of regulatory T cells in a pregnancy murine model. Am J Reprod Immunol 2009; 62:283-92.
154. Choi S, Schwartz RH. Molecular mechanisms for adaptive tolerance and other T cell anergy models. Semin Immunol 2007; 19:140-52.
155. Inobe M, Schwartz RH. CTLA-4 engagement acts as a brake on CD4+ T cell proliferation and cytokine production but is not required for tuning T cell reactivity in adaptive tolerance. J Immunol 2004; 173:7239-48.
156. Singh NJ, Schwartz RH. The strength of persistent antigenic stimulation modulates adaptive tolerance in peripheral CD4+ T cells. J Exp Med 2003; 198:1107-17.
157. Tanchot C, Barber DL, Chiodetti L, Schwartz RH. Adaptive tolerance of CD4+ T cells in vivo: multiple thresholds in response to a constant level of antigen presentation. J Immunol 2001; 167:2030-9.
158. Gallimore A, Glithero A, Godkin A, Tissot AC, Pluckthun A, Elliott T, et al. Induction and exhaustion of lymphocytic choriomeningitis virus-specific

cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. J Exp Med 1998; 187:1383-93.
159. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 2006; 443:350-4.
160. Kaufmann DE, Walker BD. Programmed death-1 as a factor in immune exhaustion and activation in HIV infection. Curr Opin HIV AIDS 2008; 3:362-7.
161. Golden-Mason L, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, McMahon BJ, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. J Virol 2009; 83:9122-30.
162. Grosso JF, Goldberg MV, Getnet D, Bruno TC, Yen HR, Pyle KJ, et al. Functionally distinct LAG-3 and PD-1 subsets on activated and chronically stimulated CD8 T cells. J Immunol 2009; 182:6659-69.
163. Breton G, Yassine-Diab B, Cohn L, Boulassel MR, Routy JP, Sekaly RP, et al. siRNA knockdown of PD-L1 and PD-L2 in monocyte-derived dendritic cells only modestly improves proliferative responses to Gag by CD8(+) T cells from HIV-1-infected individuals. J Clin Immunol 2009; 29:637-45.
164. Anderson CC. Time, space and contextual models of the immunity tolerance decision: bridging the geographical divide of Zinkernagel and Hengartner's 'Credo 2004'. Scand J Immunol 2006; 63:249-56.
165. Ehl S, Hombach J, Aichele P, Rulicke T, Odermatt B, Hengartner H, et al. Viral and bacterial infections interfere with peripheral tolerance induction and activate CD8+ T cells to cause immunopathology. J Exp Med 1998; 187:763-74.
166. Mumprecht S, Schurch C, Schwaller J, Solenthaler M, Ochsenbein AF. Programmed death 1 signaling on chronic myeloid leukemia-specific T cells results in T-cell exhaustion and disease progression. Blood 2009; 114:1528-36.
167. Zang X, Allison JP. The B7 family and cancer therapy: costimulation and coinhibition. Clin Cancer Res 2007; 13:5271-9.
168. Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. Blood 2008; 111:3635-43.
169. Derre L, Rivals JP, Jandus C, Pastor S, Rimoldi D, Romero P, et al. BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination. J Clin Invest 2010; 120:157-67.
170. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 2006; 6:295-307.
171. Tanaka H, Tanaka J, Kjaergaard J, Shu S. Depletion of CD4+ CD25+ regulatory cells augments the generation of specific immune T cells in tumor-draining lymph nodes. J Immunother 2002; 25:207-17.
172. Ko K, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, et al. Treatment of advanced tumors with agonistic anti-GITR mAb and its

effects on tumor-infiltrating Foxp3+CD25+CD4+ regulatory T cells. J Exp Med 2005; 202:885-91.
173. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science 1996; 271:1734-6.
174. Tanaka K, Albin MJ, Yuan X, Yamaura K, Habicht A, Murayama T, et al. PDL1 is required for peripheral transplantation tolerance and protection from chronic allograft rejection. J Immunol 2007; 179:5204-10.
175. Truong W, Plester JC, Hancock WW, Kaye J, Merani S, Murphy KM, et al. Negative and positive co-signaling with anti-BTLA (PJ196) and CTLA4Ig prolongs islet allograft survival. Transplantation 2007; 84:1368-72.
176. Gao W, Demirci G, Strom TB, Li XC. Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. Transplantation 2003; 76:994-9.
177. Ozkaynak E, Wang L, Goodearl A, McDonald K, Qin S, O'Keefe T, et al. Programmed death-1 targeting can promote allograft survival. J Immunol 2002; 169:6546-53.
178. Truong W, Hancock WW, Plester JC, Merani S, Rayner DC, Thangavelu G, et al. BTLA targeting modulates lymphocyte phenotype, function, and numbers and attenuates disease in nonobese diabetic mice. J Leukoc Biol 2009; 86:41-51.
179. Ito T, Ueno T, Clarkson MR, Yuan X, Jurewicz MM, Yagita H, et al. Analysis of the role of negative T cell costimulatory pathways in CD4 and CD8 T cell-mediated alloimmune responses in vivo. J Immunol 2005; 174:6648-56.
180. Sandner SE, Clarkson MR, Salama AD, Sanchez-Fueyo A, Domenig C, Habicht A, et al. Role of the programmed death-1 pathway in regulation of alloimmune responses in vivo. J Immunol 2005; 174:3408-15.
181. Sho M, Yamada A, Najafian N, Salama AD, Harada H, Sandner SE, et al. Physiological mechanisms of regulating alloimmunity: cytokines, CTLA-4, CD25+ cells, and the alloreactive T cell clone size. J Immunol 2002; 169:3744-51.
182. Shirasugi N, Ikeda Y, Akiyama Y, Matsumoto K, Hamano K, Esato K, et al. Induction of hyporesponsiveness to fully allogeneic cardiac grafts by intratracheal delivery of alloantigen. Transplantation 2001; 71:561-4.
183. Aramaki O, Shirasugi N, Takayama T, Shimazu M, Kitajima M, Ikeda Y, et al. Programmed death-1-programmed death-L1 interaction is essential for induction of regulatory cells by intratracheal delivery of alloantigen. Transplantation 2004; 77:6-12.
184. Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, et al. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. Nat Immunol 2003; 4:1093-101.
185. Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. J Immunol 2002; 168:1080-6.

186. Dudler J, Li J, Pagnotta M, Pascual M, von Segesser LK, Vassalli G. Gene transfer of programmed death ligand-1.Ig prolongs cardiac allograft survival. Transplantation 2006; 82:1733-7.
187. Kawamoto K, Tanemura M, Ito T, Deguchi T, Machida T, Nishida T, et al. Prolonged survival of pig islets xenograft by adenovirus-mediated expression of either the membrane-bound human FasL or the human decoy Fas antigen gene. Xenotransplantation 2008; 15:333-43.
188. Plege A, Borns K, Baars W, Schwinzer R. Suppression of human T-cell activation and expansion of regulatory T cells by pig cells overexpressing PD-ligands. Transplantation 2009; 87:975-82.
189. Wen X, Zhu H, Li L, Li Y, Wang M, Liu J, et al. Transplantation of NIT-1 cells expressing pD-L1 for treatment of streptozotocin-induced diabetes. Transplantation 2008; 86:1596-602.
190. Hamilton SE, Wolkers MC, Schoenberger SP, Jameson SC. The generation of protective memory-like CD8+ T cells during homeostatic proliferation requires CD4+ T cells. Nat Immunol 2006; 7:475-81.
191. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity 2008; 29:848-62.
192. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. Nat Rev Immunol 2009; 9:823-32.
193. Dummer W, Ernst B, LeRoy E, Lee D, Surh C. Autologous regulation of naive T cell homeostasis within the T cell compartment. J Immunol 2001; 166:2460-8.
194. Ge Q, Rao VP, Cho BK, Eisen HN, Chen J. Dependence of lymphopenia-induced T cell proliferation on the abundance of peptide/ MHC epitopes and strength of their interaction with T cell receptors. Proc Natl Acad Sci U S A 2001; 98:1728-33.
195. Kieper WC, Burghardt JT, Surh CD. A role for TCR affinity in regulating naive T cell homeostasis. J Immunol 2004; 172:40-4.
196. Moses CT, Thorstenson KM, Jameson SC, Khoruts A. Competition for self ligands restrains homeostatic proliferation of naive CD4 T cells. Proc Natl Acad Sci U S A 2003; 100:1185-90.
197. Brandt L, Stenstam M. Letter: Subnormal lymphocyte-counts in adult coeliac disease. Lancet 1975; 1:978-9.
198. Utsinger PD, Yount WJ. Lymphopenia in Sjogren's syndrome with rheumatoid arthritis: relationship to lymphocytotoxic antibodies, cryoglobulinemia, and impaired mitogen responsiveness. J Rheumatol 1976; 3:355-66.
199. Nishizuka Y, Sakakura T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. Science 1969; 166:753-5.
200. Datta S, Sarvetnick N. Lymphocyte proliferation in immune-mediated diseases. Trends Immunol 2009; 30:430-8.
201. Subra JF, Renier G, Reboul P, Tollis F, Boivinet R, Schwartz P, et al. Lymphopenia in occupational pulmonary silicosis with or without autoimmune disease. Clin Exp Immunol 2001; 126:540-4.

202. Moxham VF, Karegli J, Phillips RE, Brown KL, Tapmeier TT, Hangartner R, et al. Homeostatic proliferation of lymphocytes results in augmented memory-like function and accelerated allograft rejection. J Immunol 2008; 180:3910-8.
203. 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.
204. Monti P, Scirpoli M, Maffi P, Ghidoli N, De Taddeo F, Bertuzzi F, et al. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. J Clin Invest 2008; 118:1806-14.
205. 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.
206. Goldrath AW, Bevan MJ. Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. Immunity 1999; 11:183-90.
207. Guimond M, Veenstra RG, Grindler DJ, Zhang H, Cui Y, Murphy RD, et al. Interleukin 7 signaling in dendritic cells regulates the homeostatic proliferation and niche size of CD4+ T cells. Nat Immunol 2009; 10:149-57.
208. Napolitano LA, Grant RM, Deeks SG, Schmidt D, De Rosa SC, Herzenberg LA, et al. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat Med 2001; 7:73-9.
209. Calzascia T, Pellegrini M, Lin A, Garza KM, Elford AR, Shahinian A, et al. CD4 T cells, lymphopenia, and IL-7 in a multistep pathway to autoimmunity. Proc Natl Acad Sci U S A 2008; 105:2999-3004.
210. Min B, Yamane H, Hu-Li J, Paul WE. Spontaneous and homeostatic proliferation of CD4 T cells are regulated by different mechanisms. J Immunol 2005; 174:6039-44.
211. Berard M, Brandt K, Bulfone-Paus S, Tough DF. IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. J Immunol 2003; 170:5018-26.
212. Cho JH, Boyman O, Kim HO, Hahm B, Rubinstein MP, Ramsey C, et al. An intense form of homeostatic proliferation of naive CD8+ cells driven by IL-2. J Exp Med 2007; 204:1787-801.
213. Koehn BH, Ford ML, Ferrer IR, Borom K, Gangappa S, Kirk AD, et al. PD-1-dependent mechanisms maintain peripheral tolerance of donor-reactive CD8+ T cells to transplanted tissue. J Immunol 2008; 181:5313-22.
214. Krieg C, Boyman O, Fu YX, Kaye J. B and T lymphocyte attenuator regulates CD8+ T cell-intrinsic homeostasis and memory cell generation. Nat Immunol 2007; 8:162-71.
215. Lin SJ, Peacock CD, Bahl K, Welsh RM. Programmed death-1 (PD-1) defines a transient and dysfunctional oligoclonal T cell population in acute homeostatic proliferation. J Exp Med 2007; 204:2321-33.

216. Benard A, Ceredig R, Rolink AG. Regulatory T cells control autoimmunity following syngeneic bone marrow transplantation. Eur J Immunol 2006; 36:2324-35.
217. Keir ME, Latchman YE, Freeman GJ, Sharpe AH. Programmed death-1 (PD-1):PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes. J Immunol 2005; 175:7372-9.
218. Blank C, Brown I, Marks R, Nishimura H, Honjo T, Gajewski TF. Absence of programmed death receptor 1 alters thymic development and enhances generation of CD4/CD8 double-negative TCR-transgenic T cells. J Immunol 2003; 171:4574-81.
219. Mick VE, Starr TK, McCaughtry TM, McNeil LK, Hogquist KA. The regulated expression of a diverse set of genes during thymocyte positive selection in vivo. J Immunol 2004; 173:5434-44.
220. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27:1047-53.
221. Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967; 16:35-9.
222. Kemp CB, Knight MJ, Scharp DW, Lacy PE, Ballinger WF. Transplantation of isolated pancreatic islets into the portal vein of diabetic rats. Nature 1973; 244:447.
223. Kolb E, Largiader F. Clinical islet transplantation. Transplant Proc 1980; 12:205-7.
224. Najarian JS, Sutherland DE, Matas AJ, Steffes MW, Simmons RL, Goetz FC. Human islet transplantation: a preliminary report. Transplant Proc 1977; 9:233-6.
225. Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Falqui L, et al. Insulin independence after islet transplantation into type I diabetic patient. Diabetes 1990; 39:515-8.
226. 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.
227. Ault A. Edmonton's islet success tough to duplicate elsewhere. Lancet 2003; 361:2054.
228. Hirshberg B. Lessons learned from the international trial of the edmonton protocol for islet transplantation. Curr Diab Rep 2007; 7:301-3.
229. Shieh SJ, Chou FC, Yu PN, Lin WC, Chang DM, Roffler SR, et al. Transgenic expression of single-chain anti-CTLA-4 Fv on beta cells protects nonobese diabetic mice from autoimmune diabetes. J Immunol 2009; 183:2277-85.
230. Nanji SA, Hancock WW, Anderson CC, Adams AB, Luo B, Schur CD, et al. Multiple combination therapies involving blockade of ICOS/B7RP-1 costimulation facilitate long-term islet allograft survival. Am J Transplant 2004; 4:526-36.

231. Wang CJ, Chou FC, Chu CH, Wu JC, Lin SH, Chang DM, et al. Protective role of programmed death 1 ligand 1 (PD-L1)in nonobese diabetic mice: the paradox in transgenic models. Diabetes 2008; 57:1861-9.
232. Pakala SV, Ilic A, Chen L, Sarvetnick N. TNF-alpha receptor 1 (p55) on islets is necessary for the expression of LIGHT on diabetogenic T cells. Clin Immunol 2001; 100:198-207.
233. Truong W, Plester JC, Hancock WW, Merani S, Murphy TL, Murphy KM, et al. Combined coinhibitory and costimulatory modulation with anti-BTLA and CTLA4Ig facilitates tolerance in murine islet allografts. Am J Transplant 2007; 7:2663-74.
234. Li S, Zhang M, Xiang F, Zhao J, Jiang C, Zhu J. Dendritic cells expressing BTLA induces CD8(+) T cell tolerance and attenuates the severity of diabetes. Vaccine 2011.
235. Burrows SR, Fernan A, Argaet V, Suhrbier A. Bystander apoptosis induced by CD8+ cytotoxic T cell (CTL) clones: implications for CTL lytic mechanisms. Int Immunol 1993; 5:1049-58.
236. Duke RC. Self recognition by T cells. I. Bystander killing of target cells bearing syngeneic MHC antigens. J Exp Med 1989; 170:59-71.
237. Wang R, Rogers AM, Ratliff TL, Russell JH. CD95-dependent bystander lysis caused by CD4+ T helper 1 effectors. J Immunol 1996; 157:2961-8.
238. Doody DP, Stenger KS, Winn HJ. Immunologically nonspecific mechanisms of tissue destruction in the rejection of skin grafts. J Exp Med 1994; 179:1645-52.
239. Korsgren O, Jansson L. Characterization of mixed syngeneic-allogeneic and syngeneic-xenogeneic islet-graft rejections in mice. Evidence of functional impairment of the remaining syngeneic islets in xenograft rejections. J Clin Invest 1994; 93:1113-9.
240. Polakos NK, Cornejo JC, Murray DA, Wright KO, Treanor JJ, Crispe IN, et al. Kupffer cell-dependent hepatitis occurs during influenza infection. Am J Pathol 2006; 168:1169-78; quiz 404-5.
241. Schildknecht A, Brauer S, Brenner C, Lahl K, Schild H, Sparwasser T, et al. FoxP3+ regulatory T cells essentially contribute to peripheral CD8+ T-cell tolerance induced by steady-state dendritic cells. Proc Natl Acad Sci U S A 2010; 107:199-203.

Chapter 2: A version of this chapter has been published.

The citation is given below.

Thangavelu G et al., 2011. J Autoimmun. 36(3-4): 301-312.

Programmed death-1 is required for systemic self-tolerance in newly generated T cells during the establishment of immune homeostasis.

2.1. Introduction

The peripheral T cell compartment is tightly regulated by homeostatic mechanisms. Reductions in T cell numbers, as occurs in viral infections and lymphoablative therapy, can lead to a physiological process called lymphopenia induced proliferation (LIP)[1]. There is evidence for several distinct forms of LIP, including a rapid or acute proliferation (also called endogenous proliferation), a slower IL-7 dependent 'homeostatic' proliferation, and a rapid expansion of CD8 central memory cells that occurs in the presence of excess IL-15. Both the slow[2-4] and rapid[5] forms of LIP demonstrate dependence on the presence of adequate lymph node stroma, particularly in the case of CD4 LIP[2-4]. In contrast, the form of LIP that leads to proliferation of central memory CD8 T cells, as occurs in lymphopenic mice lacking NK cells (e.g. IL-2Rγ chain deficient[6, 7]), occurs even in mice substantially deficient in lymph nodes[7].

Lymphopenia can exacerbate auto-reactivity of T cells by homeostatic expansion[8, 9]. However, syngeneic HSC or T cell transfer into lymphopenic animals usually leads to LIP without autoimmunity. Either mechanisms exist to prevent LIP-induced autoimmunity or additional, as yet unknown, stimuli are required to generate disease. Although the balance between multiple co-stimulatory and co-inhibitory signals controls conventional T cell responses[10], whether co-inhibitory signals control lymphopenia driven activation leading to autoimmune disease is unknown. The PD-1 pathway is upregulated during viral infection[11] and in a subset of T cells undergoing acute homeostatic proliferation[12] suggesting its

expression may regulate lymphopenia driven activation. In addition, PD-1 deficiency leads to a narrow spectrum of late-life autoimmunity in mice[13-15] and human PD-1 polymorphisms[16] further support a role for PD-1 in self-tolerance.

Control of homeostatic activation is likely to be particularly important when the first T cells seed the periphery during the fetal/neonatal period[17, 18]. Such newly generated T cells have not yet had a chance to undergo peripheral tolerance, and therefore may have greater autoimmune potential[19]. Consistent with an increased need for control at this stage, recent thymic emigrants (RTE) are considered to have a reduced ability to attain effector function[20-22] despite having an increased capacity for LIP[23-25]. Reduced effector function of RTE could be due to their incomplete maturation[20, 21] or instead an increased negative regulation of fully competent cells. The hypothesis of the current study is that PD-1 is required in T cells to prevent lymphopenia induced autoimmunity. We examined these issues and found that a high proportion of RTE express PD-1 and that PD-1 expression is necessary to prevent lymphopenia driven multi-organ autoimmunity.

2.2. Materials and methods

2.2.1. Mice

These studies used male and female B6.129S7-Rag1^{tm1mom}/J (abbreviated as Rag$^{-/-}$), (C57BL/6J × C57BL/10SgSnAi)-[KO]*γc*-[KO]*Rag2* knockout (abbreviated as Rag$^{-/-}$ γc$^{-/-}$), CD45.1-C57BL/6, TCR transgenic Rag2$^{-/-}$ Marilyn (originally obtained from the NIAID Exchange Program)[26], and C57BL/6-*Pdcd1-/-* (PD-1$^{-/-}$;

backcrossed 11 generations to C57BL/6) mice originally generated by Prof. T. Honjo and colleagues[14]. GFP $Rag^{-/-}$, $Rag^{-/-}$ $K^{b-/-}$ $D^{b-/-}$, and $Rag^{-/-}$ $PD\text{-}1^{-/-}$ mice were generated by crossing the above $Rag^{-/-}$ mice with C57BL/6-Tg(UBC-GFP) 30Scha/J, C57BL/6 H-$2K^{btm1}$-H-$2D^{btm1}$N12 (from the NIAID Exchange Program, NIH: 004215)[27], and C57BL/6-*Pdcd1-/-* mice, respectively. C57BL/6-HY^{cd4} (abbreviated as HY^{cd4}) mice have been previously described[28]. $HY^{cd4}PD\text{-}1^{-/-}$ mice were generated by crossing C57BL/6-HY^{cd4} mice with C57BL/6-*Pdcd1*$^{-/-}$ mice. $Rag2^{-/-}$ Marilyn $PD\text{-}1^{-/-}$ mice were generated by crosses between $Rag2^{-/-}$ Marilyn with C57BL/6-*Pdcd1*$^{-/-}$ mice. B6.129S2-*Lta*^{tm1Dch}/J (abbreviated as $LT\alpha^{-/-}$) were obtained from Jackson laboratory. Animal care was in accordance with guidelines of the Canadian Council on Animal Care.

2.2.2. Stem cell transplantation

Fetal liver cells (day 15-16 fetuses) were used as a source of hematopoietic stem cells (HSC). Six-eight week old immunodeficient animals received 10-15 x 10^6 fetal liver cells i.v. (from $PD\text{-}1^{-/-}$ or wild type (WT) fetuses). Recipients were not irradiated unless indicated. In some experiments, as indicated, recipients were given 15 x 10^6 fetal liver cells containing a 1:1 mixture of $PD\text{-}1^{-/-}$ and WT CD45.1 cells. In other experiments 15 x 10^6 female (sex determined by PCR[29]) fetal liver cells from $PD\text{-}1^{-/-}$ mice were given to female Marilyn mice.

2.2.3. Definition of disease and histology

Disease included cachexia, hunched appearance, ruffled fur, reduced mobility,

skin and ocular lesions. Recipient mice were no longer considered disease free when two or more of the above symptoms were evident. Histological evaluations were completed by a Veterinary Pathologist (R.R.E.U.) blinded to the treatment groups.

2.2.4. In vivo antibody treatment

We used a total dose of 1500μg anti-mouse PD-1 mAb (J43), anti-mouse PD-L1 (10F.9g2) and isotype control (Rat IgG2b) split into six equal doses once every 5 days beginning at 30-35 days of post WT HSC transplantation; timing of the start of injections was determined by the detection of substantial T cell numbers in the peripheral blood. NK cells in $Rag^{-/-}$ mice were depleted by using anti-mouse NK1.1 mAb (PK136) at a dose of 250μg once per week from day minus 7 to day 28 relative to PD-$1^{-/-}$ HSC transplantation.

2.2.5. Flow cytometry analysis

Peripheral blood samples and splenocytes were stained after incubation with an FcR block. T cell repertoire was analyzed by using a Vβ TCR screening panel (BD Pharmingen ^{TM}). A FACSCalibur™ (BD Biosciences) with CellQuest™ Pro software and BD LSR II (BD Biosciences) with FACS Diva^{TM} software were used for most of the data acquisition and analysis, with the exception of the studies on HY^{cd4} mice. Analysis of HY^{cd4} thymocytes: Fluorochrome conjugated antibodies for cell surface staining were purchased from eBioscience. For intracellular cleaved caspase-3 staining, cells were fixed with the BD

Cytofix/Cytoperm™ Kit and then stained with an anti-cleaved caspase 3 antibody purchased from Cell Signaling Technology. Cell events were collected using a FACS Canto II™ (BD Biosciences) and FlowJo software was used for data analysis.

2.2.6. Irradiation bone marrow chimera experiments and adoptive transfer studies

$Rag^{-/-}$ $\gamma c^{-/-}$ or GFP $Rag^{-/-}$ recipients were lethally irradiated in split doses (5 hours apart) of a total of 9 to 12 Gy (Cesium source, Gamma Cell 3000). A total of 10^7 bone marrow cells from GFP $Rag^{-/-}$ or $Rag^{-/-}$ $\gamma c^{-/-}$ donors were injected i.v. into $Rag^{-/-}$ $\gamma c^{-/-}$ or GFP $Rag^{-/-}$ respectively. Approximately two months post bone marrow transplantation recipients were given 15 x 10^6 PD-$1^{-/-}$ fetal liver cells. Thymocytes or splenocytes containing 5 x 10^6 single positive T cells from 7 week old PD-$1^{-/-}$ mice were injected i.v. into immunodeficient recipients. In experiments testing the adoptive transfer of cells from diseased mice, splenocytes containing 3.5 x 10^6 T cells from diseased or healthy mice were injected into secondary $Rag^{-/-}$ $\gamma c^{-/-}$ or $Rag^{-/-}$ recipients. In other experiments WT or LT$\alpha^{-/-}$ mice were lethally irradiated in split doses 48 hrs apart to a total dose of 13Gy. Immediately after the second split dose of irradiation, all recipients were given 15 x 106 PD-1-/- or WT fetal liver cells.

2.2.7. Adoptive transfer studies and FACS sorting

Thymocytes vs. splenocytes injected i.v. contained 5 x 10^6 single positive T cells

from PD-1$^{-/-}$ mice. To test transfer of disease, splenocytes containing 3.5 x 10^6 T cells from diseased or healthy mice were injected into secondary recipients. TCR$^+$ CD24^{lo} cells were sorted aseptically from splenocytes of five week old PD-1$^{-/-}$ mice on a FACS BD influx^{TM} cell sorter (BD Biosciences). The purity of the sorted cell populations was 92%.

2.2.8. BrdU incorporation, immunofluorescence and serum cytokine analysis

Experimental mice were treated with 2mg BrdU in PBS by i.p. injection. BrdU incorporation was assessed in splenic T cells after 36 hours of injection using a BrdU flow cytometry kit (BD Pharmingen^{TM}). For immunofluorescence 5μm crosssections were cut from tissues, fixed, blocked with goat serum and stained with rat anti-mouse CD4 or CD8α (Biolegend, San Deigo, CA) followed by goat anti-rat Alexaflour 488 (Invitrogen Laboratories, Burlington, ON) and visualized on a compound fluorescent microscope (Axioplan, Axiovision 4.1 software, Carl Zeiss, Toronto, ON). Serum cytokines were analyzed by multiplex bead immunassays (BioSource-Invitrogen, Carlsbad, CA) using a Luminex 100^{TM} instrument. Standard curves with defined cytokine concentrations were used to determine the concentrations of cytokines.

2.2.9. Statistical analysis

We used Prism version 4.0a (GraphPad Software Inc., SanDiego, CA, USA) for statistical analysis. A student's t-test (unpaired or paired) and one-way analysis of variance (ANOVA) determined statistically significant differences ($P< 0.05$)

between two and three groups respectively. The log-rank test was used to compare survival curves. All data are expressed as mean ± SEM.

2.3. Results

2.3.1. Requirement for PD-1 in RTE cells to prevent systemic autoimmunity

PD-1 is expressed on an increased proportion of T cells with an effector memory phenotype compared to a central memory phenotype ([30], and **Figure 2-1A**). To begin to examine if PD-1 may also have a role in newly generated T cells, we examined PD-1 expression on $CD24^{hi}$ peripheral T cells, as CD24 is a marker of RTE[20, 25]. A higher proportion of $CD24^{hi}$ T cells co-expressed PD-1 when compared to $CD24^{lo}$ T cells in four out of four wild type C57BL/6 (B6) mice tested (P=0.046**; Figure 2-1A** and **2-2**). To examine whether homeostatic activation of RTE might be under the control of PD-1, we transferred syngeneic B6 HSC into lymphocyte-deficient $Rag^{-/-}$ recipients and examined PD-1 expression in the newly generated T cells. We monitored the development of T cells by checking their appearance in the peripheral blood of all HSC recipients at different time points after HSC injection. In the majority of the recipients, T cells were detected only after 30 days post HSC injection (data not shown), as would be expected due to the time required to establish the HSC in the bone marrow followed by subsequent migration of precursors to the thymus and the ensuing maturation and selection processes. We found that PD-1 was expressed on a much higher proportion of T cells newly generated from HSC than on circulating or resident splenic T cells of adult B6 mice (P=0.009; **Figure 2-1B,C**). Consistent

with a key role for PD-1 in RTE, the frequency of T cells expressing PD-1 declined significantly over time post HSC transplantation (day 40 vs. 180, *P*=0.014; **Figure 2-1C**). In addition, a higher proportion of $CD24^{hi}$ T cells co-expressed PD-1 when compared to $CD24^{lo}$ T cells in all HSC recipients, both early and late post HSC transplantation (day 40, *P*=0.006; day 180, *P*=0.002; **Figure 2-1C**).

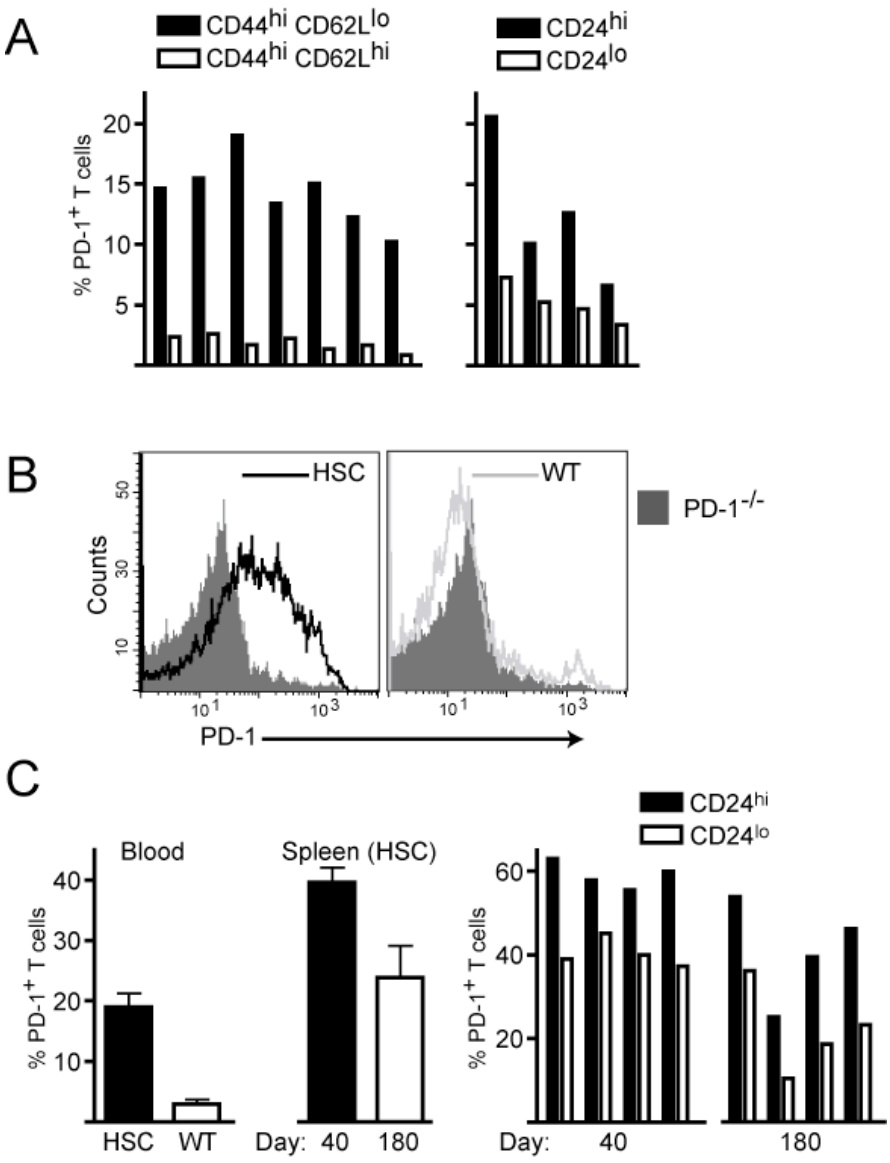


Figure 2-1. PD-1 is expressed on a high proportion of newly generated T cells. (A) *Left:* The frequency of PD-1^{+} cells in splenic T cells from individual (n=7) WT B6 mice gated on TCR^{+} and CD44^{hi} and CD62L^{lo} (effector memory) vs. CD62L^{hi} (central memory) cells. *Right:* The frequency of PD-1^{+} cells in splenic T cells from individual (n=4) WT B6 mice gated on TCR^{+} and CD24^{hi} vs. CD24^{lo} cells. (B, C) PD-1 expression on TCR^{+} gated cells from adult control B6 mice (WT) and Rag$^{-/-}$ mice 50-56 days or day 40 and 180 (where indicated) post WT HSC injection (HSC). (B) Representative analysis of T cells in the spleen. PD-1 staining of TCR^{+} gated spleen cells from PD-1$^{-/-}$ mice (filled grey) is also shown. (C) *Left:* Frequency of PD-1^{+} T cells in the blood and spleen; mean and SE (Blood: HSC, n=16; WT, n=3; Spleen: d40, n=7; d180, n=4). *Right:* The frequency, in individual mice (n=4), of splenic PD-1^{+} TCR^{+} cells that were either CD24^{hi} vs. CD24^{lo} at 40 or 180 days post HSC transplantation.

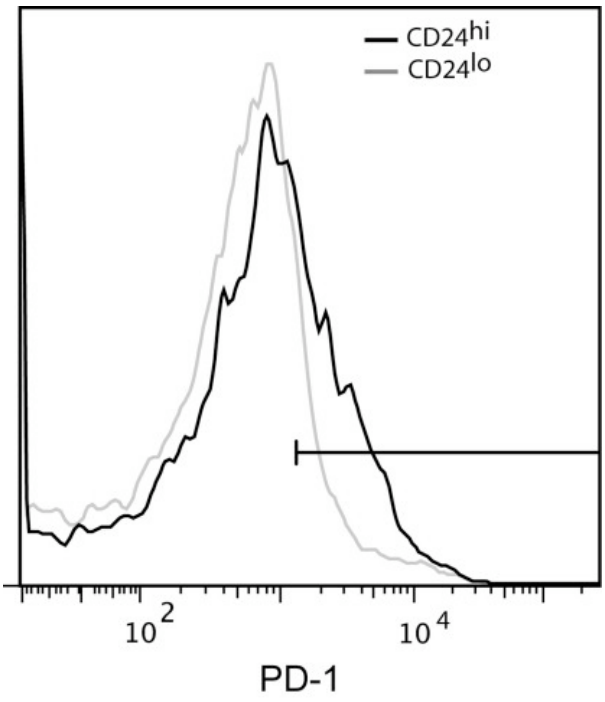


Figure 2-2. Increased frequency of PD-1 expressing T cells in recent thymic emigrants. A representative comparison of PD-1 expression on recent thymic emigrants ($CD24^{hi}$) vs. established ($CD24^{lo}$) T cells from spleens of WT B6 mice is shown. The horizontal line defines the region of PD-1 positive cells. Values for individual mice are shown in the right panel of Figure 2-1A.

To test whether PD-1 was needed to prevent LIP-induced autoimmunity in established T cells or specifically in RTE cells, we transferred mature PD-$1^{-/-}$ lymphocytes or their HSC precursors, respectively, into $Rag^{-/-}$ mice. Mature PD-$1^{-/-}$ lymphocytes did not induce any evident overt illness in lymphopenic animals **(Figure 2-3A)**. In stark contrast, animals given PD-$1^{-/-}$ HSC developed lethal multi-organ disease (100% of animals; n = 60 from more than ten experiments) within several days to at most a few weeks post appearance of T cells in the periphery, while, consistent with our previous data[29], none of the animals given wild type (WT) HSC developed disease **(Figure 2-3A)**. WT HSC could produce disease if the recipients were treated with mAbs targeting PD-1 or PD-L1 **(Figure 2-3B)**. Consistent with the idea that RTE have a unique disease inducing potential, PD-$1^{-/-}$ thymocytes but not resident splenic T cells could induce disease **(Figure 2-3A)**. Transfer of one million FACS sorted TCR^{+} $CD24^{low}$ splenic T cells from PD-$1^{-/-}$ mice was also unable to cause any overt disease when transferred into $Rag^{-/-}$ mice (days of disease free survival: >100 x 4). On physical examination **(Figure 2-3C)**, diseased animals were not thrifty and were characterized by cachexia, ruffled fur, scaly ulcerative skin and ocular lesions (exudate and reddened conjunctiva). Diseased animals showed lymphocytic infiltration in major organs such as the liver, kidney, pancreas, heart, eye **(Figure 2-3C, E)** as well as the lung and esophagus (data not shown), and rapidly lost weight **(Figure 2-3D)**. Infiltration in these tissues included CD4+ and CD8+ T cells in recipients of PD-$1^{-/-}$ but not WT HSC (one representative example is shown in **Figure 2-3C)**. Cytokines play an important role in inflammation and

autoimmunity. A number of serum cytokine/chemokines such as IFN-γ, IL-13, TNF-α, IP-10, MIG, MCP-1, VEGF in PD-$1^{-/-}$ HSC recipients were significantly increased compared to WT HSC recipients **(Figure 2-4A)**.

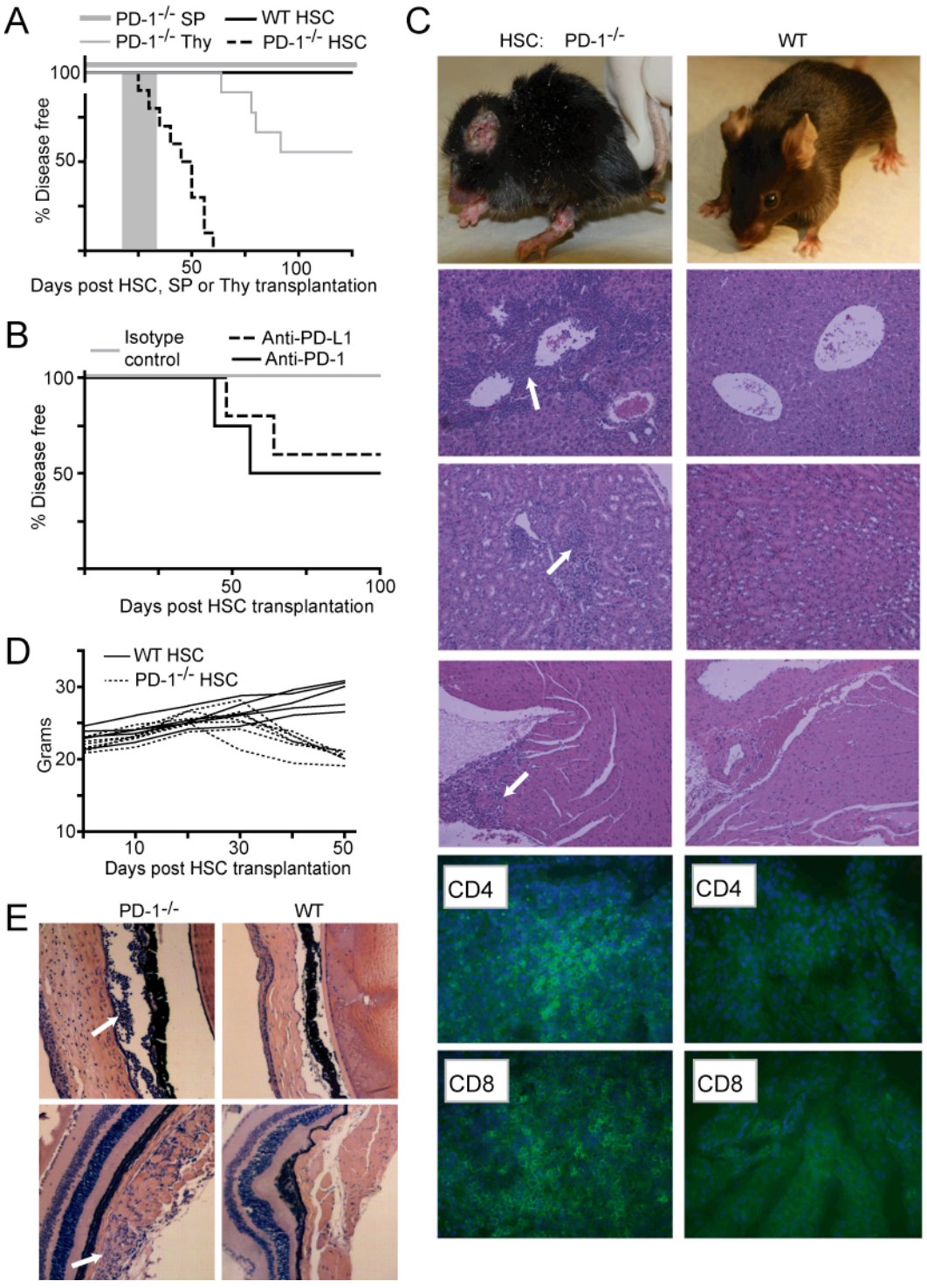


Figure 2-3. Newly generated but not established PD-1$^{-/-}$ T cells cause lethal multi-organ inflammatory disease in lymphopenia. (A) Adult Rag$^{-/-}$ mice were given HSC from PD-1$^{-/-}$ or WT fetuses (n=10 per group) or PD-1$^{-/-}$ thymocytes or splenocytes (Thy or SC; n=8-9 per group; both contained 5 x 10^{6} single positive T cells) and monitored for disease incidence. The grey rectangle indicates the range, in days, at which the first T cells were detected in the peripheral blood after HSC

injection. (B) Adult $Rag^{-/-}$ mice (n=4-5) were given WT HSC and treated with either anti-PD-1 or anti-PD-L1 beginning 30-35 days post HSC. (C) *Top:* Macroscopic pictures of representative recipients of $PD\text{-}1^{-/-}$ and WT HSC. Middle (six panels): Histology (hematoxylin and eosin staining; original magnification x100) of the liver, kidney and heart of recipients of $PD\text{-}1^{-/-}$ (left) and WT (right) HSC. Arrows point to areas of infiltration. *Lower* (four panels)*:* Immunofluorescence (original magnification x400) of pancreas from individual recipients of $PD\text{-}1^{-/-}$ and WT HSC (similar data was found for other organs). Blue: staining with the nuclear marker 4',6'-diamidino-2-phenylindole (DAPI); green: CD4 or CD8 staining. (D) Body weight measurements in individual recipients (n=5) of $PD\text{-}1^{-/-}$ or WT HSC. (E) Anterior uveitis and episcleritis were observed in recipients of $PD\text{-}1^{-/-}$ HSC. Histology (H&E) of eyes from the recipients of $PD\text{-}1^{-/-}$ or WT HSC 45 days post HSC.

Consistent with the multi-organ nature of the disease, there was no obvious oligoclonal expansion of T cells **(Figure 2-4B)**. Importantly, PD-1 and Rag double knockout recipients of $PD\text{-}1^{-/-}$ HSC **(Figure 2-4C)** also developed disease, showing that disease was not due to a response to potential histocompatibility antigens linked to the *Pdcd-1* locus.

In neonatal mice, previous studies suggested that CD8 +T cells traffic into tissues where they encounter self tissue antigens presented by MHC class I on parenchymal cells and become tolerant[31]. Should this trafficking into tissue be defective in our model, leading to a class I restricted autoimmunity within tissues, it would predict that MHC class I deficient recipients would be protected from disease. To test this possibility we transferred $PD\text{-}1^{-/-}$ HSC into recipients lacking MHC class I ($Rag^{-/-}$ $K^{b-/-}$ $D^{b-/-}$) and found that they were fully susceptible to disease **(Figure 2-4C)**. Thus, the disease could not be explained by an inability of CD8 T cells to traffic into adult tissues and become tolerant to MHC class I presented antigens on parenchymal tissues. We confirmed that disease is indeed

dependent on T cells by transferring PD-1$^{-/-}$ HSC into thymectomized Rag$^{-/-}$ recipients. All successfully thymectomized Rag$^{-/-}$ recipients were free from disease (n= 4; days of disease free survival of >100 x 4), whereas euthymic Rag$^{-/-}$ recipients developed disease (data not shown).

2.3.2. PD-1 deficiency does not prevent negative selection in the thymus

The high frequency of PD-1 positive cells within RTE and the lack of disease with established PD-1$^{-/-}$ T cells suggested that PD-1 expression by RTE was required to control their function. However, the autoimmunity caused by PD-1 deficient RTE could also reflect a requirement for PD-1 in negative selection in the thymus. While some studies have suggested a role for PD-1 in positive selection[32, 33], data assessing PD-1 in negative selection have been limited to the alloreactive 2C TCR transgenic model[14, 34]. However, PD-1 expression has been observed on a subset of double positive (DP) thymocytes undergoing negative selection using the physiological HY^{cd4} model system suggesting that PD-1 signaling may be important during deletional tolerance[35]. To examine the contribution of PD-1 to thymic negative selection, HY^{cd4} PD-1$^{-/-}$ mice were generated. There were no differences in the CD4 by CD8 profiles in male or female HY^{cd4} mice comparing PD-1$^{-/-}$ mice to their PD-1 expressing counterparts **(Figure 2-5A)**. Furthermore, there was a similar reduction in the absolute number of T3.70^{+} DP and CD8 single positive (SP8) thymocytes in HY^{cd4} male mice compared to female mice regardless of PD-1 expression, indicating that potentially auto-reactive thymocytes were being deleted similarly. Equivalent

negative selection in the absence of PD-1 was further confirmed by a similar increase in the activation of caspase-3 in $T3.70^{+}$ DP thymocytes from HY^{cd4} male mice compared to female mice in the presence or absence of PD-1 **(Figure 2-5B)**. To evaluate the effect of PD-1 deficiency on negative selection of CD4 +T cells, we generated Marilyn (anti-HY/I-A^{b})[26] PD-$1^{-/-}$ mice. Consistent with the data on CD8+ T cell selection, Marilyn thymocytes underwent a similar level of negative selection in male mice in the presence vs. absence of PD-1 **(Figure 2-5C,D)**. Taken together, these data strongly suggest that the autoimmunity observed in the above experiments is not the result of a defect in thymic negative selection when PD-$1^{-/-}$ is absent during T cell development.

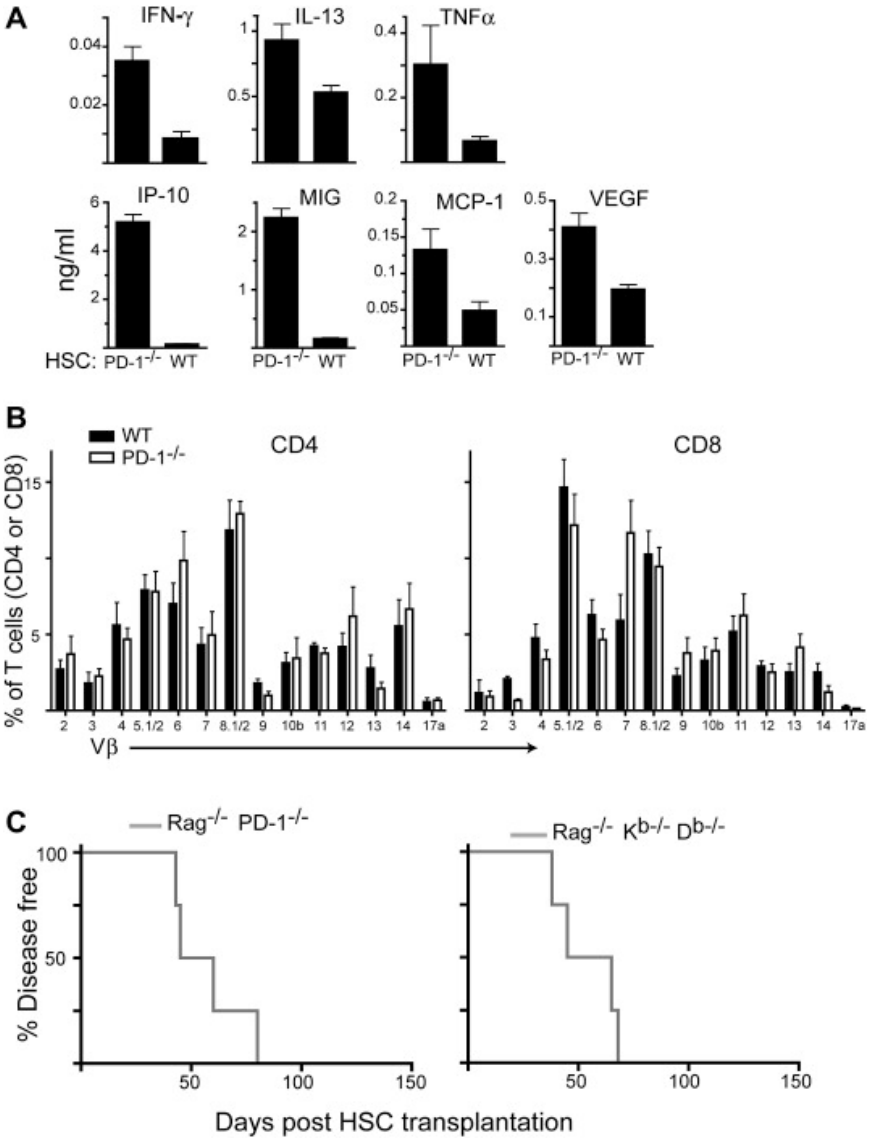


Figure 2-4. PD-1$^{-/-}$ HSC cause increased circulating cytokines/chemokines without oligoclonal expansion and disease does not depend on host PD-1 or MHC class I. (A) Serum cytokines were quantified from Rag$^{-/-}$ recipients of PD-1$^{-/-}$ or WT (n=4 per group) HSC (50 days post HSC). The serum cytokine concentrations of IFN-γ, IL-13, TNF-α, IP-10, MIG, MCP-1, VEGF were significantly ($P< 0.01$) higher in PD-1$^{-/-}$ than WT HSC recipients. Serum concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17 were not significantly ($P> 0.05$) different between PD-1$^{-/-}$ and WT HSC recipients (not shown). (B) CD4^{+} and CD8^{+} T cells from spleens of recipients of PD-1$^{-/-}$ or WT HSC (n=4 per group) were analyzed 50 days post HSC for TCR diversity. (C) *Left:* Adult Rag$^{-/-}$ PD-1$^{-/-}$ (n=4) mice were given PD-1$^{-/-}$ HSC. *Right:* Adult Rag$^{-/-}$ K$^{b-/-}$ D$^{b-/-}$ (n=4) mice were given PD-1$^{-/-}$ HSC.

2.3.3. Control of lymphopenia driven activation and autoimmunity through competition

Lymphopenia driven activation results from reduced competition for resources (MHC on dendritic cells and cytokines) that occurs within lymphopenic lymph nodes, and can be blocked by reducing lymph node stroma[5] or by increasing the number of competitor T cells[1]; polyclonal naïve T cells compete with each other to bind with self-MHC/peptide complexes and cytokines for their survival and homeostatic proliferation[36]. Therefore, we further tested our conclusion that PD-1 was needed to control homeostatic activation of RTE cells by using two separate approaches to increase the competition for resources. We used hosts that have greatly reduced lymph node stroma when compared to the adult $Rag^{-/-}$ recipients and lymph node sufficient $Rag^{-/-}$ hosts with added competitor T cells. To test whether disease could be inhibited by provision of a competitor (bystander) T cell population[3] we co-transfered WT stem cells with PD-$1^{-/-}$ stem cells. Since a hallmark of lymphopenia driven activation is the conversion of naïve T cells into memory cells, we also examined in these chimeras the frequency of PD-$1^{-/-}$ T cells with a memory phenotype. We found that 40% or more WT circulating T cells was enough to reduce the frequency of effector memory phenotype T cells (naïve and central memory T cell proportions increased) and prevent disease development **(Figure 2-6 A, B and Table 2-1).**

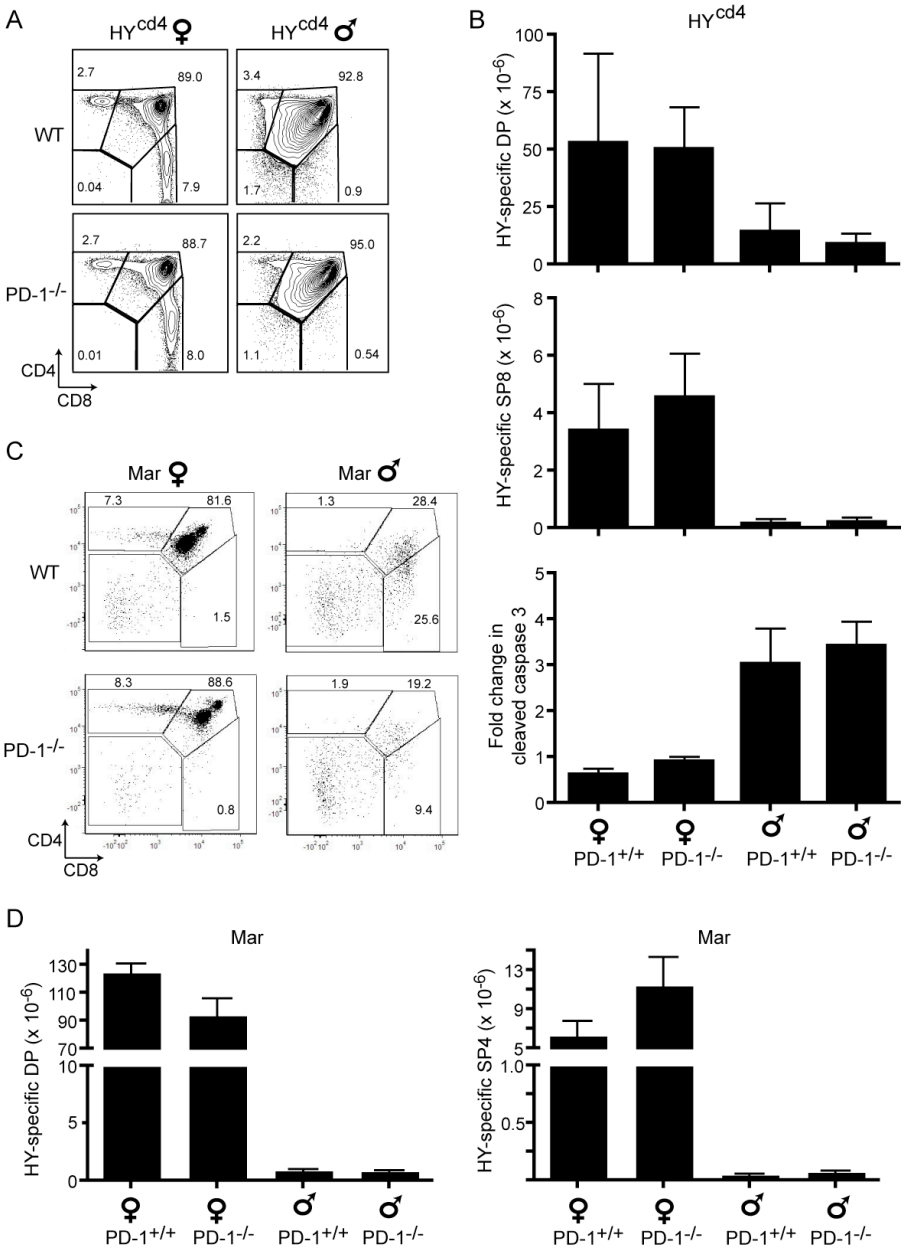


Figure 2-5. PD-1 deficiency does not impair negative selection. (A) Representative CD4 by CD8 profiles of T3.70^{+} thymocytes from HY^{cd4} female vs. male mice that are WT or PD-1$^{-/-}$. (B) Absolute numbers of T3.70^{+} of DP and SP8 thymocytes (Females, HY^{cd4} PD-1$^{+/+}$: n=15, HY^{cd4} PD-1$^{-/-}$: n=3, Males, HY^{cd4} PD-1$^{+/+}$: n=20, HY^{cd4} PD-1$^{-/-}$: n=6), top and middle panels respectively. *Bottom panel:* Fold increase in percentage of cleaved caspase-3 T3.70^{+} DP thymocytes from indicated strain relative to percent cleaved caspase-3 DP thymocytes from C57BL/6 mice (HY^{cd4}F: n=3, HY^{cd4}F PD-1$^{-/-}$: n=5, HY^{cd4}M: n=4, HY^{cd4}M PD-1$^{-/-}$: n=6). (C) Representative CD4 by CD8 profiles of TCR gated thymocytes from Marilyn (Mar) female vs. male mice that are WT or PD-1$^{-/-}$. (D) Absolute

numbers of TCR^+ of DP and SP4 thymocytes (Females, Mar PD-$1^{+/+}$: n=3, Mar PD-$1^{-/-}$: n=4, Males, Mar PD-$1^{+/+}$: n=3, Mar PD-$1^{-/-}$: n=4).

We next tested whether disease could be inhibited by reducing the recipient's lymphoid stroma rather than providing competitor T cells. Two of the recipients with a relative deficiency in lymph node stroma were lymphotoxin deficient ($LT\alpha^{-/-}$) mouse[37], which lacks most secondary lymphoid tissue except spleen, and adult Rag and common cytokine receptor gamma chain (γc) double knockout recipients. Although $LT\alpha^{-/-}$ mice lack lymph nodes, they have normal development of T cells and B cells. $Rag^{-/-}$ $\gamma c^{-/-}$ mice lack a functional IL-7R, substantially blocking lymph node development[38]. Adult $Rag^{-/-}$ $\gamma c^{-/-}$ recipients were completely resistant to disease caused by PD-$1^{-/-}$ HSC transfer **(Figure 2-6C)**, and lacked or had reduced infiltration in major organs **(Figure 2-7A)**. Lymph nodes were generally absent in $Rag^{-/-}$ $\gamma c^{-/-}$ recipients, and the few lymph nodes that were found were greatly reduced in size compared to $Rag^{-/-}$ recipients, even 175 days after receipt of HSC (as expected[38]; data not shown). Lack of disease in $Rag^{-/-}$ $\gamma c^{-/-}$ recipients suggested that inhibition of lymphopenia driven activation of RTE, due to a paucity of lymph node stroma, was dominant over increased homeostatic activation that may result from the loss of IL-7 mediated negative feedback on dendritic cells[39]. Lack of disease was not due to an inability to develop a functional T cell response post HSC transfer to lymph node deficient recipients, as we have previously shown that such mice robustly reject even well healed skin grafts[40]. In addition, lack of disease was not due to a resistance of $Rag^{-/-}$ $\gamma c^{-/-}$ tissues to immune attack; transferred splenocytes from diseased

animals (PD-1$^{-/-}$ HSC to Rag$^{-/-}$ recipients) caused disease in both Rag$^{-/-}$ and Rag$^{-/-}$ γc$^{-/-}$ secondary recipients **(Figure 2-6D and Figure 2-7B)**, consistent with effector function and proliferation of memory T cells being independent of lymphoid organs. In contrast, splenocytes from disease free mice (PD-1$^{-/-}$ HSC to Rag$^{-/-}$ γc$^{-/-}$ recipients) did not induce disease in either type of recipient **(Figure 2-6D)**. We next examined markers of activation and proliferation in Rag$^{-/-}$ vs. Rag$^{-/-}$ γc$^{-/-}$ recipients of WT vs. PD-1$^{-/-}$ HSC. BrdU labeling demonstrated that T cells of PD-1$^{-/-}$ HSC recipients undergo much more proliferation than recipients of WT HSC **(Figure 2-6 E)**. Rag$^{-/-}$ γc$^{-/-}$ recipients had significantly lower numbers of splenic T cells, and a lower frequency of memory phenotype and BrdU positive T cells than Rag$^{-/-}$ recipients **(Figure 2-6 E)**. Remarkably, T cells of Rag$^{-/-}$ recipients of PD-1$^{-/-}$ HSC were almost universally effector memory cells phenotypically, unlike T cells of recipients of WT HSC or Rag$^{-/-}$ γc$^{-/-}$ recipients of PD-1$^{-/-}$ HSC **(Figure 2-6 F).**

While disease resistance in Rag$^{-/-}$ γc$^{-/-}$ recipients was consistent with a requirement for lymph node stroma in disease induction, defects in innate immune cells of these recipients might instead have prevented disease. For example, host NK cells might be required for autoimmunity. To test these possibilities, we created reciprocal bone marrow chimeras between Rag$^{-/-}$ and Rag$^{-/-}$ γc$^{-/-}$ recipients prior to transfer of PD-1$^{-/-}$ HSC. Lack of disease was not due to potential defects in Rag$^{-/-}$ γc$^{-/-}$ innate cells, as protection from disease required γc deficiency in host radioresistant cells rather than a deficiency of γc in the bone marrow cells **(Figure**

2-8 and 2-9). The presence of disease in reciprocal bone marrow chimeras did not correlate with the bone marrow's capacity to produce NK cells; GFP $Rag^{-/-}$ to $Rag^{-/-}$ $\gamma c^{-/-}$ chimeras had bone marrow capable of NK cell production but nevertheless remained disease free, while $Rag^{-/-}$ $\gamma c^{-/-}$ to GFP $Rag^{-/-}$ chimeras had bone marrow incapable of producing NK cells and yet rapidly succumbed to disease. Furthermore, $Rag^{-/-}$ recipients chronically depleted of NK cells were also fully susceptible to disease **(Figure 2-8C)**.

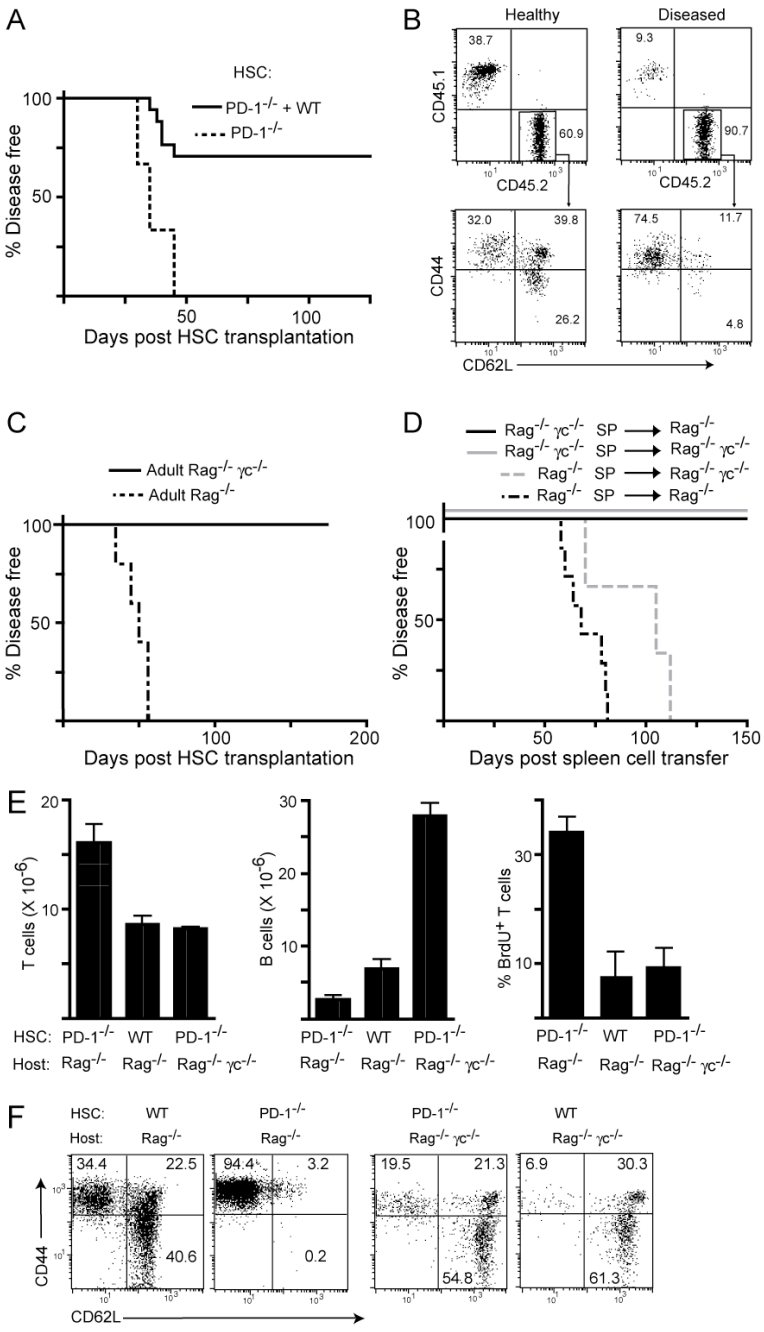


Figure 2-6. Protection from lymphopenia driven activation and disease by WT competitor HSC or by using IL-2Rg deficient recipients. (A) CD45.2 PD-1$^{-/-}$ and CD45.1 WT HSC at a 1:1 ratio were given to adult Rag$^{-/-}$ mice (n=13). Controls were given PD-1$^{-/-}$ cells alone. (B) *Top:* Representative flow cytometry gated on TCRβ^{+} cells in blood from healthy vs. diseased recipients of a mixture of CD45.2 PD-1$^{-/-}$ + CD45.1 WT HSC; 45 days post HSC. *Bottom:* Representative flow cytometry (gated on TCRb^{+} CD45.2^{+} cells) in recipients of PD-1$^{-/-}$ plus WT HSC 45 days post HSC (See also supplementary Table 1). **(**C) Adult Rag$^{-/-}$ γc$^{-/-}$ mice (n=7) or adult Rag$^{-/-}$ (n=5) mice were given PD-1$^{-/-}$ HSC. Also see

supplemental Figure 1 for histology. **(**D) Disease incidence post adoptive transfer of splenocytes (SP) from diseased mice (PD-1$^{-/-}$ HSC → Rag$^{-/-}$; abbreviated as Rag$^{-/-}$ SP) or healthy mice (PD-1$^{-/-}$ HSC → Rag$^{-/-}$ γc$^{-/-}$; abbreviated as Rag$^{-/-}$ γc$^{-/-}$ SP) into secondary Adult Rag$^{-/-}$ γc$^{-/-}$ (n=3 per group) or Rag$^{-/-}$ recipients (SP from diseased mice, n=7; SP from healthy mice, n=4). Also see supplemental Figure 1 for histology. (E) Enumeration of splenic T cells and B cells in adult Rag$^{-/-}$ recipients of PD-1$^{-/-}$ or WT HSC and adult Rag$^{-/-}$ γc$^{-/-}$ recipients of PD-1$^{-/-}$ HSC (splenic T cells in Rag$^{-/-}$ recipients of PD-1$^{-/-}$ were increased vs. WT HSC, n=5, and vs. Rag$^{-/-}$ γc$^{-/-}$ recipients of PD-1$^{-/-}$ HSC, n=3, $P< 0.01$; splenic B cells were significantly lower, $P< 0.05$, than those of Rag$^{-/-}$ recipients of WT HSC or Rag$^{-/-}$ γc$^{-/-}$ recipients of PD-1$^{-/-}$ HSC). The percent BrdU^{+} T cells in spleen of Rag$^{-/-}$ recipients of PD-1$^{-/-}$ (n=3) HSC was significantly higher than that of Rag$^{-/-}$ recipients of WT HSC (n=3; $P< 0.01$) and Rag$^{-/-}$ γc$^{-/-}$ recipients of PD-1$^{-/-}$ HSC (n=3; $P< 0.01$) examined 50 days post HSC. (F) Representative flow cytometry showing the frequency of 'memory' T cells (gated on TCRb^{+} cells) in peripheral blood. The percent effector memory T cells (CD44^{hi} CD62L^{lo}) in Rag$^{-/-}$ recipients of PD-1$^{-/-}$ HSC (n=15; 92.28 ± 0.83) was significantly higher ($P< 0.0001$) than that of Rag$^{-/-}$ recipients of WT HSC (n=10; 58.35 ± 6.8) and Rag$^{-/-}$ γc$^{-/-}$ recipients of PD-1$^{-/-}$ HSC (40.08± 6.9) and Rag$^{-/-}$ γc$^{-/-}$ recipients of WT HSC (n=3; 7.8 ± 0.49), analyzed 40-50 days post HSC injection.

Table 2-1: Frequency of WT T cells required to prevent disease

Mouse number	% CD45.1 of TCRβ$^+$ cells (WT T cells)	% CD45.2 of TCRβ$^+$ cells (PD-1$^{-/-}$ T cells)	Outcome
1	11.76	87.80	Sick
2	24.15	75.00	Sick
3	20.99	78.89	Sick
4	9.27	90.65	Sick
5	ND[a]	ND	Sick
6	38.72	60.85	Disease free
7	44.03	55.61	Disease free
8	61.75	37.51	Disease free
9	67.18	32.61	Disease free
10	98.68	0.05	Disease free
11	38.48	61.17	Disease free
12	38.95	60.28	Disease free
13	56.87	42.69	Disease free

Frequency of WT vs. PD-1$^{-/-}$ T cells determined by flow cytometry in blood from healthy vs. diseased recipients of a mixture of CD45.2 PD-1$^{-/-}$ + CD45.1 WT HSC; 45 days post HSC (see also Fig. 5A,B).
[a]ND (not done); this mouse was euthanized prior to the time of analysis due to the severity of illness.

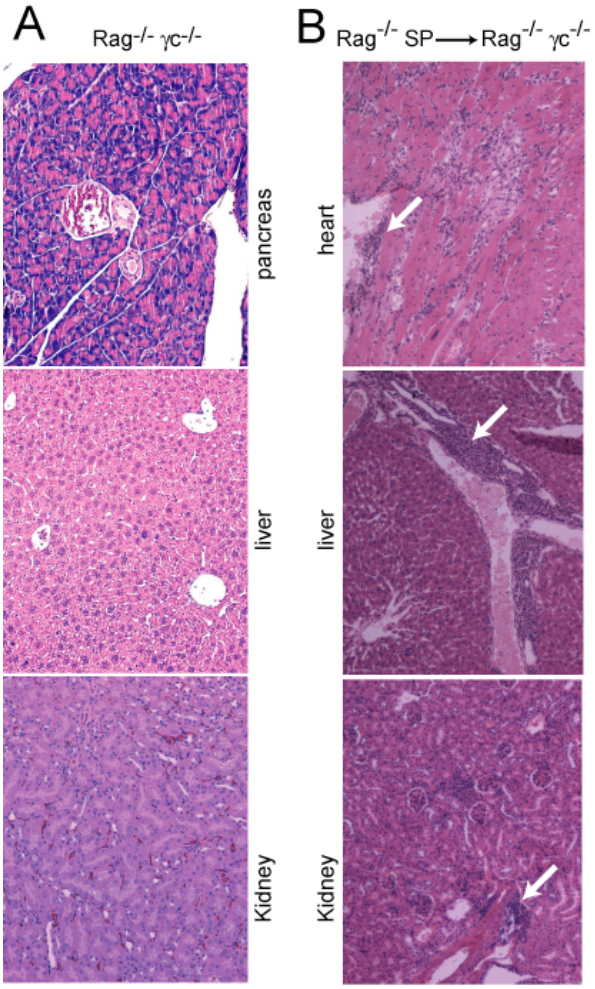


Figure 2-7. Loss of host γ-chain protects from the generation of disease, but not sensitivity to the effectors of disease. H and E staining (A) of pancreas, liver and kidney of $Rag^{-/-}$ $\gamma c^{-/-}$ recipients of PD-$1^{-/-}$ HSC; day 175 post HSC. (B) Lymphocytic infiltration indicated by white arrows in heart, liver and kidney of adoptively transferred $Rag^{-/-}$ $\gamma c^{-/-}$ recipients ($Rag^{-/-}$ SP → $Rag^{-/-}$ $\gamma c^{-/-}$) shown in Figure 5D. Representative sections 120 days post splenocyte transfer.

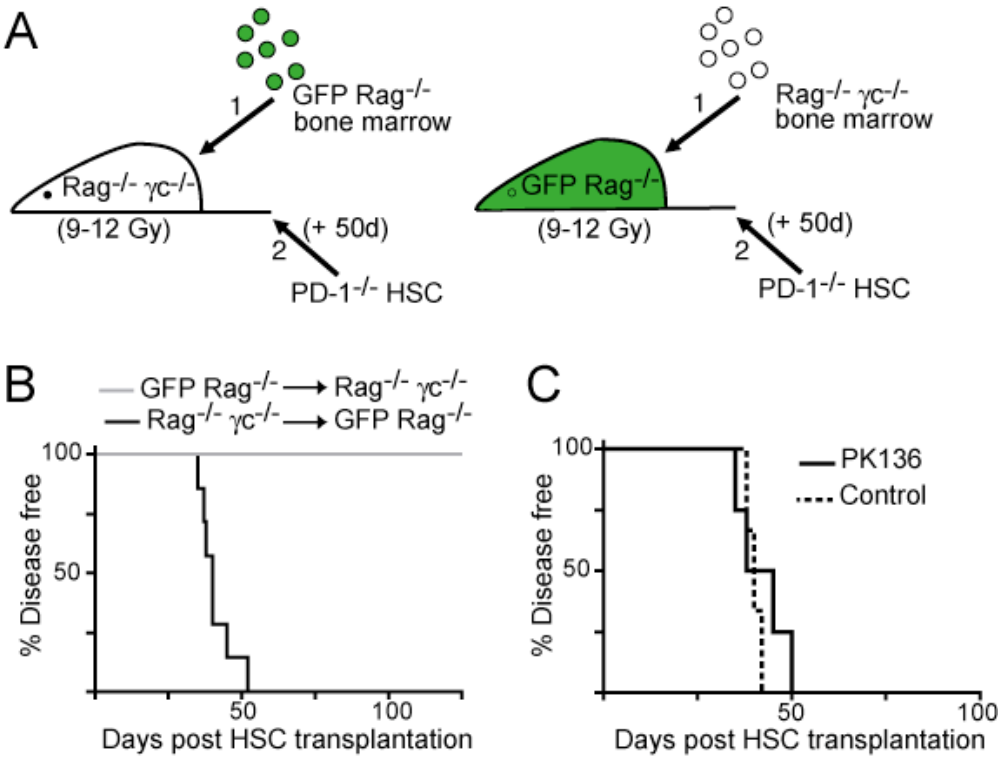


Figure 2-8. Loss of host IL-2Rγ-chain protects from disease; role of host hematopoietic cells vs. radioresistant cells (A) *Top:* Experimental design for reciprocal bone marrow chimeras used in A-C. Adult $Rag^{-/-}\gamma c^{-/-}$ (n=6) or GFP $Rag^{-/-}$ recipients (n=7) were lethally irradiated and given bone marrow cells from GFP $Rag^{-/-}$ or $Rag^{-/-}\gamma c^{-/-}$ mice respectively. 50 days later they were given PD-$1^{-/-}$ HSC. See also supplementary Figure 2 for characterization of bone marrow chimeras by flow cytometry. (B) Disease incidence in bone marrow chimeras (see (A)) given PD-$1^{-/-}$ HSC. (C) Failure of NK cell depletion to prevent disease. Adult $Rag^{-/-}$ recipients were treated with NK cell depleting antibody (PK136) and were given PD-$1^{-/-}$ HSC (n = 4).

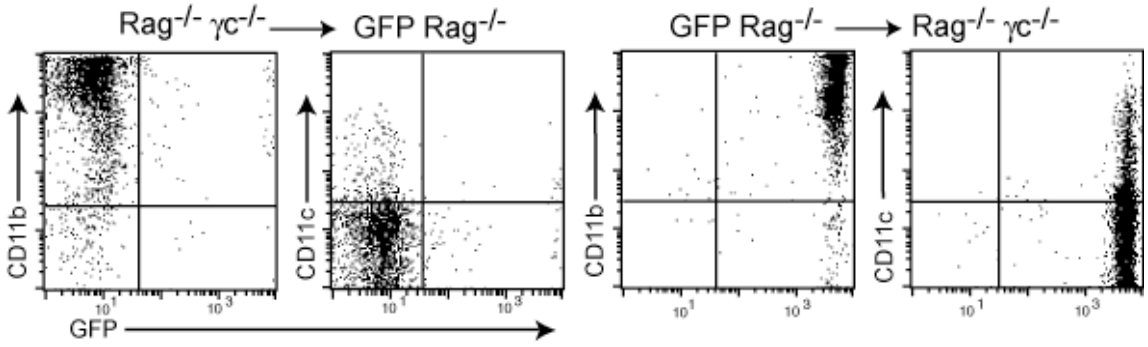


Figure 2-9. Loss of host γ-chain protects from disease; role of host hematopoietic cells vs. radioresistant cells. Representative flow cytometry of peripheral blood from reciprocal bone marrow chimeras (used in Figure 6) 50 days post bone marrow transplantation (prior to receipt of PD-1$^{-/-}$ HSC).

Similar to the Rag$^{-/-}$ γc$^{-/-}$ recipients, disease was absent in irradiated LTα$^{-/-}$ recipients. In contrast, disease was present in irradiated WT (LTα$^{+/+}$) mice **(Figure 2-10A).** Moreover, the frequency of memory phenotype cells in irradiated LTα$^{+/+}$ recipients was significantly higher than those of irradiated LTα$^{-/-}$ recipients **(Figure 2-10B)**. Together these data indicated that reducing lymphoid stroma could block disease, and suggested a possible explanation for the paradox that PD-1 deficient mice themselves do not succumb to a rapidly lethal autoimmunity. The ability to prevent disease by reducing lymphoid stroma, suggests that PD-1 deficient mice might be spared from strong autoimmunity because their T cell repertoire is generated during the fetal/neonatal period when lymphoid stroma is very limited. To test the role of recipient age, we compared transfer of PD-1$^{-/-}$ HSC into neonatal vs. adult Rag$^{-/-}$ mice. Unlike adult recipients, nearly all of the neonatal recipients remained disease free **(Figure 2-10C)**. The frequency of memory phenotype T cells was also greatly reduced in all

of the neonatal recipients that remained disease free **(Figure 2-10D)**. Taken together these data strongly suggested that a systemic lack of tolerance with PD-1$^{-/-}$ HSC occurred due to reduced competition and the presence of adequate lymphoid tissue to support lymphopenia driven T cell activation[3] of RTE.

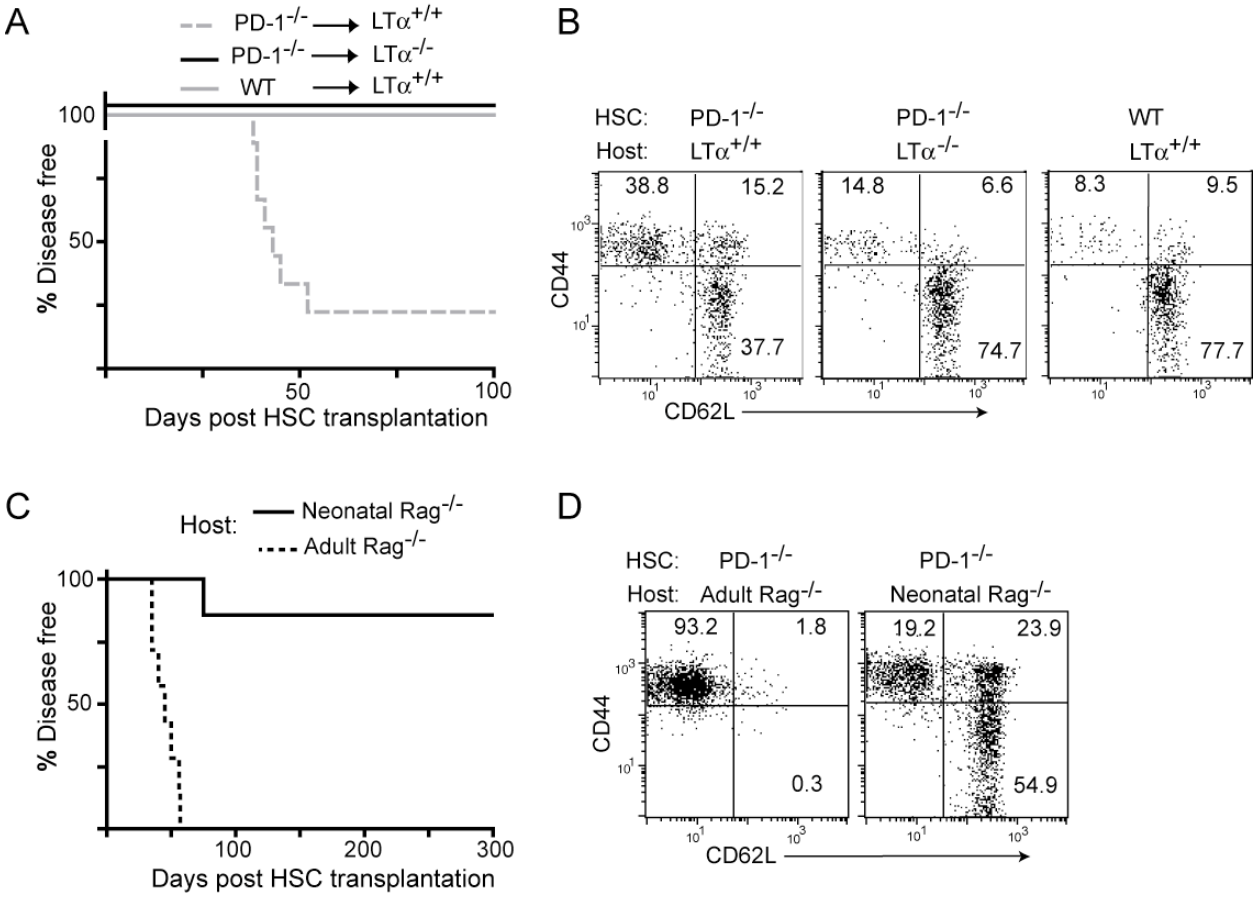


Fig. 2-10. Resistance to disease and lymphopenia driven activation in adult adult LTα$^{-/-}$ and neonatal Rag$^{-/-}$ recipients. (A) Lethally irradiated LTα$^{+/+}$ (PD-1$^{+/+}$ or PD-1$^{-/-}$; n=8) or LTα$^{-/-}$ (n=5) mice were given PD-1$^{-/-}$ HSC. Controls were irradiated WT (n=5) mice that were given WT HSC. (B) Representative flow cytometry showing the frequency of 'memory' T cells (gated on TCRβ^{+} cells) in peripheral blood of mice shown in A. The frequency of CD44^{hi} CD62L^{lo} T cells in LTα$^{+/+}$ (n=8; 41.8± 4.5) recipients of PD-1$^{-/-}$ HSC was significantly higher (*P*<

0.01) than that of LTα$^{-/-}$ recipients (n= 5; 12.3 ± 3.1) or control (n=5; 7.0 ± 0.45) recipients that were given WT HSC. (C) Day 1 Rag$^{-/-}$ neonates (n=7) or adult Rag$^{-/-}$ (n=7) mice were given PD-1$^{-/-}$ HSC and development of disease monitored. (D) The percent CD44^{hi} CD62L^{lo} T cells in neonates (n=7; 30.8± 5.7) was significantly lower (P< 0.0001) than that of adult recipients (n=7; 89.5 ± 1.5) analyzed 40-50 days post PD-1$^{-/-}$ HSC.

2.4. Discussion

Numerous co-inhibitory pathways have been identified and while each may contribute to self-tolerance, their specific functions are almost certainly unique[41]. Identification of their unique functions should provide more refined strategies for tolerance induction in autoimmunity and transplantation. Previous studies have suggested a relatively limited role for PD-1 in self tolerance, with a deficiency in PD-1 signaling leading to tissue specific autoimmunity, targeting a single tissue[15, 42, 43], or a variable late life lupus like disease[14]. In the current study, we found that PD-1 deficiency can lead to a profound loss of self tolerance, leading to rapid lethality associated with lymphocyte infiltration of many organs. This widespread loss of self tolerance with PD-1 deficient cells, suggests that PD-1's most critical function is in the control of the autoimmune potential of newly generated T cells in the setting of lymphopenia. We found no evidence for a reduced ability of PD-1$^{-/-}$ thymocytes, either those generating MHC class I or II restricted T cells, to undergo negative selection to conventional self peptide/MHC complexes in the thymus. These data further emphasize that control of tolerance by PD-1 is most likely to occur in the periphery. Welsh and colleagues recently showed that PD-1 is highly expressed on a subpopulation of established peripheral CD8+ T cells transferred into lymphopenic hosts, and was associated with apoptosis and

reduced their effector output[12]. They also suggested that PD-1 may have a key role in preventing lymphopenia driven autoimmune disease, although this hypothesis was not examined. Some support for this hypothesis has come from recent studies showing CD4 T cells in PD-L1 deficient lymphopenic hosts cause autoimmunity in the lung[43]. However, this relatively limited autoimmunity was only demonstrated in the artificial setting of a complete deficiency of $Foxp3^+$ Treg cells (Foxp3 depleted cells), raising doubts about the physiologic significance. Our data indicated that PD-1 is critical to prevent a much more generalized and rapid autoimmune disease induced by lymphopenia, but that this function was critical for RTE rather than established peripheral T cells. Importantly, the RTE generated multi-organ autoimmunity we observed occurred without any artificial depletion of specific cell populations such as Treg cells. Together with the inability of established peripheral PD-$1^{-/-}$ T cells to cause the disease, the ability of thymocytes to cause disease and the presence of a thymus dependent disease within several days of the appearance of PD-$1^{-/-}$ T cells in periphery post HSC transplantation, indicated that RTE were responsible for disease. These data also suggest that confirmation of an intact PD-1 pathway may be needed in the setting of lymphoablation and bone marrow transplantation, particularly with autologous hematopoietic stem cell transplants used to treat autoimmunity[44-47], where alterations in this pathway can be expected.

Suggestive of a potential role for RTE in autoimmunity, a recent report implicated RTE as a major component in the infiltrates of human autoimmune thyroiditis[48].

Furthermore, studies showing thymocytes or neonatal spleen cells but not adult spleen cells are able to cause oophoritis and gastritis in lymphopenic mice[19] are consistent with a greater autoimmune potential in RTE. RTE have a particular importance in a number of settings where homeostatic expansion can occur. RTE are critical to regeneration of the T cell repertoire following lymphodepletion caused by viral infections or intentionally generated by conditioning therapies used in hematopoietic stem cell transplantation. Thus, homeostatic activation of RTE and the potential for autoimmunity to be triggered by immune reconstitution post-lymphopenia have been major concerns. However, these concerns have been tempered by observations suggesting that RTE remain immature and deficient in effector function for a significant period after migration into the periphery[20-22]. We found that RTE are not functionally impotent but instead harbor a heightened disease-inducing potential that is under the control of negative regulatory pathways. It has also been shown that the interactions between PD-1 and PD-L1 influences CD8+T cells early after antigen encounter[49]. CD8+ T cells rapidly up regulated PD-1 expression upon encounter with antigen, when antigen was expressed as neo-self antigen but not as a microbial antigen. Blocking the PD-1/PD-L1 pathway with either anti-PD-1 or anti-PD-L1 or using PD-$1^{-/-}$ T cells during neo-self-antigen encounter prevented tolerance[49, 50]. Thus, PD-1 may have an important role in controlling the early T cell response to self-antigen and in the response to homeostatic signals in RTE.

Our data, showing that approximately 95% of T cells phenotypically become effector memory cells when they lack PD-1 and go through the RTE stage in a lymphopenic host, strongly suggests that the disease is driven in a polyclonal fashion. The autoimmunity may in addition require autoantigen recognition, a question that could be addressed using the elegant approach employed recently for disease due to CTLA-4 deficiency[51]. Furthermore, the ability to inhibit disease with competitor HSC or by reduction of lymphoid stroma is associated with greatly reduced polyclonal activation. Mechanistically, the lack of PD-1 in LIP might have changed the Treg:Teffector ratio by increasing Teffectors or because of a reduced ability to generate induced Treg cells[43, 52], a possibility we are investigating.

Homeostatic activation is conventionally considered to be activation resulting from reduced competition for resources (binding to self MHC, cytokines from APCs/Stroma etc.)[53]. However, the lack of autoimmunity and LIP in $Rag^{-/-}$ $\gamma c^{-/-}$ recipients of $PD\text{-}1^{-/-}$ HSC initially seemed surprising given that previous studies have shown CD8+ T cells can have enhanced LIP in $\gamma c^{-/-}$ recipients[6, 7]; enhanced LIP was attributed to the lack of NK cells in these mice and hence reduced competition for IL-15 between pre-existing central memory CD8+ T cells and NK cells[7]. However, our studies specifically assessed the requirement for γc in autoimmunity and LIP of newly generated T cells (lacking any pre-existing central memory cells). In the setting of newly generated T cells, LIP and autoimmunity was clearly dependent on the presence of γc. Our data are

consistent with studies showing that some forms of LIP are highly dependent on lymph node stroma[2-5], and lymph nodes are almost completely absent in $Rag^{-/-}$ $\gamma c^{-/-}$ [54] recipients that we found were protected from disease. In addition, the rules of LIP may be different for T cells that are self antigen specific. Consistent with this possibility a recent study showed that a self antigen specific transgenic CD8 T cell underwent substantial LIP in $\gamma c^{-/-}$ but not $Rag^{-/-}$ $\gamma c^{-/-}$ recipients[55]. Thus, LIP rules defined by experiments done in $\gamma c^{-/-}$ recipients do not necessarily hold for studies done in completely T cell deficient $Rag^{-/-}$ $\gamma c^{-/-}$ recipients.

The relative lack of LIP and autoimmunity in lymph node deficient recipients and in neonates raises the question of how precisely to define lymphopenia. Lymphopenia is a relative deficiency in lymphocytes, but relative to what exactly? For example, the neonatal period in mice is often discussed as being a naturally lymphopenic period relative to the adult stage. Supporting this idea, neonates but not adults support homeostatic proliferation[18]. However, the most relevant definition of lymphopenia might be a low ratio of lymphocytes to available lymphoid stroma. From this viewpoint, neonates, having both low T cell numbers and a paucity of lymphoid stroma are not highly lymphopenic, at least not in comparison adult immunodeficient mice. Consistent with this view, the homeostatic proliferation seen in neonates[18] occurs for a much smaller fraction of input T cells than when T cells are transferred to $Rag^{-/-}$ adult recipients[7]. Our data in neonatal $Rag^{-/-}$ recipients of PD-$1^{-/-}$ HSC suggest that the T cell repertoire that is naturally generated in a PD-$1^{-/-}$ animal (during the fetal/neonatal period), in the

relative absence of LIP, is able to develop sufficient peripheral tolerance to prevent rapid multi-organ autoimmunity.

Thus, a primary role for PD-1 is in the control of homeostatic activation, a process most strong in RTE cells as revealed during states of lymphopenia[56]. This perspective suggests a model whereby the relatively mild autoimmunity seen in PD-1$^{-/-}$ mice may be explained by a partial loss in the ability to control homeostatic activation; the restricted lymphoid environment present in the neonatal period, when T cell homeostasis is established, prevents a complete loss of control. As the animal grows, and the lymphoid stroma increases, newly generated PD-1$^{-/-}$ T cells are controlled by the tolerant population of T cells already present within the lymphoid tissue, a feedback mechanism. Successful feedback may involve generation of the appropriate balance of regulatory and effector cells[43, 57] when the first waves of T cells seed the periphery. The natural paucity of lymphoid stroma early in life that appears to control homeostatic activation of PD-1 deficient T cells, also raises an important question for understanding the role of co-inhibitors. Future studies should address how the absence of CTLA-4, but not PD-1, signaling is able overcome the restricted lymphoid environment of the neonatal period to generate lethal multi-organ autoimmune disease.

Contribution

I performed almost all of the experiments and analyzed the data except the HY^{cd4} studies.

2.5. References

1. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. Nat Rev Immunol 2009; 9:823-32.
2. Dai Z, Lakkis FG. Cutting edge: Secondary lymphoid organs are essential for maintaining the CD4, but not CD8, naive T cell pool. J Immunol 2001; 167:6711-5.
3. Dummer W, Ernst B, LeRoy E, Lee D, Surh C. Autologous regulation of naive T cell homeostasis within the T cell compartment. J Immunol 2001; 166:2460-8.
4. Ploix C, Lo D, Carson MJ. A ligand for the chemokine receptor CCR7 can influence the homeostatic proliferation of CD4 T cells and progression of autoimmunity. J Immunol 2001; 167:6724-30.
5. Do JS, Min B. Differential requirements of MHC and of DCs for endogenous proliferation of different T-cell subsets in vivo. Proc Natl Acad Sci U S A 2009; 106:20394-8.
6. Ramsey C, Rubinstein MP, Kim DM, Cho JH, Sprent J, Surh CD. The lymphopenic environment of CD132 (common gamma-chain)-deficient hosts elicits rapid homeostatic proliferation of naive T cells via IL-15. J Immunol 2008; 180:5320-6.
7. Zecher D, Li Q, Oberbarnscheidt MH, Demetris AJ, Shlomchik WD, Rothstein DM, et al. NK cells delay allograft rejection in lymphopenic hosts by downregulating the homeostatic proliferation of CD8+ T cells. J Immunol 2010; 184:6649-57.
8. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 2004; 117:265-77.
9. Le Campion A, Gagnerault MC, Auffray C, Becourt C, Poitrasson-Riviere M, Lallemand E, et al. Lymphopenia-induced spontaneous T-cell proliferation as a cofactor for autoimmune disease development. Blood 2009; 114:1784-93.
10. Sinclair NR, Anderson CC. Co-stimulation and co-inhibition: equal partners in regulation. Scand J Immunol 1996; 43:597-603.
11. Radziewicz H, Dunham RM, Grakoui A. PD-1 tempers Tregs in chronic HCV infection. J Clin Invest 2009; 119:450-3.
12. Lin SJ, Peacock CD, Bahl K, Welsh RM. Programmed death-1 (PD-1) defines a transient and dysfunctional oligoclonal T cell population in acute homeostatic proliferation. J Exp Med 2007; 204:2321-33.
13. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008; 26:677-704.
14. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999; 11:141-51.
15. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001; 291:319-22.

16. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. Int Immunol 2007; 19:813-24.
17. Le Campion A, Bourgeois C, Lambolez F, Martin B, Leaument S, Dautigny N, et al. Naive T cells proliferate strongly in neonatal mice in response to self-peptide/self-MHC complexes. Proc Natl Acad Sci U S A 2002; 99:4538-43.
18. Min B, McHugh R, Sempowski GD, Mackall C, Foucras G, Paul WE. Neonates support lymphopenia-induced proliferation. Immunity 2003; 18:131-40.
19. Smith H, Lou YH, Lacy P, Tung KS. Tolerance mechanism in experimental ovarian and gastric autoimmune diseases. J Immunol 1992; 149:2212-8.
20. Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ. Continued maturation of thymic emigrants in the periphery. Nat Immunol 2004; 5:418-25.
21. Makaroff LE, Hendricks DW, Niec RE, Fink PJ. Postthymic maturation influences the CD8 T cell response to antigen. Proc Natl Acad Sci U S A 2009; 106:4799-804.
22. Haines CJ, Giffon TD, Lu LS, Lu X, Tessier-Lavigne M, Ross DT, et al. Human CD4+ T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function. J Exp Med 2009; 206:275-85.
23. Hassan J, Reen DJ. Human recent thymic emigrants--identification, expansion, and survival characteristics. J Immunol 2001; 167:1970-6.
24. Dardalhon V, Jaleco S, Kinet S, Herpers B, Steinberg M, Ferrand C, et al. IL-7 differentially regulates cell cycle progression and HIV-1-based vector infection in neonatal and adult CD4+ T cells. Proc Natl Acad Sci U S A 2001; 98:9277-82.
25. Opiela SJ, Koru-Sengul T, Adkins B. Murine neonatal recent thymic emigrants are phenotypically and functionally distinct from adult recent thymic emigrants. Blood 2009; 113:5635-43.
26. Lantz O, Grandjean I, Matzinger P, Di Santo JP. Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation. Nat Immunol 2000; 1:54-8.
27. Perarnau B, Saron MF, Reina San Martin B, Bervas N, Ong H, Soloski MJ, et al. Single H2Kb, H2Db and double H2KbDb knockout mice: peripheral CD8+ T cell repertoire and anti-lymphocytic choriomeningitis virus cytolytic responses. Eur J Immunol 1999; 29:1243-52.
28. Baldwin TA, Sandau MM, Jameson SC, Hogquist KA. The timing of TCR alpha expression critically influences T cell development and selection. J Exp Med 2005; 202:111-21.
29. Chan WF, Perez-Diez A, Razavy H, Anderson CC. The ability of natural tolerance to be applied to allogeneic tissue: determinants and limits. Biol Direct 2007; 2:10.

30. Shimada Y, Hayashi M, Nagasaka Y, Ohno-Iwashita Y, Inomata M. Age-associated up-regulation of a negative co-stimulatory receptor PD-1 in mouse CD4+ T cells. Exp Gerontol 2009; 44:517-22.
31. Alferink J, Tafuri A, Vestweber D, Hallmann R, Hammerling GJ, Arnold B. Control of neonatal tolerance to tissue antigens by peripheral T cell trafficking. Science 1998; 282:1338-41.
32. Keir ME, Latchman YE, Freeman GJ, Sharpe AH. Programmed death-1 (PD-1):PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes. J Immunol 2005; 175:7372-9.
33. Nishimura H, Honjo T, Minato N. Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice. J Exp Med 2000; 191:891-8.
34. Blank C, Brown I, Marks R, Nishimura H, Honjo T, Gajewski TF. Absence of programmed death receptor 1 alters thymic development and enhances generation of CD4/CD8 double-negative TCR-transgenic T cells. J Immunol 2003; 171:4574-81.
35. Baldwin TA, Hogquist KA. Transcriptional analysis of clonal deletion in vivo. J Immunol 2007; 179:837-44.
36. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity 2008; 29:848-62.
37. De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 1994; 264:703-7.
38. Coles MC, Veiga-Fernandes H, Foster KE, Norton T, Pagakis SN, Seddon B, et al. Role of T and NK cells and IL7/IL7r interactions during neonatal maturation of lymph nodes. Proc Natl Acad Sci U S A 2006; 103:13457-62.
39. Guimond M, Veenstra RG, Grindler DJ, Zhang H, Cui Y, Murphy RD, et al. Interleukin 7 signaling in dendritic cells regulates the homeostatic proliferation and niche size of CD4+ T cells. Nat Immunol 2009; 10:149-57.
40. Anderson CC, Matzinger P. Immunity or tolerance: opposite outcomes of microchimerism from skin grafts. Nat Med 2001; 7:80-7.
41. Thangavelu G, Smolarchuk C, Anderson CC. Co-inhibitory molecules: Controlling the effectors or controlling the controllers? Self/Nonself 2010; 1:77-88.
42. Kido M, Watanabe N, Okazaki T, Akamatsu T, Tanaka J, Saga K, et al. Fatal autoimmune hepatitis induced by concurrent loss of naturally arising regulatory T cells and PD-1-mediated signaling. Gastroenterology 2008; 135:1333-43.
43. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 2009; 206:3015-29.
44. Yaniv I, Ash S, Farkas DL, Askenasy N, Stein J. Consideration of strategies for hematopoietic cell transplantation. J Autoimmun 2009; 33:255-9.
45. Hugle T, Daikeler T. Stem cell transplantation for autoimmune diseases. Haematologica 2010; 95:185-8.

46. Daikeler T, Tyndall A. Autoimmunity following haematopoietic stem-cell transplantation. Best Pract Res Clin Haematol 2007; 20:349-60.
47. Loh Y, Oyama Y, Statkute L, Quigley K, Yaung K, Gonda E, et al. Development of a secondary autoimmune disorder after hematopoietic stem cell transplantation for autoimmune diseases: role of conditioning regimen used. Blood 2007; 109:2643-548.
48. Armengol MP, Sabater L, Fernandez M, Ruiz M, Alonso N, Otero MJ, et al. Influx of recent thymic emigrants into autoimmune thyroid disease glands in humans. Clin Exp Immunol 2008; 153:338-50.
49. Goldberg MV, Maris CH, Hipkiss EL, Flies AS, Zhen L, Tuder RM, et al. Role of PD-1 and its ligand, B7-H1, in early fate decisions of CD8 T cells. Blood 2007; 110:186-92.
50. Keir ME, Freeman GJ, Sharpe AH. PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues. J Immunol 2007; 179:5064-70.
51. Ise W, Kohyama M, Nutsch KM, Lee HM, Suri A, Unanue ER, et al. CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. Nat Immunol 2010; 11:129-35.
52. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. Proc Natl Acad Sci U S A 2008; 105:9331-6.
53. Kieper WC, Burghardt JT, Surh CD. A role for TCR affinity in regulating naive T cell homeostasis. J Immunol 2004; 172:40-4.
54. Chappaz S, Finke D. The IL-7 signaling pathway regulates lymph node development independent of peripheral lymphocytes. J Immunol 2010; 184:3562-9.
55. Johnson LD, Jameson SC. Self-specific CD8+ T cells maintain a semi-naive state following lymphopenia-induced proliferation. J Immunol 2010; 184:5604-11.
56. Singh NJ, Schwartz RH. The lymphopenic mouse in immunology: from patron to pariah. Immunity 2006; 25:851-5.
57. Winstead CJ, Fraser JM, Khoruts A. Regulatory CD4+CD25+Foxp3+ T cells selectively inhibit the spontaneous form of lymphopenia-induced proliferation of naive T cells. J Immunol 2008; 180:7305-17.

Chapter 3: Role of B and T lymphocyte (BTLA) attenuator in controlling lymphopenia induced autoimmunity

3.1. Introduction

Having demonstrated the role of PD-1 in controlling lymphopenia induced autoimmunity, I tested the importance of other co-inhibitory molecules such as B and T lymphocyte attenuator (BTLA) in controlling lymphopenia-induced autoimmunity. The outcome of the immune response is influenced by the balance between co-stimulation and co-inhibition[1, 2]. Besides lymphocyte receptor signaling, which is due to antigen encounter with the antigen receptor, co-stimulation acts as a second signal and causes increased proliferation of lymphocytes and their survival[3]. In contrast, co-inhibition terminates or dampens the lymphocyte response by providing negative signals[4].

BTLA (CD272) is a co-inhibitory receptor expressed by T cells, B cells, NK cells, NKT cells and antigen presenting cells[5, 6]. It has two cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The interaction of BTLA with its ligand herpesvirus entry mediator (HVEM) attenuates T cell proliferation by inducing tyrosine phosphorylation and associating with SHP-1 and SHP-2[6]. A previous study reported that the induction of peripheral tolerance of T cells was dependent on BTLA[7]. Deficiency of BTLA in an autoimmune background caused severe lymphocytic infiltration in major organs[8], indicating that BTLA deficiency has the potential to synergize with other factors to generate autoimmunity. In this report, we have investigated whether lymphopenia may be a co-factor for autoimmunity together with BTLA deficiency.

Homeostatic proliferation is a physiological process that occurs after lymphopenic states created by infections[9] or lymphoablation[10, 11]. Lymphopenia induced homeostatic proliferation can be associated with autoimmunity[12-14]. However, syngeneic stem cell transplantation in lymphopenic animals fails to induce autoimmunity despite the presence of homeostatic proliferation of T cells. Of late, we have shown that programmed cell death-1 plays a critical role in recent thymic emigrants in preventing lymphopenia induced autoimmunity[15]. The hypothesis of the current study is that BTLA is required in recent thymic emigrants to prevent lymphopenia induced autoimmunity. In this study we tested this hypothesis by using a hematopoietic stem cells transplantation model and found that BTLA but not Fas plays a critical role in newly generated T cells in the prevention of lymphopenia induced autoimmunity.

3.2. Materials and Methods

3.2.1. Mice

Male and female B6.129S7-Rag1^{tm1mom}/J (abbreviated as Rag$^{-/-}$), (C57BL/6J × C57BL/10SgSnAi)-[KO] γ*c*-[KO]*Rag2* knockout (abbreviated as Rag$^{-/-}$ γc$^{-/-}$), CD45.1-C57BL/6 (B6), RAG2p-GFP Tg[16] and C57BL/6-BTLA$^{-/-}$ (BTLA$^{-/-}$) mice[6] were used in the experiments described in this chapter. B6.MRL-*Tnfrsf6*^{lpr}/J (*lpr*) were purchased from Jackson Laboratory (Bar Harbor, ME). Animal care was in accordance with guidelines of the Canadian Council on Animal Care.

3.2.2. Stem cell transplantation

Fetal liver cells, a source of hematopoietic stem cells, were harvested from day15-16 B6 or BTLA$^{-/-}$ fetuses. A single-cell suspension was made by gently pipetting the fetal livers and filtration through a cell strainer[15]. Female or male six to eight week old immunodeficient mice were used as recipients and each recipient received 10-15 x 10^6 fetal liver cells. In mixed stem cell experiments, recipients were given 15 x 10^6 fetal liver cells total containing a 1:1 mixture of BTLA$^{-/-}$ and B6 wild type (WT) CD45.1 cells.

3.2.3. Definition of disease and histology

Disease symptoms included cachexia, hunched appearance, ruffled fur, reduced mobility, skin and ocular lesions. Recipient mice were no longer considered disease free when two or more of the above symptoms were evident. Tissues from multiple organs collected from recipient mice were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histological evaluations were completed by a Veterinary Pathologist (R.R.E.U.) blinded to the treatment groups.

3.2.4. Flow cytometry analysis

Fluorochrome conjugated antibodies for cell surface molecules were purchased from eBioscience. Peripheral blood samples and splenocytes were stained after incubation with FcR block, which is a cocktail of anti-CD16/32 antibody (2.4G2; Bio Express, West Lebanon, NH) and mouse, rat and hamster sera, used to reduce

background staining and in turn increase the specificity of antibody binding. The antibody staining was done at 4°C for 15 minutes followed by washing and resuspension in PBS. A FACSCalibur™ (BD Biosciences) with CellQuest™ Pro software and BD LSR II (BD Biosciences) with Flowjo software were used for data acquisition and analyses.

3.2.5. Thymocyte transfer and FACS sorting

Thymocytes or splenocytes (2-2.5 x 10^6 single positive T cells) from B6 or BTLA$^{-/-}$ mice were injected into immunodeficient recipients. Briefly, thymuses and spleens were removed from the donors and mashed with glass slides to make a single cell suspension followed by filtration with a cell strainer (BD Falcon ^{TM}). TCR$^+$ CD24^{low} cells were sorted aseptically from splenocytes of six week old BTLA$^{-/-}$ mice on a FACS BD influx^{TM} cell sorter (BD Biosciences). The purity of the sorted cell populations was 92%.

3.2.6. BrdU incorporation and immunofluorescence

Experimental mice were treated with 2mg BrdU in PBS by i.p. injection. BrdU incorporation was assessed in splenic T cells 24 hours after injection using a BrdU flow cytometry kit (BD Pharmingen^{TM}). For immunofluorescence 5μm cross sections were cut from OCT embedded cryo tissues, fixed, blocked with goat serum and incubated with rat anti-mouse CD4 or CD8a (Biolegend, San Deigo, CA) followed by secondary antibody, which was goat anti-rat Alexaflour 488 (Invitrogen Laboratories, Burlington, ON) and visualized on a compound

fluorescent microscope (Axioplan, Axiovision 4.1 software, Carl Zeiss, Toronto, ON).

3.2.7. Measurement of liver enzymes

The levels of asparate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in serum were determined using test strips with a VetTest analyzer (IDEXX laboratories, USA).

3.2.8. Statistical analysis

Prism version 4.0a (GraphPad Software Inc., SanDiego, CA, USA) was used for statistical analysis. Statistical significance was evaluated using a Student's *t*-test or a one-way ANOVA was used to compare two more groups. The log-rank test was used to compare survival curves. All data were expressed as mean ± SEM. A value of $p < 0.05$ was considered a statistically significant difference between groups.

3.3. Results

3.3.1. BTLA is required in RTE to prevent lymphopenia induced autoimmunity

We recently showed that programmed death-1 (PD-1) is critical in recent thymic emigrants (RTE) to prevent lymphopenia-induced autoimmunity using a syngeneic hematopoietic stem cell (HSC) model[15]. It was therefore of interest to determine whether other inhibitory signaling receptors in lymphocytes are also

necessary for RTE or if instead this role is unique to PD-1. To examine whether RTE might have a higher frequency of $BTLA^{high}$ cells compared to established T cells, similar to our previous findings with PD-1, we used mice that express GFP under the control of the promoter of Rag2[16]. Interestingly, we found that RTE (GFP^+) in these Rag2-GFP mice had a higher proportion of BTLA high T cells when compared to mature (GFP^-) T cells (Figure 1; left top) The percent of RTE (8.8 ± 2.1) that express BTLA was significantly higher ($p < 0.05$) than those of mature T cells (2.6 ± 0.7). We next investigated whether BTLA is required in RTE to prevent lymphopenia-induced autoimmunity by using syngeneic $BTLA^{-/-}$ HSC. $BTLA^{-/-}$ mature T or WT HSC cells were used as control groups. In addition, $BTLA^{-/-}$ thymocytes were used as a second source of RTE. The majority of the recipients of $BTLA^{-/-}$ thymocytes or HSC became sick, while the control groups remained disease free **(Figure 3-1; middle)**. Fas is a death receptor and has a capacity to induce apoptosis of cells. We used *lpr* HSC as a control to test whether a lack of death signal would induce disease in lymphopenia. Interestingly, there was no overt disease in *lpr* HSC recipients and this suggests that not all the receptors that provide negative signals to T cells in this model are needed to control lymphopenia induced autoimmunity. Collectively, these data suggested a requirement for BTLA in RTE to control lymphopenia-induced autoimmunity.

The sick mice showed cachexia, hunched back **(Figure 3-1)**, ruffled fur, dermatitis on the tail and diarrhea. At necropsy, we noticed that a small

percentage (10%) of mice developed colitis. Histological analysis of tissue sections obtained from sick mice revealed lymphocytic infiltration in major organs including the liver, kidney and pancreas **(Figure 3-2; top)**.

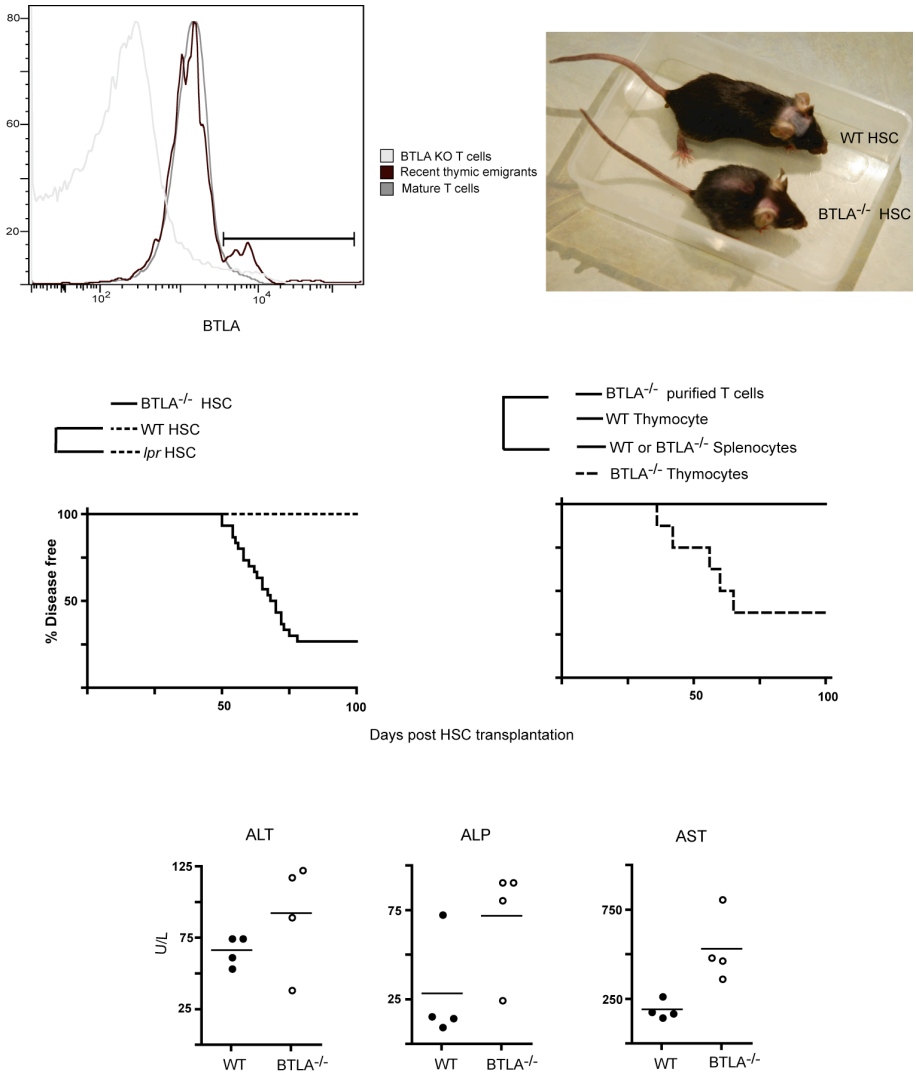


Figure 3-1. BTLA$^{-/-}$ HSC and thymocytes induce lethal multi-organ inflammatory disease in lymphopenia. *Top left:* BTLA expression was higher in recent thymic emigrants (RTE; gated on GFP^{+} TCR^{+}). The expression of BTLA was higher in RTE (n=3) than those of mature T cells (GFP^{-} TCR^{+}). *Top right:* Macroscopic picture of recipients of either BTLA$^{-/-}$ or WT HSC. *Middle left:* Adult Rag$^{-/-}$ mice were given fetal liver cells (HSC) from BTLA$^{-/-}$ (n=30) or WT (n=10) or *lpr* fetuses (n=3) and disease was monitored. *Middle right:* Adult Rag$^{-/-}$ mice were given BTLA$^{-/-}$ (n=5) or WT thymocytes (n=4) or WT/BTLA$^{-/-}$ splenocytes or purified BTLA$^{-/-}$ splenic T cells (n=3 for each group) and monitored for disease incidence. Disease was only observed in BTLA$^{-/-}$ HSC or thymocyte group, with all other groups surviving to day 100 disease free. Serum was collected from the sick recipients of BTLA$^{-/-}$ HSC between day 40 to 65 post HSC transfer. Also, serum was collected from the control recipients of WT HSC at the same time; n = 4 /group. Data are expressed as mean ± SE. AST was higher ($P < 0.01$) in sera of BTLA$^{-/-}$ HSC recipients (532.8 ± 96.4) than those of WT

HSC recipients (193.0 ± 26.2). Other enzymes such as ALT (92.50 ± 19.2) or ALP (72.00 ± 15.8) in BTLA$^{-/-}$ HSC recipients were not significantly different from those levels of WT HSC recipients (ALT: 66.5 ± 10.3; ALP: 28.50 ± 14.8).

Lymphocytic infiltration in the liver included CD4^{+} and CD8^{+} T cells in recipients of BTLA$^{-/-}$ HSC but not WT HSC (Figure 3-2; bottom).

A previous study reported the occurrence of autoimmune hepatitis in old 129 SvEV BTLA$^{-/-}$ mice[17]. Moreover, liver was the major organ affected in Rag$^{-/-}$ recipients of BTLA HSC. Hence, we measured the level of liver enzymes in sera of sick mice. The level of AST in sera was significantly higher in recipients of BTLA$^{-/-}$ HSC than those of WT HSC **(Figure 3-1)**. Although other liver enzymes such as ALT, ALP were also elevated in sera of BTLA$^{-/-}$ HSC recipients, the differences did not reach statistical significance **(Figure 3-1)**.

3.3.2. BTLA$^{-/-}$ T cells have a greater capacity to undergo homeostatic proliferation and autoimmunity can be controlled by reducing lymphopenia driven activation through competition and reduced lymphoid space

Homeostatic proliferation of T cells was confirmed using BrdU labeling and evaluation of activation markers. The percent of BrdU+ T cells was higher in the recipients of BTLA$^{-/-}$ HSC than those of WT HSC **(Figure 3- 3; top)**. Moreover, the percent of effector memory T cells in sick mice was higher than that of WT HSC recipients **(Figure 3- 3; bottom)**. These results suggest that T cells of BTLA$^{-/-}$ HSC recipients undergo much more proliferation than those of WT HSC recipients.

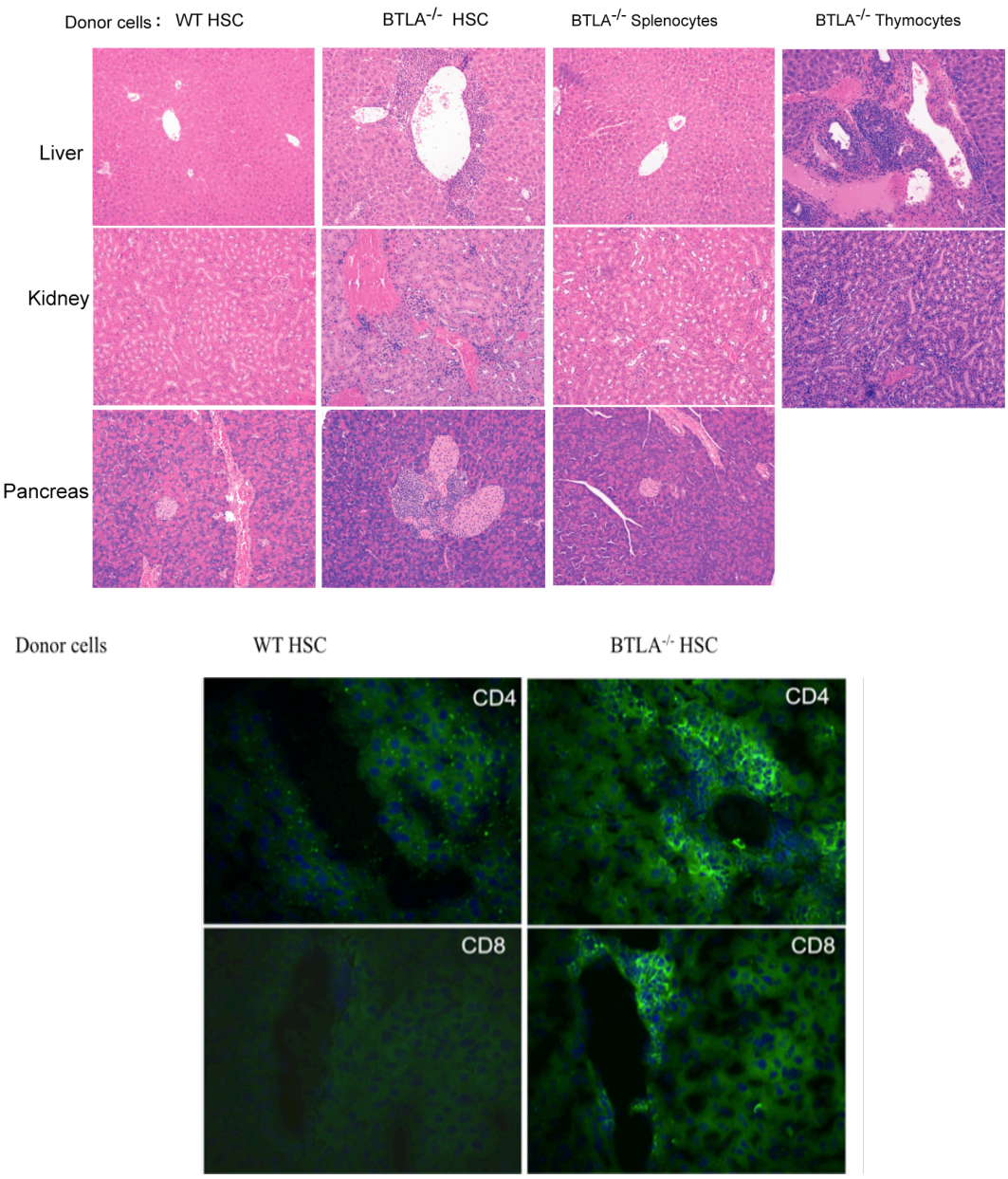


Figure3- 2: Lymphocytic infiltration in organs of BTLA$^{-/-}$ HSC recipients. *Top:* Representative histology (hematoxylin and eosin staining) of the liver, kidney and pancreas of recipients of WT HSC (left), BTLA$^{-/-}$ HSC (second left), BTLA$^{-/-}$ splenocytes (second right) and BTLA$^{-/-}$ thymocytes (right). *Bottom* (four panels): Immunofluorescence (original magnification ×400) of liver from individual recipients of BTLA $^{-/-}$ and WT HSC. Blue: staining with the nuclear marker 4',6'-diamidino-2-phenylindole (DAPI); green: CD4 or CD8 staining.

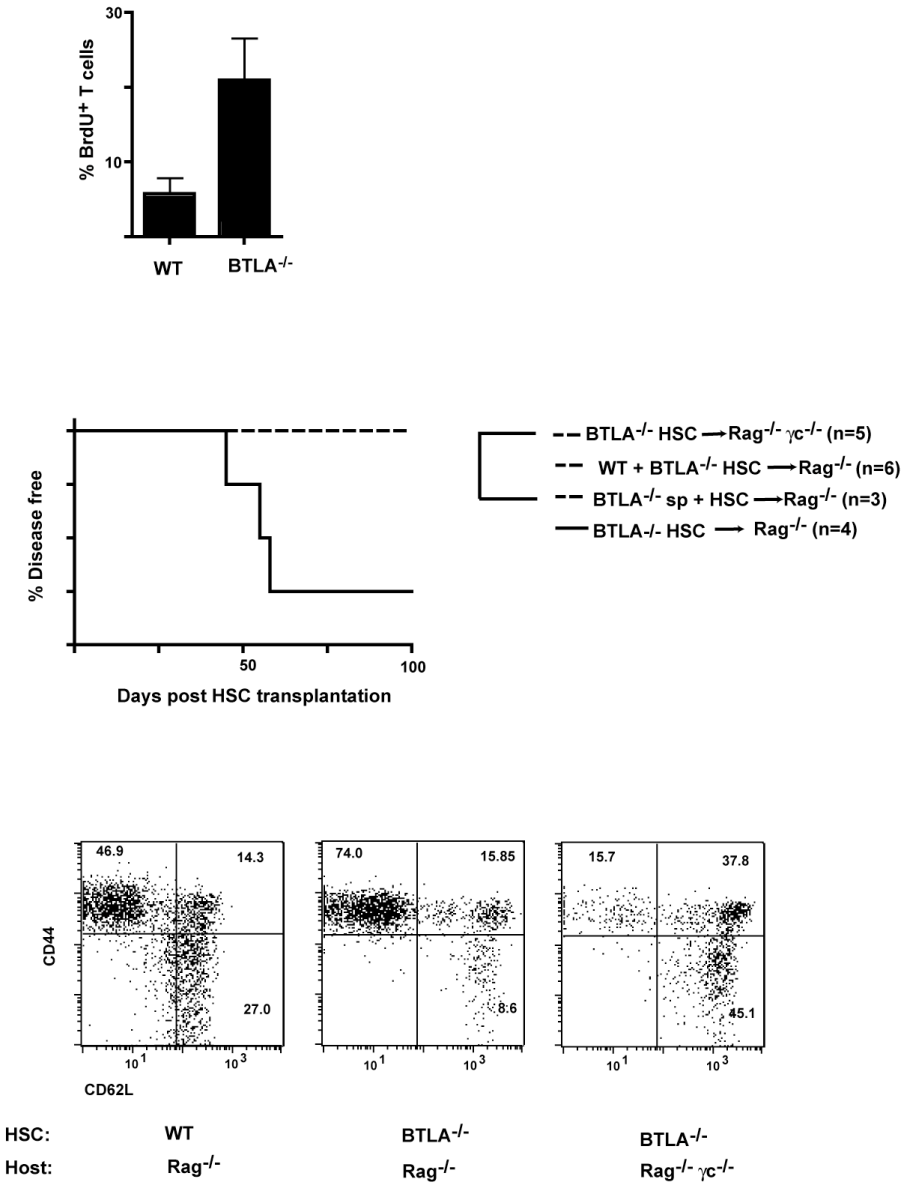


Figure 3-3: T cells from BTLA$^{-/-}$ HSC undergo more homeostatic proliferation and the disease induced by BTLA$^{-/-}$ HSC can be controlled by reducing lymphoid space or by providing polyclonal competitor T cells. *Top:* The mean percentage of BrdU+ T cells in spleen of Rag$^{-/-}$ recipients (n=4; each group) of BTLA$^{-/-}$ HSC (20.93 ± 5.6) was significantly higher ($P < 0.05$) than that of WT HSC (5.76 ± 2.1) examined 65 days post HSC. *Middle:* Adult γc$^{-/-}$ Rag$^{-/-}$ mice (n=5) were given BTLA$^{-/-}$ HSC. Adult Rag$^{-/-}$ mice were given BTLA$^{-/-}$ HSC alone or together with WT HSC (1:1; n= 6) or BTLA$^{-/-}$ splenocytes (sp; n=3). *Bottom:* The percent of effector memory T cells (TCR+ CD44^{hi} CD62L^{lo}) in Rag$^{-/-}$ recipients of BTLA$^{-/-}$ HSC (n = 4; 73.28 ± 3.1) was significantly higher ($P < 0.05$) than that of Rag$^{-/-}$ recipients of WT HSC (n = 4; 52.29 ± 6.9) and γc$^{-/-}$ Rag$^{-/-}$

recipients of BTLA$^{-/-}$ HSC (n=3; 25.73 ± 7.2), analyzed 55 days post HSC injection.

Having demonstrated that BTLA is required in newly generated T cells to prevent lymphopenia-induced autoimmunity we used two strategies to test whether disease could be blocked by reducing lymphopenia driven activation. Firstly, we co-transferred WT HSC with BTLA$^{-/-}$ HSC to provide a competitor T cell population. Secondly, recipients with reduced lymph node stroma (Rag$^{-/-}$ γc$^{-/-}$ mice) were used as HSC recipients. Interestingly, T cells from BTLA$^{-/-}$ HSC failed to induce disease in both models **(Figure 3- 3; middle)**. Co-transfer of WT HSC with BTLA$^{-/-}$ HSC also reduced the effector memory T cells of BTLA$^{-/-}$ HSC (n=3; day 50 post HSC; 25 ± 6.6). Taken together, these results suggested that autoimmunity can be controlled by reducing lymphopenia driven activation of BTLA$^{-/-}$ T cells, similar to our findings with autoimmunity caused by PD-1$^{-/-}$ T cells.

3.4. Discussion

Previous studies have demonstrated that co-inhibitory molecules, such as CTLA-4 and PD-1, play essential roles in the maintenance of immune homeostasis and self-tolerance[18-20]. Although accumulating evidence suggests that BTLA could also be involved in the maintenance of self-tolerance, its main role is still unclear. The development of autoimmunity in BTLA$^{-/-}$ mice is influenced by the genetic background of mice. For example, old BTLA$^{-/-}$ mice on a 129SvEV background develop autoantibodies to nuclear antigen and an autoimmune hepatitis-like

disease[17]. On the other hand, C57BL/6 BTLA$^{-/-}$ mice appear free from autoimmune disease[21], which might be interpreted as a reduced importance of BTLA in self-tolerance. Interestingly, our study demonstrated a critical role for BTLA in the prevention of lymphopenia induced multi-organ autoimmunity. The effect of BTLA deficiency was restricted to newly generated T cells rather than mature T cells and the present study supported the idea that the central role of co-inhibitory molecules in the setting of lymphopenia is to control the autoimmune potential of newly generated T cells[15]. In addition, it emphasized the involvement of multiple, and yet specific, co-inhibitory molecules in the regulation of this process. Thus, synergism of lymphopenia and BTLA deficiency can instigate newly developed T cells to induce autoimmunity.

The majority of the lymphopenic recipients that were transplanted with BTLA$^{-/-}$ HSC showed lymphocytic infiltration in the major organs. There was an increase in the frequency of effector memory T cells in recipients of BTLA$^{-/-}$ HSC compared to WT HSC recipients. Kaye and colleagues reported that T cells from BTLA$^{-/-}$ mice had an advantage in undergoing more homeostatic proliferation, compared to T cells from wild type mice[22]. Although T cells from WT HSC can undergo homeostatic proliferation in lymphopenic mice, the presence of intact BTLA may inhibit the development of effector memory T cells and disease. Moreover, a recent study demonstrated that a single dose of agonistic anti-BTLA antibody inhibited the development of graft versus host disease[23]. BTLA has been shown to act as a prosurvival signal for T cells under certain conditions[24, 25] and

this may be the reason that the disease onset took a longer time in BTLA$^{-/-}$ HSC recipients, in comparison to those of PD-1$^{-/-}$ HSC in which the onset of the disease was earlier[15].

Liver enzymes were elevated in BTLA$^{-/-}$ compared to WT HSC recipients. The spontaneous development of autoimmune hepatitis-like disease in aged BTLA$^{-/-}$ 129SvEV was due to the accumulation of CD4+ T cells and NKT cells in the liver[17]. In the present study, lymphocytic infiltration was observed in the liver of the BTLA$^{-/-}$ HSC recipients. However, the mechanism of liver damage may be different in our model because the liver damage occurred earlier than BTLA$^{-/-}$ 129SvEV mice and there was also infiltration of the liver with CD8+ T cells.

Homeostatic proliferation of T cells occurs due to the availability of more lymphoid space in lymphopenic recipients. Previous studies have reported that lymphopenia induced homeostatic proliferation can be inhibited by either using competitor T cells[26] or reducing lymph node stroma[27]. As a first step, we investigated whether co-transfer of WT HSC with BTLA$^{-/-}$ HSC can prevent the disease. Interestingly, co-transfer of WT HSC completely prevented the disease by reducing the activation of T cells and frequency of effector memory T cells. This result suggested that T cells can compete with each other for resources (MHC on APC, cytokines etc.)[28, 29] to undergo homeostatic proliferation. Furthermore, we tested whether disease can be prevented by reducing lymph node stroma. We took advantage of the Rag$^{-/-}$ $\gamma c^{-/-}$ mouse, which is lymph node

deficient, as a recipient of $BTLA^{-/-}$ HSC. In contrast to $Rag^{-/-}$ recipients, all of the $Rag^{-/-}$ $\gamma c^{-/-}$ recipients were free from the development of disease. The frequency of effector memory cells was also lower in $Rag^{-/-}$ $\gamma c^{-/-}$ recipients. These data suggested that a paucity of lymph node stroma can prevent the disease development by reducing lymhopenia induced homeostatic proliferation of newly developing T cells.

A previous study demonstrated the importance of BTLA signaling in the regulation of mucosal inflammation[30] in which the authors showed that signaling through BTLA prevented the acceleration of colitis induced by WT CD4+ T cells in $HVEM^{-/-}$ $Rag^{-/-}$ recipients. In addition, transfer of WT CD4+ T cells into $BTLA^{-/-}$ $Rag^{-/-}$ also accelerated the colitis. In the present study, we observed that a small proportion of $BTLA^{-/-}$ HSC recipients developed colitis, which further supports the role of BTLA in the prevention of colitis. However, our studies suggest that in addition to tissue specific tolerance requirements, BTLA can become critical for tolerance more generally in the context of lymphopenia and newly generated T cells.

In addition, the results from this study suggest that a requirement for intact BTLA function should be one of the criteria to be considered in patients who undergo lympho-ablation followed by autologous stem cell transplantation[31, 32].

Contribution

I performed all the experiments and analyzed the data.

3.5. References

1. Sinclair NR, Anderson CC. Co-stimulation and co-inhibition: equal partners in regulation. Scand J Immunol 1996; 43:597-603.
2. Thangavelu G, Smolarchuk C, Anderson CC. Co-inhibitory molecules: Controlling the effectors or controlling the controllers? Self Nonself 2010; 1:77-88.
3. Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nat Rev Immunol 2003; 3:609-20.
4. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 2004; 4:336-47.
5. Han P, Goularte OD, Rufner K, Wilkinson B, Kaye J. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. J Immunol 2004; 172:5931-9.
6. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 2003; 4:670-9.
7. Liu X, Alexiou M, Martin-Orozco N, Chung Y, Nurieva RI, Ma L, et al. Cutting edge: A critical role of B and T lymphocyte attenuator in peripheral T cell tolerance induction. J Immunol 2009; 182:4516-20.
8. Oya Y, Watanabe N, Kobayashi Y, Owada T, Oki M, Ikeda K, et al. Lack of B and T lymphocyte attenuator exacerbates autoimmune disorders and induces Fas-independent liver injury in MRL-lpr/lpr mice. Int Immunol 2011; 23:335-44.
9. Margolick JB, Donnenberg AD. T-cell homeostasis in HIV-1 infection. Semin Immunol 1997; 9:381-8.
10. Goldrath AW, Bevan MJ. Selecting and maintaining a diverse T-cell repertoire. Nature 1999; 402:255-62.
11. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. Nat Rev Immunol 2009; 9:823-32.
12. Gleeson PA, Toh BH, van Driel IR. Organ-specific autoimmunity induced by lymphopenia. Immunol Rev 1996; 149:97-125.
13. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 2004; 117:265-77.
14. Le Campion A, Gagnerault MC, Auffray C, Becourt C, Poitrasson-Riviere M, Lallemand E, et al. Lymphopenia-induced spontaneous T-cell proliferation as a cofactor for autoimmune disease development. Blood 2009; 114:1784-93.
15. Thangavelu G, Parkman JC, Ewen CL, Uwiera RR, Baldwin TA, Anderson CC. Programmed death-1 is required for systemic self-tolerance in newly generated T cells during the establishment of immune homeostasis. J Autoimmun 2011; 36:301-12.
16. 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.

17. Oya Y, Watanabe N, Owada T, Oki M, Hirose K, Suto A, et al. Development of autoimmune hepatitis-like disease and production of autoantibodies to nuclear antigens in mice lacking B and T lymphocyte attenuator. Arthritis Rheum 2008; 58:2498-510.
18. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999; 11:141-51.
19. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001; 291:319-22.
20. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995; 270:985-8.
21. Deppong C, Degnan JM, Murphy TL, Murphy KM, Green JM. B and T lymphocyte attenuator regulates T cell survival in the lung. J Immunol 2008; 181:2973-9.
22. Krieg C, Boyman O, Fu YX, Kaye J. B and T lymphocyte attenuator regulates CD8+ T cell-intrinsic homeostasis and memory cell generation. Nat Immunol 2007; 8:162-71.
23. Albring JC, Sandau MM, Rapaport AS, Edelson BT, Satpathy A, Mashayekhi M, et al. Targeting of B and T lymphocyte associated (BTLA) prevents graft-versus-host disease without global immunosuppression. J Exp Med 2010; 207:2551-9.
24. Hurchla MA, Sedy JR, Murphy KM. Unexpected role of B and T lymphocyte attenuator in sustaining cell survival during chronic allostimulation. J Immunol 2007; 178:6073-82.
25. Sakoda Y, Park JJ, Zhao Y, Kuramasu A, Geng D, Liu Y, et al. Dichotomous regulation of GVHD through bidirectional functions of the BTLA-HVEM pathway. Blood 2011; 117:2506-14.
26. Dummer W, Ernst B, LeRoy E, Lee D, Surh C. Autologous regulation of naive T cell homeostasis within the T cell compartment. J Immunol 2001; 166:2460-8.
27. Do JS, Min B. Differential requirements of MHC and of DCs for endogenous proliferation of different T-cell subsets in vivo. Proc Natl Acad Sci U S A 2009; 106:20394-8.
28. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity 2008; 29:848-62.
29. Wang Q, Strong J, Killeen N. Homeostatic competition among T cells revealed by conditional inactivation of the mouse Cd4 gene. J Exp Med 2001; 194:1721-30.
30. Steinberg MW, Turovskaya O, Shaikh RB, Kim G, McCole DF, Pfeffer K, et al. A crucial role for HVEM and BTLA in preventing intestinal inflammation. J Exp Med 2008; 205:1463-76.
31. Zeher M, Papp G, Szodoray P. Autologous haemopoietic stem cell transplantation for autoimmune diseases. Expert Opin Biol Ther 2011.

32. Eide MB, Lauritzsen GF, Kvalheim G, Kolstad A, Fagerli UM, Maisenholder M, et al. High dose chemotherapy with autologous stem cell support for patients with histologically transformed B-cell non-Hodgkin lymphomas. A Norwegian multi centre phase II study. Br J Haematol 2011; 152:600-10.

Chapter 4: A version of this chapter has been published.

The citation is given below.

Thangavelu et al., 2011. Immunobiology. 216(8) 918-924.

The role of co-inhibitory signals in spontaneous tolerance of weakly mismatched transplants

4.1. Introduction

Although tolerance in newly generated T cells can be highly PD-1/BTLA dependent, its not clear whether naïve T cells are also dependent on PD-1/BTLA for tolerance upon their first encounter with the antigen. The latter possibility would suggest that PD-1/BTLA would have a role in tolerance of naïve T cells even in adult immunocompetent animals. To test this we used male (HY) antigen as a model in female mice, in which T cells are naïve to male antigen. The success of MHC matched transplants is impeded by immune responses to minor-H antigens[1]. For instance, bone marrow transplants between HLA matched siblings induced GVH disease due to immune responses against minor-H antigens[2]. Autosomal and Y-chromosome genes encode H-antigens[3]. The male specific antigen (HY) triggers rejection of male syngeneic skin grafts in certain inbred strains of female mice[4]. Naïve H-2^b females can reject male skin grafts and thus are referred to as strong responders. Although H-2^b females reject male skin or bone marrow[3, 5], they could not reject male islet[6, 7], kidney[8] or cardiac grafts[9]. Spontaneous acceptance of male islet grafts may also induce dominant tolerance to male antigens[7]. Adoptive transfer of T cells from tolerant mice to female neonates allowed acceptance of male skin grafts in the adoptive recipients, although third party grafts were not tested. Islet or heart grafts given time to heal into the recipient before the recipient's immune system develops can also lead to tolerance of donor antigens[10]. This 'natural tolerance' of a transplant given pre-immunocompetence is designed to mimic the conditions that allow establishment of tolerance to peripheral self antigens. However, spontaneous

tolerance of male islet grafts by adult mice, i.e. tolerance of grafts given post-immunocompetence of the recipient, may not involve precisely the same mechanisms, due to the inflammatory conditions associated with grafts given to immunocompetent adult recipients and the presence of an established adaptive immune system. It is therefore of interest to determine whether the primary mechanisms of peripheral self tolerance, such as those mediated by co-inhibitory signals, are also involved in spontaneous tolerance of weakly mismatched grafts in immunocompetent recipients.

The balance between co-stimulation and co-inhibition influences the outcome of immune responses by allowing strong immune response against appropriate foreign antigens and maintaining tolerance to self-antigens[11, 12]. Cytotoxic lymphocytic antigen-4 (CTLA-4; CD152), programmed death-1 (PD-1; CD279) and B and T lymphocyte attenuator (BTLA; CD272) are some of the major co-inhibitory molecules that have been shown to be involved in immunological tolerance. CTLA-4 is expressed by activated T cells and Tregs and can compete with the co-stimulatory molecule CD28 to bind with its ligands B7.1 (CD80) and B7.2 (CD86). Lack of CTLA-4 induced a fatal lymphoprolifertive disorder in mice[13] that was recently shown to be due to autoreactive T cells[14]. PD-1 shares 23% amino acid sequence homology with CTLA-4 and is expressed by activated T cells, B cells and myeloid cells. Lack of PD-1 leads to a narrow spectrum of autoimmune disease that varies based on the strain background[15, 16]. PD-1 binds PD-L1(B7H1; CD274) and PD-L2 (B7DC; CD273), with PD-L1 being widely

expressed in hematopoietic and non-hematopoietic cells, whereas PD-L2 expression is restricted to dendritic cells and macrophages[17, 18]. Deficiency of BTLA induced autoimmune hepatitis and anti-nuclear antibodies in aged mice[19] and increased susceptibility to experimental autoimmune encephalomyelitis[20] and allergic airway inflammation[21].

We tested whether the "spontaneous tolerance" of weakly mismatched transplants is mediated by co-inhibitory pathways. Herein, we show that PD-1 plays a critical role in the tolerance of single minor mismatched islet transplants.

4.2. Materials and methods

4.2.1. Mice

Adult wild type C57BL/6, (B6; H-2^b) mice were obtained from NCI (Frederick, MD). B6.129S7-$Rag1^{tm1mom}$/J (abbreviated as $Rag^{-/-}$) mice from Jackson were bred in house. We generated MHC class-I and Rag deficient mice (C57BL/6 H-$2K^{btm1}$-H-$2D^{btm1}$N12 $Rag^{-/-}$; abbreviated as class-$I^{-/-}$ $Rag^{-/-}$) by crossing the two knockout lines obtained originally from Jackson. MHC class-II deficient B6.129-*H2-Ab1*^{tm1Gru} (abbreviated as class-$II^{-/-}$) mice were from Taconic Farms. C57BL/6-*Pdcd1-/-* (PD-$1^{-/-}$) mice, originally generated by Prof. T. Honjo and colleagues[15, 22] in embryonic stem cells of the 129 background and backcrossed 11 generations to C57BL/6, and C57BL/6-$BTLA^{-/-}$ ($BTLA^{-/-}$ mice[20]) were bred at the University of Alberta. All protocols on care and handling of animals were carried out in CCAC accredited facilities at the University of Alberta.

4.2.2. Diabetes induction and islet transplantation

Diabetes was chemically induced by a single intraperitoneal injection of STZ; 200 mg/kg; Sigma-Aldrich) in female recipients. Diabetes was confirmed as blood glucose of >20 mmol/L twice on consecutive days. Diabetic recipients were transplanted with 400 male donor islets into the renal subcapsular space. The function of the graft was monitored by blood glucose; rejection was defined as blood glucose exceeding 15 mmol/L on two consecutive days. Nephrectomy was performed to assess whether transplanted islets were responsible for the normoglycemic state.

4.2.3. In vivo antibody treatment and immunization

We used 250 μg anti-mouse PD-1 mAb (J43), anti-mouse PD-L1 (10F.9g2), anti-mouse PD-L2 (TY25), anti-mouse BTLA (6F7) and isotype control (Rat IgG2b) given every other day, beginning on the day of transplantation, for a total of six injections (last injection on day 10). Anti-CTLA-4 (4F10), at a dose of 100 μg of blocking antibody was injected every 2 days from the day of transplantation for a total of six injections. Anti-mouse PD-1, was used at 250 μg mAb (J43) per injection, and given twice, with six days between injections, to block PD-1 signaling. For sensitization, $4x10^6$ male B6 or PD-$1^{-/-}$ splenocytes were injected into female B6 or PD-$1^{-/-}$ mice respectively. Fourteen days post immunization, all recipients were made diabetic, as described above, and transplanted with male islet grafts. WT B6 recipients received male islets from WT B6 donors, and

PD-1$^{-/-}$ recipients received islets from PD-1$^{-/-}$ male donors.

4.2.4. Pentamer studies

We used PE labelled H-2D^{b} (WMHHNMDLI; UTY 246-254; Proimmune, Bradenton, FL) pentamers together with antibodies to mouse CD8, pan TCRβ and CD19 (eBioscience, San Diego, CA) to detect the frequency of anti-HY CD8 T cells. Non-specific binding was blocked with anti-CD16/32 antibody (2.4G2; Bio Express, West Lebanon, NH), and mouse, rat and hamster sera. Data acquisition and analysis was on a FACSCalibur™ flow cytometer (BD Biosciences).

4.2.5. Histology and Immunofluorescence

Kidney with islet grafts were fixed in formalin, embedded in paraffin, sectioned, and stained with anti-insulin, hematoxylin and eosin. For immunofluorescence 5μm crosssections were fixed with acetone and blocked with 20% normal goat serum (Jackson ImmunoResearch Westgrove, PN). Staining was with rat anti-mouse CD4 or CD8α, (1:200; Biolegend, San Deigo, CA) followed by goat anti-rat Alexaflour 488 (1:200; Invitrogen Laboratories, Burlington, ON). Slides were coverslipped using Prolong Gold Anti-fade with DAPI (Invitrogen), and visualized on a compound flourscent microscope (Axioplan, Axiovision 4.1 software, Carl Zeiss, Toronto, ON).

4.2.6. Statistical analysis

Statistical analyses were done using GraphPad Prism Software (San Diego, CA). The Kaplan-Meier method and the log rank test were used for graft survival and a Student's t-test for pentamer studies.

4.3. Results

4.3.1. Tolerance induced by spontaneously accepted islet grafts is PD-1 dependent

Female WT mice accepted male islet grafts indefinitely **(Figure 4-1A)**, in agreement with previous studies[6, 7]. We tested whether targeting co-inhibitory pathways prevents spontaneous acceptance of male islets, by treating mice with mAb that block either CTLA-4 or PD-1[23, 24]. While we have never observed rejection of 'syngeneic' male islets by WT mice, one of the recipients treated with anti-PD-1 rejected its male islet graft **(Figure 4-1A)**, suggesting that PD-1 may have some role in spontaneous acceptance. A preliminary analysis using anti-PD-L1 or anti-PD-L2 treatment did not provide further support for this hypothesis, as these antibodies did not prevent spontaneous graft acceptance **(Figure 4-1B)**. Therefore, we tested the role of PD-1 by two additional approaches. In the first approach, we gave female PD-$1^{-/-}$ mice male islets. In order to ensure the only antigenic mismatches were derived from the Y chromosome, we used PD-$1^{-/-}$ mice as the donors of male islets. Again, lack of PD-1 function appeared to have at most a small effect, with only one out of seven recipients rejecting their graft **(Figure 4-1C)**. We also tested the role of BTLA, using $BTLA^{-/-}$ recipients. There

was no increase in rejection of male islet grafts in BTLA$^{-/-}$ females **(Figure 4-1C).**

Our previous studies[6] indicated that diabetes (as induced by STZ) could suppress the anti-HY immune response, and this diabetes-induced immunosuppression might have reduced the ability to detect a role for these receptors in spontaneous graft acceptance. We therefore tested whether the ability to maintain the graft after immunization with donor antigen may depend on PD-1 function. We immunized female WT, PD-1$^{-/-}$ or BTLA$^{-/-}$ recipients with long-term accepted grafts (100-105 days) to test if the grafts had induced tolerance to HY or if instead the immunization would trigger islet rejection. Interestingly, almost all of the PD-1$^{-/-}$ recipients, but not WT or BTLA$^{-/-}$ recipients, rejected their long-term established graft **(Figure 4-1D)**. Together these results indicate that despite the spontaneous islet allograft acceptance not being strongly PD-1/PD-L1/PD-L2 dependent, the tolerance induced by the long-term presence of the islet graft is highly dependent on PD-1 but not BTLA function.

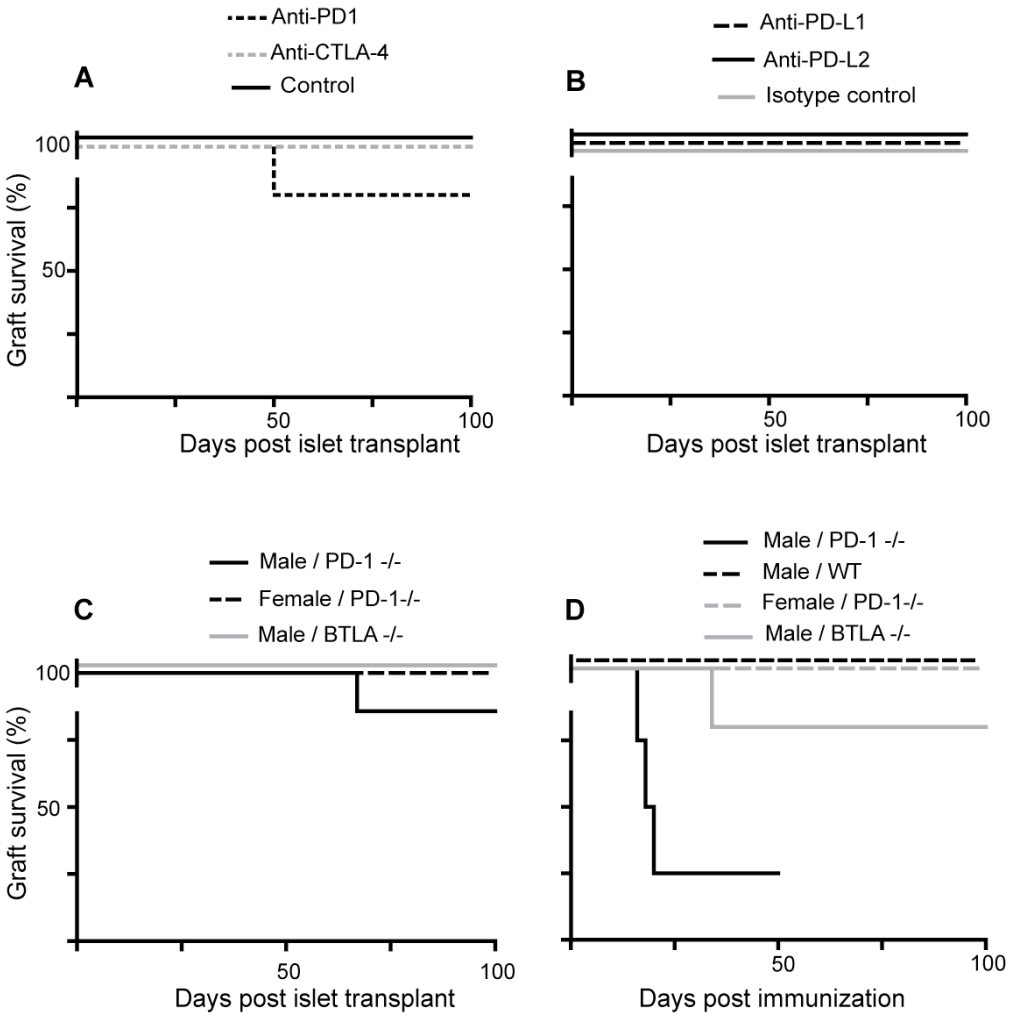


Figure 4-1. PD-1 is required for tolerance but not acceptance of a weakly mismatched islet allograft. (A) Male B6 islets were transplanted to syngeneic female mice that were either untreated (black solid line; n=4; Control) or treated with blocking antibodies to CTLA-4 (grey dashed line; n=5) or PD-1 (black dashed line; n=5). (B) B6 male islets were transplanted to syngeneic female mice that were treated with anti-PD-L1 (black dashed line; n=5), anti-PD-L2 (black solid line; n=5) or isotype control (grey solid line; n=5). (C) Female PD-$1^{-/-}$ mice were transplanted with male PD-$1^{-/-}$ or WT islet grafts (black solid line; n=7) or female PD-$1^{-/-}$ islet grafts (black dashed line; n=3). Also, female $BTLA^{-/-}$ mice were transplanted with male $BTLA^{-/-}$ islet grafts (grey solid line; n=5). (D) 100-105 days post transplantation, female recipients were immunized with 4 x 10^6 male splenocytes. The groups included female PD-$1^{-/-}$ mice transplanted with male (black solid line; n=4) or female (grey dashed line; n=3) islets or female B6

(WT) mice transplanted with male islets (black dashed line; n=4) and female BTLA$^{-/-}$ mice transplanted with male islets (grey solid line; n=5).

4.3.2. Spontaneous graft acceptance in sensitized recipients is PD-1 dependent

Having found that immunization with male antigen breaks the long-term acceptance of male islet grafts in female PD-1$^{-/-}$ mice, it suggested the possibility that PD-1 may play a more critical role in controlling the response of sensitized recipients. We therefore tested the effect of sensitization with donor antigen prior to transplantation on spontaneous graft acceptance in WT vs. PD-1$^{-/-}$ or BTLA$^{-/-}$ recipients. Female WT, PD-1$^{-/-}$ or BTLA$^{-/-}$ mice were immunized with male splenocytes before islet transplantation. Similar to the immunization post transplantation data, there was consistent rejection (100%) of male, but not control female, islet grafts only in the sensitized female PD-1$^{-/-}$ mice **(Table 4-1).** Sensitized female PD-1$^{-/-}$ recipients rejected donor male islet from either WT B6 or PD-1$^{-/-}$ B6 donors, indicating that rejection was not due to potential additional minor antigen mismatches between WT B6 and PD-1$^{-/-}$ B6 mice. Lack of female islet rejection showed the response was donor specific and not due to potential autoreactivity in PD-1$^{-/-}$ mice. Similarly, we found that the PD-1$^{-/-}$ recipients that rejected male islets would accept a female islet graft and reject a second male islet graft when re-transplanted in contralateral kidney (data not shown). Histological examination confirmed the rejection of male islets in sensitized PD-1$^{-/-}$ but not WT recipients, the latter showing strong insulin staining and only a peri-islet infiltrate **(Figure 4- 2B)**. Male islet grafts were infiltrated with CD4 and CD8 cells in sensitized PD-1$^{-/-}$ mice **(Figure 4-2C).**

Blockade of PD-1 signaling can result in the expansion of anti-donor CD8+ T cells[25]. We examined the frequency of anti-HY CD8 T cells using pentamers, in sensitized female WT or PD-1$^{-/-}$ mice. There was an increase in the percentage of anti-HY CD8 T cells in sensitized female PD-1$^{-/-}$ mice compared to WT mice ($P<$ 0.05; **Figure 4-2A**), suggesting a role for PD-1 in dampening the priming of anti-donor T cells.

Table 4-1. PD-1 is required to prevent rejection of male islet grafts in sensitized recipients.

Group	Islet Donor	Recipients	Anti-PD-1 treatment	Graft Survival (d)	% Graft survival
1	Male	PD-1$^{-/-}$	N/A[a]	10 x 2, 14, 18, 20 x 2, 28, 76[b]	0
2	Female	PD-1$^{-/-}$	N/A	> 100 x 5	100
3	Male	BTLA$^{-/-}$	None	44, > 100 x 3	75
4	Male	WT	None	> 100 x 7	100
5	Male	WT	At Immunization	15, 16, > 100 x 3	60
6	Male	WT	At Transplantation	20, > 100 x 4	80
7	Male	WT	At Immunization & Transplantation	17, 19, 28, > 100	25

Female WT or PD-1$^{-/-}$ or BTLA$^{-/-}$ mice were immunized with male splenocytes two weeks before transplantation of islet grafts. The sensitized PD-1$^{-/-}$ mice given male islets (group 1) were significantly different ($P<$ 0.01) from groups 2, 3, and 4. For groups 5-7, female WT mice were treated with anti-PD-1 at the time of immunization with male splenocytes or at the time of transplantation of male islets, 2 weeks after immunization or at both stages. Group 7 vs. 4 ($P<$ 0.05).
[a]N/A, not applicable

[b]Group 1 recipients (n=8) of male islets received islets from WT B6 (n=4) or PD-1$^{-/-}$ B6 (n=4) donors.

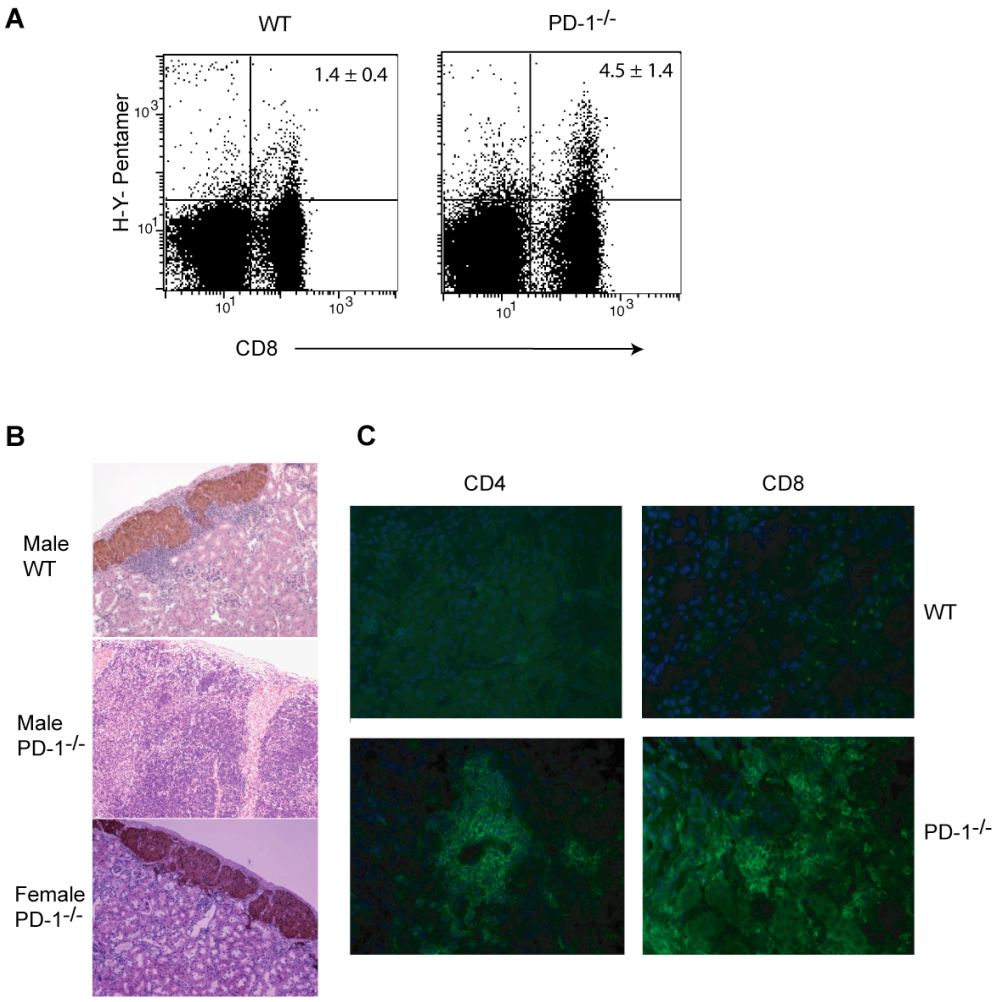


Figure 4-2. Lack of PD-1 increased the frequency of anti-HY CD8 T cells. (A) Female B6 or PD-1$^{-/-}$ mice were immunized with male splenocytes and pentamer staining was performed in splenocytes 2 weeks after immunization. Plots show the frequency of TCR gated pentamer positive anti-HY CD8 T cells. (B) Histological examination of representative islet grafts from sensitized female WT (not rejected; top) showed numerous intact islets that contain insulin granules (brown) surrounded with a mononuclear cell infiltrate. Male islet grafts in sensitized female PD-1$^{-/-}$ recipients (rejected; middle) had substantial infiltrates that penetrated the islets and little if any insulin staining. Female islet grafts with insulin granules (brown) in sensitized female PD-1$^{-/-}$ recipients (not rejected; bottom). Histology was assessed 5 days post-islet graft rejection and at 105 days post islet transplantation for mice that accepted the islet graft. (C) Immunofluorescence pictures of male islet grafts in

sensitized B6 (not rejected) or PD-1-/- (rejected) mice. Blue: staining of nucleus with 4',6'-diamidino-2- phenylindole (DAPI); green: CD4 or CD8 staining. Immunoflluorescence was assessed 2 weeks post islet graft rejection and 130 days post islet transplantation for mice that accepted the islet graft.

Our data indicated that PD-1 is important during the priming stage, with increased accumulation of donor specific CD8 T cells when PD-1 is absent during immunization. However, ligands for PD-1 are also expressed within the islets themselves[26] and therefore lack of PD-1 signaling during the response to the transplant could potentially also contribute to the rejection. To examine this question, we targeted PD-1 with mAb specifically at the immunization stage before transplantation or at the transplantation stage or at both stages. Targeting the PD-1 pathway at both the stages tended to be more effective than blocking at either one of the stages alone **(Table 4-1)**. Taking these results together, PD-1 may be required at both the priming and transplantation stages for spontaneous allograft acceptance in sensitized recipients.

4.3.3. Loss of PD-1 leads to donor MHC class-I but not class-II dependent rejection

Whether direct presentation of donor antigens by islets is critical for their rejection is controversial. One study suggested donor MHC class-I is a major pathway in islet rejection, as class-I deficient islets were accepted in the majority of recipients[27]. In contrast, Gill and colleagues[28] showed that absence of donor class-I and II, but not either alone, prevented rejection. Given the increased frequency of anti-donor CD8 T cells in sensitized PD-$1^{-/-}$ recipients **(Figure 4-2 A)**, we hypothesized that PD-1 deficiency would lead to rejection targeting donor

class-I molecules. We compared class-I$^{-/-}$ vs. class-II$^{-/-}$ donor islet grafts in pre-sensitized PD-1$^{-/-}$ recipients. Acute graft rejection was indeed dependent on donor class-I but not class-II **(Figure 4-3)**. However, the recipients of class-I$^{-/-}$ donors did exhibit blood glucose levels close to the rejection level **(Figure 4-3)**, suggesting some attack on the islets could occur even in the absence of donor class-I.

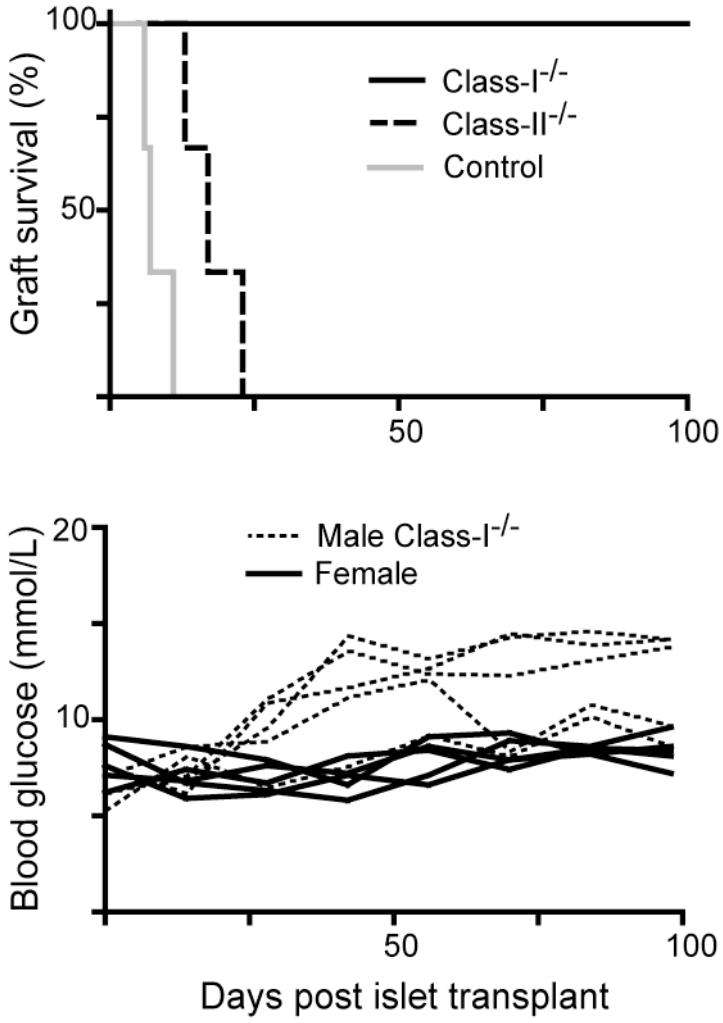


Figure 4-3. Absence of donor class-I prevented the rejection of male islet grafts in sensitized female PD-1$^{-/-}$ recipients. *Top:* Chemically induced diabetic

female PD-1$^{-/-}$ mice, previously sensitized with male spleen cells were transplanted with male class-I$^{-/-}$ Rag$^{-/-}$ (black solid line; n=5), class-II$^{-/-}$ (black dashed line; n=3) or control Rag$^{-/-}$ (Class-I & II$^{+/+}$) islet grafts (grey solid line; n=3). *Bottom:* Islets from male class-I$^{-/-}$ Rag$^{-/-}$ mice were transplanted to sensitized diabetic female B6 PD-1$^{-/-}$ recipients (black dashed lines; n=5). Data shown are blood glucose levels of individual mice, and values for sensitized diabetic female B6 PD-1$^{-/-}$ recipients of control female islets (black solid lines; n=5) are shown for comparison.

4.4. Discussion

PD-1 has been shown to play an important role in the maintenance of immunological tolerance[15, 16]. Previous studies have reported that deficiency or blockade of the PD-1/PD-L1 pathway prevented the prolongation or acceptance of MHC mismatched skin[29] and cardiac[30, 31] allografts, which were achieved with various tolerogenic regimens. Whether such induced transplant acceptance and spontaneous acceptance would involve the same tolerance mechanisms was unknown. We have shown here the significance of the PD-1 pathway in the spontaneous acceptance of weakly mismatched transplants. Female H-2^{b} mice spontaneously accepted syngeneic male islet grafts and an earlier study[7] reported that the spontaneous acceptance of male islet grafts could induce tolerance to male antigen. We tested whether co-inhibitory molecules are involved in the induction of this spontaneous acceptance of male islet grafts. Our studies represent only an initial test of the role of co-inhibitory molecules such as CTLA-4 and PD-1 by using specific blocking antibodies. While only anti-PD-1 had any discernable effect in allowing rejection of male islets by naïve recipients, and CTLA-4 seemed not to be involved, our studies using anti-CTLA-4 are too limited to completely exclude a role for this pathway in spontaneous allograft

acceptance. A larger study and examination of pre-sensitized recipients is required to fully evaluate this possibility. In the case of BTLA deficiency, only a weak effect was discernable, and even then only in the sensitized recipients.

The frequency of T cells against HY antigen in naïve female mice is low[32] and CD4 T cell help is critical in the CD8 T cell response to HY[33, 34]. Blocking or loss of PD-1 signaling in naïve female mice did not induce rejection of male islet grafts in the majority of naïve female mice. This may indicate that the HY antigens alone are insufficient to trigger islet rejection. However, an earlier study[6] from our laboratory had shown that non-diabetic female recipients induced stronger anti-HY immune responses and more peri-islet infiltration of grafts than those of diabetic female recipients. Thus, lack of rejection may also be due to the immunosuppressive effects of STZ induced diabetes on anti-HY immune responses[6]. Hence, we tested whether immunization with donor antigen in the absence of PD-1 signaling would break the spontaneous acceptance of male islet grafts. Immunization did indeed trigger rejection of accepted grafts in PD-$1^{-/-}$ recipients. This rejection was not a result of potential additional minor antigens on the immunizing male spleen cells, as the immunizing cells were also from PD-$1^{-/-}$ mice. A second objective of our experiment was to mimic the situation of islet transplant recipients, in which the recipient's immune system may already be sensitized to islet and/or donor antigens. Interestingly, we found that PD-1 has a crucial role in both the long-term acceptance of the graft after immunization with donor antigen and in initial graft acceptance in pre-sensitized recipients.

There are at least two possibilities that may explain the rejection of male islet grafts in the absence or blockade of PD-1. The first possibility is by increasing the frequency of anti-HY CD8 T cells, as we observed using HY/D^b pentamers. Increased CD8 T cells could be due to reduced PD-1 signals in the CD8s themselves, reduced PD-1 signals to HY specific helper T cells that promote CD8 expansion, or due to a reduced ability to generate adaptive Treg cells[35]. In accord with our results, previous studies have shown that loss of PD-1 or blockade of the PD-1 pathway increased the clonal expansion or percentage of the anti-donor T cell population, respectively[25, 36]. A second possibility is the absence of PD-1/PD-L1 ligation in the target of rejection, the islets. PD-L1 is expressed in many cells, including beta cells of the islets[26]. Lack of PD-L1/PD-1 in NOD mice[26, 37], or in an induced diabetes model[38], potentiated the onset of autoimmune diabetes, which suggested that PD-L1 and PD-1 interaction has a protective role in islets. Therefore, we speculated that the absence of PD-1/PD-L1 interactions within parenchymal tissues might play a role in the rejection of male islet grafts. An earlier study[39] had shown the importance of PD-1 at both priming and effector stages of CD8 T cell responses, supporting this possibility. Our data suggest that both an increased frequency of anti-donor T cells and a lack of PD-1 signals at the graft site contribute to the loss of spontaneous tolerance. Consistent with a role for CD8 T cells, class-I expression in donor islets was required for acute male islet allograft rejection in sensitized female PD-$1^{-/-}$ recipients. However, a previous study demonstrated that class-I deficient MHC mismatched islet allografts could be rejected in wild-type mice[28]. A potential explanation for the different outcomes

could be the degree of mismatches and the high frequency of donor islet reactive T cells in the previous study. In addition, we did see increased blood glucose levels in several recipients of class-I deficient islets, suggesting some attack on the islets had occurred. Blockade of PD-1 can increase DTH like immune responses[40] that may have contributed to killing of islets by a donor class-I independent mechanism.

Although sensitized WT B6 recipients had a much lower frequency of HY specific CD8 T cells compared to the sensitized PD-$1^{-/-}$ recipients, they nevertheless had a relatively high frequency of these cells. Thus, it is not yet fully clear why sensitized WT B6 mice are unable to reject a male islet graft. However, increased frequencies of HY specific T cells, compared to naïve mice, has been observed in mice made tolerant to HY through peptide administration[41, 42]. These data suggest that HY specific T cell function (e.g. cytokine production or regulatory function) is likely to be just as important as the frequency of specific T cells in determining whether HY expressing target cells are eliminated.

Our studies also have implications for understanding the regulation of immune responses to chronically persistent antigen. Failure of immune responses to clear microbes may lead to persistent or chronic infections, which is mainly associated with T cell dysfunction. The PD-1/PD-L1 pathway is involved in the impairment of T cell function during chronic viral infections; blocking the PD-1/PD-L1 pathway reversed the T cell dysfunction[43, 44]. These studies of chronic antigen

exposure, involve responses to systemic viral antigens. Under certain conditions, systemic alloantigens can stimulate immunity and yet not be cleared by the immune system. Examples include systemically injected donor hematopoietic cells and host alloantigens targeted by donor T cells during GVH reactions[45, 46]. Such persistent systemic histocompatibility antigens can switch alloimmunity (anti-donor or anti-host) into tolerance; a tolerance that may involve a number of mechanisms, including loss of CD4 co-receptor expression[45], and tolerogenic signals from PD-1[46]. Liver allografts can lead to systemic donor antigens via migration of passenger leukocytes[47], and the spontaneous tolerance of liver allografts also appears to be dependent on co-inhibitory signals, including both PD-1 and CTLA-4[48, 49]. From these virus and alloantigen studies, it might be assumed that it is the systemic nature of persistent antigens that triggers the tolerogenic co-inhibitory pathway. Our results suggest that spontaneous PD-1 dependent tolerance may not be limited to situations with high levels of systemic antigens. We found that donor alloantigen, in the form of an islet graft under the kidney capsule, resists rejection and induces a form of tolerance that is highly dependent on PD-1 function. Success of allograft transplantation is influenced by various factors. The current studies have shown that PD-1 plays a critical role in the spontaneous acceptance of weakly mismatched allografts and thus supports the idea that potentiation of naturally induced co-inhibitory signals[11], such as via PD-1[50], could be exploited as a mechanism to achieve transplantation tolerance.

Contribution

I performed all the experiments and analyzed the data

4.5. References

1. Dierselhuis M, Goulmy E. The relevance of minor histocompatibility antigens in solid organ transplantation. Curr Opin Organ Transplant 2009; 14:419-25.
2. Simpson E, Scott D, James E, Lombardi G, Cwynarski K, Dazzi F, et al. Minor H antigens: genes and peptides. Transpl Immunol 2002; 10:115-23.
3. Simpson E, Roopenian D. Minor histocompatibility antigens. Curr Opin Immunol 1997; 9:655-61.
4. Eichwald EJ, Silmser CR. Skin. Transplant Bull 1955; 2:148-9.
5. Sireci G, Barera A, Macaluso P, Di Sano C, Bonanno CT, Pio La Manna M, et al. A continuous infusion of a minor histocompatibility antigen-immunodominant peptide induces a delay of male skin graft rejection. Immunobiology 2009; 214:703-11.
6. Luo B, Chan WF, Lord SJ, Nanji SA, Rajotte RV, Shapiro AM, et al. Diabetes induces rapid suppression of adaptive immunity followed by homeostatic T-cell proliferation. Scand J Immunol 2007; 65:22-31.
7. Yoon IH, Choi SE, Kim YH, Yang SH, Park JH, Park CS, et al. Pancreatic islets induce CD4(+) [corrected] CD25(-)Foxp3(+) [corrected] T-cell regulated tolerance to HY-mismatched skin grafts. Transplantation 2008; 86:1352-60.
8. Moxham VF, Karegli J, Phillips RE, Brown KL, Tapmeier TT, Hangartner R, et al. Homeostatic proliferation of lymphocytes results in augmented memory-like function and accelerated allograft rejection. J Immunol 2008; 180:3910-8.
9. He C, Schenk S, Zhang Q, Valujskikh A, Bayer J, Fairchild RL, et al. Effects of T cell frequency and graft size on transplant outcome in mice. J Immunol 2004; 172:240-7.
10. Chan WF, Perez-Diez A, Razavy H, Anderson CC. The ability of natural tolerance to be applied to allogeneic tissue: determinants and limits. Biol Direct 2007; 2:10.
11. Thangavelu G, Smolarchuk C, Anderson CC. Co-inhibitory molecules: Controlling the effectors or controlling the controllers? Self/Nonself 2010; 1:77-8.
12. Sinclair NR, Anderson CC. Co-stimulation and co-inhibition: equal partners in regulation. Scand J Immunol 1996; 43:597-603.
13. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995; 270:985-8.
14. Ise W, Kohyama M, Nutsch KM, Lee HM, Suri A, Unanue ER, et al. CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. Nat Immunol 2010; 11:129-35.
15. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999; 11:141-51.
16. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001; 291:319-22.

17. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008; 26:677-704.
18. Chen C, Qu QX, Huang JA, Zhu YB, Ge Y, Wang Q, et al. Expression of programmed-death receptor ligands 1 and 2 may contribute to the poor stimulatory potential of murine immature dendritic cells. Immunobiology 2007; 212:159-65.
19. Oya Y, Watanabe N, Owada T, Oki M, Hirose K, Suto A, et al. Development of autoimmune hepatitis-like disease and production of autoantibodies to nuclear antigens in mice lacking B and T lymphocyte attenuator. Arthritis Rheum 2008; 58:2498-510.
20. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 2003; 4:670-9.
21. Deppong C, Juehne TI, Hurchla M, Friend LD, Shah DD, Rose CM, et al. Cutting edge: B and T lymphocyte attenuator and programmed death receptor-1 inhibitory receptors are required for termination of acute allergic airway inflammation. J Immunol 2006; 176:3909-13.
22. Nishimura H, Minato N, Nakano T, Honjo T. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. Int Immunol 1998; 10:1563-72.
23. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 1996; 8:765-72.
24. van Wijk F, Nierkens S, de Jong W, Wehrens EJ, Boon L, van Kooten P, et al. The CD28/CTLA-4-B7 signaling pathway is involved in both allergic sensitization and tolerance induction to orally administered peanut proteins. J Immunol 2007; 178:6894-900.
25. Koehn BH, Ford ML, Ferrer IR, Borom K, Gangappa S, Kirk AD, et al. PD-1-dependent mechanisms maintain peripheral tolerance of donor-reactive CD8+ T cells to transplanted tissue. J Immunol 2008; 181:5313-22.
26. Wang J, Yoshida T, Nakaki F, Hiai H, Okazaki T, Honjo T. Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes. Proc Natl Acad Sci U S A 2005; 102:11823-8.
27. Markmann JF, Bassiri H, Desai NM, Odorico JS, Kim JI, Koller BH, et al. Indefinite survival of MHC class I-deficient murine pancreatic islet allografts. Transplantation 1992; 54:1085-9.
28. Kupfer T, Beilke JN, Pham K, Buhrman J, Gill RG. "Indirect" acute islet allograft destruction in nonobese diabetic mice is independent of donor major histocompatibility complex and requires host B lymphocytes. Transplant Proc 2008; 40:462-3.
29. Dai H, Zhu H, Lei P, Yagita H, Liu J, Wen X, et al. Programmed death-1 signaling is essential for the skin allograft protection by alternatively activated dendritic cell infusion in mice. Transplantation 2009; 88:864-73.
30. Wang L, Han R, Hancock WW. Programmed cell death 1 (PD-1) and its ligand PD-L1 are required for allograft tolerance. Eur J Immunol 2007; 37:2983-90.

31. Wang W, Carper K, Malone F, Latchman Y, Perkins J, Fu Y, et al. PD-L1/PD-1 signal deficiency promotes allogeneic immune responses and accelerates heart allograft rejection. Transplantation 2008; 86:836-44.
32. Simpson E. Review lecture. Immunology of H-Y antigen and its role in sex determination. Proc R Soc Lond B Biol Sci 1983; 220:31-46.
33. Guerder S, Matzinger P. A fail-safe mechanism for maintaining self-tolerance. J Exp Med 1992; 176:553-64.
34. Keene JA, Forman J. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. J Exp Med 1982; 155:768-82.
35. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. Proc Natl Acad Sci U S A 2008; 105:9331-6.
36. Kroner A, Schwab N, Ip CW, Ortler S, Gobel K, Nave KA, et al. Accelerated course of experimental autoimmune encephalomyelitis in PD-1-deficient central nervous system myelin mutants. Am J Pathol 2009; 174:2290-9.
37. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 2006; 203:883-95.
38. Rajasalu T, Brosi H, Schuster C, Spyrantis A, Boehm BO, Chen L, et al. Deficiency in B7-H1 (PD-L1)/PD-1 coinhibition triggers pancreatic beta cell-destruction by insulin-specific, murine CD8 T cells. Diabetes 2010; 59:1966-73.
39. Keir ME, Freeman GJ, Sharpe AH. PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues. J Immunol 2007; 179:5064-70.
40. Salama AD, Chitnis T, Imitola J, Ansari MJ, Akiba H, Tushima F, et al. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. J Exp Med 2003; 198:71-8.
41. Chai JG, James E, Dewchand H, Simpson E, Scott D. Transplantation tolerance induced by intranasal administration of HY peptides. Blood 2004; 103:3951-9.
42. James E, Scott D, Chai JG, Millrain M, Chandler P, Simpson E. HY peptides modulate transplantation responses to skin allografts. Int Immunol 2002; 14:1333-42.
43. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 2006; 439:682-7.
44. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, et al. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. Nat Med 2006; 12:1198-202.
45. Chan WF, Razavy H, Anderson CC. Differential susceptibility of allogeneic targets to indirect CD4 immunity generates split tolerance. J Immunol 2008; 181:4603-12.
46. Rivas MN, Weatherly K, Hazzan M, Vokaer B, Dremier S, Gaudray F, et al. Reviving function in CD4+ T cells adapted to persistent systemic antigen. J Immunol 2009; 183:4284-91.

47. Starzl TE, Demetris AJ, Trucco M, Murase N, Ricordi C, Ildstad S, et al. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. Hepatology 1993; 17:1127-52.
48. Li W, Zheng XX, Kuhr CS, Perkins JD. CTLA4 engagement is required for induction of murine liver transplant spontaneous tolerance. Am J Transplant 2005; 5:978-86.
49. Morita M, Fujino M, Jiang G, Kitazawa Y, Xie L, Azuma M, et al. PD-1/B7-H1 interaction contribute to the spontaneous acceptance of mouse liver allograft. Am J Transplant 2010; 10:40-6.
50. Gao W, Demirci G, Strom TB, Li XC. Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. Transplantation 2003; 76:994-9.

Chapter 5: Control of *in vivo* bystander killing

5.1. Introduction

A key function of the immune system is to protect the host by mounting an immune response against invading pathogenic organisms. Cellular responses mediated by T cells play a major role in clearing infections by killing infected cells. Bystander killing occurs when activated T cells kill host bystander cells that do not express the target antigen but are in sufficiently close proximity to the antigen expressing targets. The autoimmunity seen in deficiencies of coinhibitors may be due to bystander killing. In this study, we proposed that co-inhibitory molecules are required to limit bystander killing. Previous studies have documented the ability of cytolytic T lymphocytes (CTL) to cause bystander killing *in vitro*[1, 2]. The bystander killing was dependent on Fas[2, 3] or tumor necrosis factor (TNF)[4] and major histocompatability complex (MHC)[2]. However, the killing was also independent of the MHC of bystanders[5]. Transplantation experiments, either using a mixture of syngeneic and allogeneic or xenogeneic islets[6, 7], or allophenic skin grafts[8-10], supported the concept of specific killing, as it appeared that only the donor cells were killed. In contrast, other studies have demonstrated that syngeneic skin grafts from tolerant animals were rejected or attacked by syngeneic animals, resembling a delayed type hypersensitivity response[11, 12] and bystander rejection of mixed syngeneic and xenogeneic islets appeared to occur in primed recipients[13]. However, with the exception of some tumor transplant and graft versus host (GVH) studies, the studies cited above, to our knowledge, represent the extent of knowledge in transplantation relating to rejection of bystander cells/tissues lacking expression of the target antigen. It is

therefore not yet clear how common or generalizable bystander rejection is, nor is it clear what mechanisms *in vivo* mediate bystander rejection or control it sufficiently to allow host survival.

Co-inhibitory molecules are co-signaling molecules expressed by lymphocytes and also innate cells of the immune system. Programmed death-1 (PD-1) and B and T lymphocyte attenuator (BTLA) are examples of co-inhibitory molecules and have been shown to be involved in the maintenance of self-tolerance mechanism[14, 15]. We hypothesized that CD4 or CD8 T cells have capacity to induce bystander killing in islet transplantation.

Our results showed that bystander killing can occur *in vivo* and that both subsets of T cells have the capacity to induce bystander killing. In addition, we also explored the factors that increase the specificity of the immune response by limiting the occurrence of bystander killing.

5.2. Materials and methods

5.2.1. Mice

Adult C57BL/6 (B6; H-2^b) and C3H/HeJCr (H-2^K) mice were obtained from NCI (Fredrick, MD). Adult B10.BR (H-2^K), C57BL/10 (B10; H-2^b), B6.MRL-*Tnfrsf6*^{lpr}/J (*lpr*) were purchased from Jackson Laboratory (Bar Harbor, ME). Marilyn[16], Matahari[17], OT-1 Rag$^{-/-}$, GFP Rag$^{-/-}$ mice were bred at the University of Alberta. GFP Rag$^{-/-}$ mice were developed by crossing B6.129S7-*Rag1*$^{tm1mom\ -/-}$

mice with C57BL/6-Tg(UBC-GFP) 30Scha/J mice[18]. B6 mOVA Rag$^{-/-}$ mice were developed by crossing Act-mOVA transgenic mice[19] with B6 Rag$^{-/-}$ mice. B6 Rag$^{-/-}$ C-*H2-Ab1*^{bm12} (H-2^{bm12}) mice were developed by crossing B6.129S7-*Rag1*$^{tm1mom\ -/-}$ mice with B6.C-*H2-Ab1*^{bm12} (H-2^{bm12})[20]. C57BL/6-*Pdcd1*$^{-/-}$ (PD-1$^{-/-}$) mice were originally generated by Prof. T. Honjo and colleagues[21]. C57BL/6-BTLA$^{-/-}$ (BTLA$^{-/-}$[22] mice were generated by Prof. Ken Murphy and bred at the University of Alberta. BALB/c (H-2^{d}) mice were purchased from the Health Sciences Laboratory Animal Services and Charles River Laboratories (Montreal, Quebec). Marilyn mice lacking *PD-1* were generated in our laboratory by crossing Rag2$^{-/-}$ Marilyn with C57BL/6-*Pdcd1*$^{-/-}$ mice. Experimental animals were handled and cared for according to the guidelines of the Canadian Council on Animal Care.

5.2.2. Diabetes induction and islet transplantation

Recipient mice were administered streptozotocin (Sigma-Aldrich, Mississauga, ON) at 185-190 mg/kg body weight to induce diabetes. Recipients were considered diabetic after two consecutive measurements of higher blood glucose levels (> 20 mmol/L) and measurements were done using OneTouch Ultra glucometer (Lifescan Canada, Burnaby, BC). Diabetic recipients were transplanted with mixed islets (800) from two different donor types in equal numbers (400 each). Bystander killing was defined as consecutive blood glucose measurements of >15 mmol/L (hyperglycemia). Graft survival was expressed as median survival time (MST).

5.2.3. Target and bystander islet grafts

Male or ova$^{+/+}$ islet grafts and female or ova$^{-/-}$ islet grafts were used as targets and bystanders in anti-HY or anti-ova TCR transgenic mice, respectively. Targets from Rag$^{-/-}$ GFP (green fluorescent protein) mice were used in anti-HY TCR transgenic recipients, to allow rejection of target islets to be monitored by GFP presence vs. absence. In some experiments, major histocompatability complex (MHC) mismatched (H-2^{k}) bystanders were also used in TCR transgenic recipients. In addition, MHC class-II mutant (H-2^{bm12}) bystanders were also used in CD4 TCR transgenic recipients. In case of B6 wild type or PD-1$^{-/-}$ recipients, BALB/c allografts were used as targets, whereas respective syngeneic islet grafts (wild type or PD-1$^{-/-}$) were used as bystander islets.

5.2.4. Histology

Graft-bearing kidneys were harvested and processed. Paraffin tissue sections of 5 μm thickness were stained with hematoxylin-eosin and insulin as previously described[23].

5.2.5. Immunofluorescence

In some experiments, graft bearing kidneys were harvested at the end of the experiment followed by fixation in 4% formaldehyde overnight at 4°C.After fixing, grafts were washed in PBS and then incubated in a 15% sucrose–PBS solution and subsequently in a 30% sucrose–PBS solution at 4°C. Finally, grafts

were stored at -80°C after embedded in OCT compound. Cross-sections (5 μm) were cut from kidney grafts, fixed, blocked with goat serum and stained with guinea pig anti-insulin (1:1000 dilution; Dako, ON, Canada) and rabbit polyclonal anti-GFP antibody (1:100 dilution; Santacruz Biotechnology, CA, USA). The secondary antibodies such as goat anti-rat Alexaflour 488 and Alexaflour 594 (1:200 dilution; Invitrogen Laboratories, ON, Canada) were used for GFP and insulin respectively. Sections without primary antibodies were used as negative controls. Slides were visualized on a compound fluorescent microscope (Axioplan, Axiovision 4.1 software, Carl Zeiss, ON, Canada).

5.2.6. Immunization and IFN-γ neutralization antibody treatment

In sensitization experiments, anti-HY TCR Tg or OT-1 recipients were immunized with two million live male cells or $ova^{+/+}$ spleen cells, respectively, a week before transplantation. In the experiments involving sensitization of WT or PD-$1^{-/-}$ mice, recipients were immunized with allogeneic (BALB/c) live spleen cells two weeks before transplantation. In one of the experiments, Marilyn recipients were given neutralizating anti-IFN-γ (XMG 1.2) antibody at a dose of 0.5 mg/mouse every 5 days starting from day 3 post transplantation and the treatment was stopped at day 33 post transplantation.

5.2.7. Statistical analysis

Statistical analyses were done using Prism 4 (GraphPad Software) and the log rank test was used to compare survival curves. A value of $p < 0.05$ was considered significant.

5.3. Results

5.3.1. CD4 T cells can induce bystander killing *in vivo*

We investigated whether bystander killing can occur *in vivo* using islet transplantation models in T cell receptor (TCR) transgenic mice. Chemically induced diabetic female CD4 or CD8 TCR transgenic mice were transplanted either with islets from male or female B6 donors (H-2^b), or a mixture of the two sets of islets. Marilyn mice were used to test whether bystander killing can occur *in vivo* in islet transplantation and also the capacity of CD4 T cells to induce bystander killing. Female Marilyn mice have monoclonal CD4 T cells that recognize male (HY) peptide (NAGFNSNRANSSRSS) in the context of I-A^b. Consistent with the results from our previous study[24], Marilyn recipients of male islets (antigen bearing cells; targets alone) rejected their transplants whereas 100% of recipients of female islets (non-antigen bearing cells; bystanders alone) accepted their islet transplant long term **(Table 5-1A)**. We also tested whether recipient T cells can kill the female bystander islets when they kill the male target cells. Interestingly, majority of the animals with mixed male and female B6 islets rejected their grafts, indicative of bystander killing (hyperglycemia; **Table 5-1A**). Histological examination of the rejected islet grafts revealed mononuclear

infiltration of the islets. Furthermore, these grafts showed an absence or severe reduction in the number of insulin-positive cells **(Figure 5-1)** compared to those from recipients of bystanders alone. In addition, infiltration of the kidney parenchyma was observed. Together, these studies indicated that target islets capable of direct presentation of donor antigen to CD4 T cells were capable of stimulating bystander killing. To examine whether such bystander killing was unique to directly stimulated CD4 T cells, or if even the lower level of antigen presentation that occurs indirectly might be capable of stimulating bystander rejection, we used BALB/c male islets as targets. Marilyn CD4 T cells are not able to directly recognize HY in H-2^d BALB/c targets and hence, will only recognize processed HY from BALB/c islets presented in I-A^b. Interestingly, Marilyn T cells had the capacity to kill female bystander cells indirectly stimulated by the presence of male BALB/c targets **(group9; Table 5-1A)**. In addition, this experiment demonstrated that the bystander killing was not due to the transfer of MHC from the targets to bystanders. We next evaluated the capacity of CD8 T cells to induce bystander killing using Matahari mice. Matahari has monoclonal CD8 T cells, which express a transgenic T-cell receptor specific for the HY peptide (WMHHNMDLI) in the context of D^b.

There was no bystander killing in naïve Matahari recipients (data not shown). The blood glucose levels in naïve recipients were high despite not reaching the cutoff threshold (> 15 mmol/L), which suggested that there was some degree of bystander injury mediated by CD8 T cells in this group. We also tested this

conclusion with naïve OT-1 T cells, which recognize ova peptide (chicken ovalbumin 257-264) in the context of K^b. Similar to naïve Matahari mice, we did not observe bystander rejection in naïve OT-1 mice **(Table 5-1B)**.

5.3.2. Role of MHC and IFN- γ in bystander killing

Having demonstrated that CD4 T cells can induce bystander killing *in vivo*, we investigated the factors that can influence bystander killing. To determine the role of MHC in bystander killing, we used bystander cells that express different MHC alleles from the recipients ($H\text{-}2^b$). As shown in the **Table 5-1A**, bystander killing was reduced ($P< 0.05$) when there was a change in the MHC molecule of the bystander cells. We also used MHC congenic B10.BR mice to elucidate that the bystander killing was due to the change in the MHC molecule of the bystander cells rather than the background of the strain. Histological examination of the grafts from the above recipients revealed the presence of insulin-positive cells **(Figure 5-1)**. In B10.BR bystander recipients, there is a possibility that the bystander islets somehow prevent killing of target islets and thus those mice **(Figure 5-2)**. IFN-γ can modulate the immune response by up-regulating and class-II expression[26] in cells. Moreover, the combination of IFN-γ and TNF-α enhanced the up-regulation of MHC class-II expression in islets[25, 27]. To determine the role of IFN-γ in bystander killing, mixed islet graft recipients were treated with anti- IFN-γ neutralizing antibody. Interestingly, anti- IFN-γ treatment prevented the bystander killing **(Table 5-1A)**.

5.3.3. Fas is required for bystander killing but not target killing

The Fas-FasL pathway plays an important role in the maintenance of peripheral tolerance[28]. In the case of bystander killing, *in vitro* studies suggested that the Fas-FasL pathway may be required to induce bystander killing[2, 3]. We next analyzed whether Fas is required in bystander killing *in vivo* using Fas mutant (*lpr*) MHC matched bystander islets. Consistent with an essential role for Fas, *lpr* bystanders were resistant to being killed *in vivo* **(Table 5-1 A)** as the recipients maintained normoglycemia until the end of the experiment. However, male *lpr* targets alone were rejected by Marilyn T cells, indicating that targets and bystander islets are killed by different mechanisms. We next investigated the role of Fas in bystander killing of the pre-sensitized recipients. Consistent with the result in naïve recipients, bystander killing was Fas dependent even in sensitized mice **(Table 5-2)**. Interestingly, unlike sensitized CD4 T cells, sensitized CD8 T cells rejected *lpr* bystanders **(Table 5-2A)** suggesting that other mediators of killing such as IFN-γ or granzymes may be involved in the bystander killing.

Table 5-1A: CD4 T cells can induce bystander killing and the bystander killing is dependent on the MHC of the bystanders, Fas and IFN-γ

Group	Islet grafts	Survival of islet grafts	% Graft survival
1	Targets only	14, 15	0
2	H-2^{b} Bystanders only	> 100 x 5	100[a]
3	Targets and H-2^{b} bystanders	12 x 5, 13, 21 x 5, > 32*, > 100 x 3	26.6[b]
4[c]	Targets and H-2^{k} bystanders	24, > 100 x 7	87.5[a]
5	Targets and H-2^{bm12} bystanders	> 100 x 4	100[a]
5.5	Targets and H-2^{bm12} bystanders	11 x 2, 12	0
6	*lpr* Targets only	12, 16 x 3	0[b]
7	Targets and *lpr* H-2^{b} bystanders	> 100 x 4	100[a]
8	Targets and H-2^{b} bystanders + anti-IFNγ	> 100 x 4	100[a]
9[d]	H-2^{d} Targets and H-2^{b} bystanders	13, 21 x 2, > 100 x 1	25[b]

Group 1-5 and 6-9: Diabetic female Marilyn recipients were transplanted with either targets alone (male islet grafts), bystanders alone (female islet grafts) or mixed islets of targets and bystanders. Group 5.5 differed only in that the recipient was a PD-1$^{-/-}$ Marilyn.
*Animal found dead
[a] and [b] are significantly different ($p < 0.05$)
[c] fully MHC mismatched (H-2^{k}) bystanders (C3H or B10.BR bystanders -4 recipients/group; total n=8)
[d] BALB/c targets (indirect stimulation)

Table 5-1 B: Inability to detect bystander killing (rejection) by CD8 T cells

Group	Islet grafts	Survival of islet grafts	% Graft survival
1	Targets only	2, 3 x 2	0^{a}
2	Bystanders only	> 100 x 3	100^{b}
3	Targets and bystanders ($H\text{-}2^{b}$)	> 100 x 3	100^{b}
4	Targets and bystanders ($H\text{-}2^{k}$)	> 100 x 3	100^{b}

Group 1-4: Diabetic OT-1 recipients were transplanted with either targets ($ova^{+/+}$ islet graft) and/or bystanders ($ova^{-/-}$ islet graft).
[a] and [b] are significantly different ($p < 0.05$).

5.3.4. Sensitized recipients

In vivo, bystander killing has more often been observed in recipients previously primed to the target antigen(s)[13]. In the clinical setting, islet transplant recipients are autoimmune type-1 diabetics and are sensitized to islet auto-antigens, and in some cases alloantigens, prior to transplantation. To mimic the clinical setting, we investigated the nature of bystander killing in sensitized recipients. Recipient mice were sensitized to target antigen by immunizing them with target cells prior to islet transplantation. In contrast to naïve CD4 T cells (non-sensitized recipients), primed CD4 T cells (sensitized recipients) had the capacity to kill MHC mismatched bystander cells **(Table 5-2)**.

We also tested whether bystander killing can occur in sensitized CD8 TCR transgenic recipients. Interestingly, bystander killing was observed in both Matahari and OT-1 recipients. In addition, activated OT-1 T cells can induce bystander killing of MHC mismatched bystanders **(Table 5-2)**.

Evidence of bystander killing was demonstrated by hematoxylin and eosin (H & E) staining of islet grafts, where mononuclear infiltration was observed. Furthermore, there was an absence of insulin positive cells in rejected grafts **(Figure 5-1)**

Table 5-2: CD8 and CD4 T cells from sensitized mice can induce bystander killing

Group	Islet grafts	Survival of islet grafts	% Graft survival
1	Bystanders only	> 100 x 2	100[a]
2	Targets and bystanders (H-2^k)	14 x 2, 18 x 2	0[b]
3	Targets and *lpr* bystanders (H-2^b)	7, > 100 x 3	80[a]
4	Bystanders only	> 100 x 3	100[a]
5	Targets and bystanders (H-2^b)	2 x 2, 3	0[b]
6	Targets and bystanders (H-2k)	2, 3 x 2	0[b]
7	Targets and *lpr* bystanders (H-2^b)	2, 3	0[b]

Group 1-3: Diabetic female Marilyn recipients were transplanted with either bystanders (female islet grafts) or targets (male islet grafts) and bystanders.
Group 4-7: Diabetic OT-1 recipients were transplanted with either bystanders ($ova^{-/-}$ islet graft) or targets ($ova^{+/+}$ islet graft) and bystanders.
[a] and [b] are significantly different ($p < 0.05$)

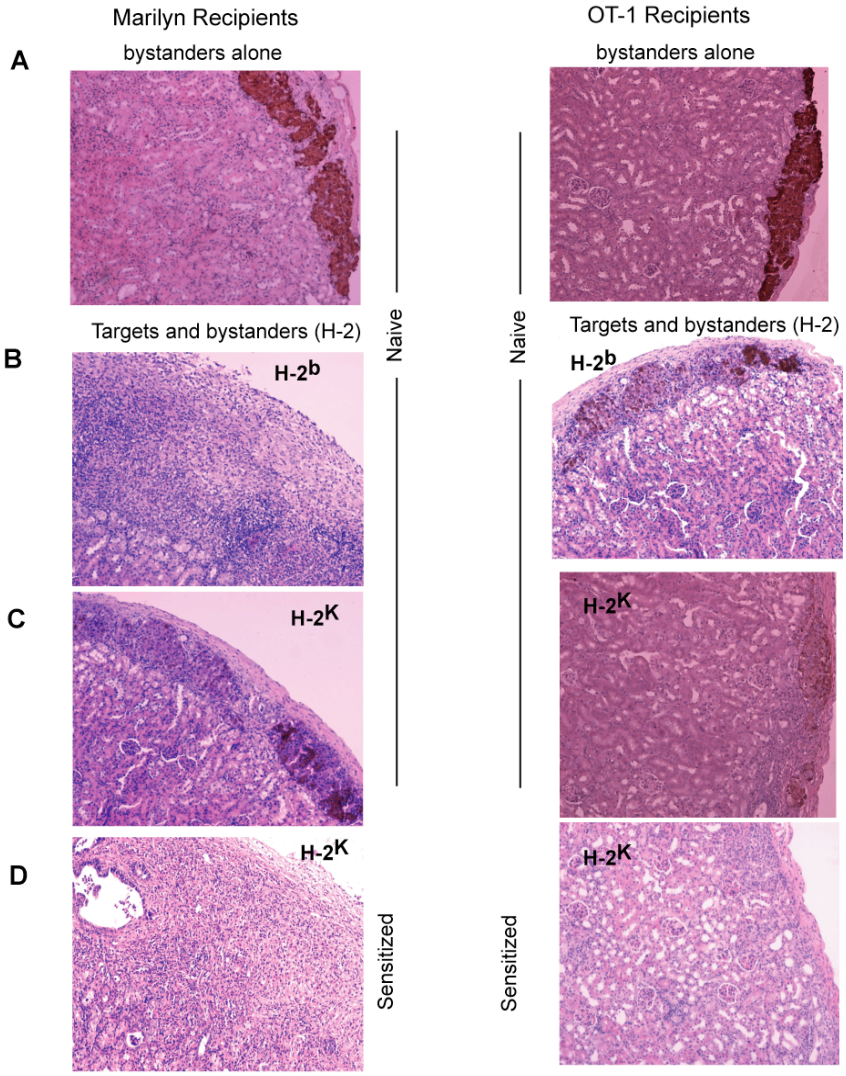


Figure 5-1: Absence of insulin positive cells and presence of monocytic infiltration in mixed islet grafts where bystander islets are rejected. Representative H&E-stained islet grafts from different groups of CD4 (Marilyn) and CD8 TCR (OT-1) transgenic recipients (all stained with H&E and anti-insulin antibody; ×100). **A:** Diabetic recipients were transplanted with bystanders alone and the grafts were harvested 100 days post transplantation. Insulin positive cells (brown color; not rejected) were present in the grafts. **B-C:** Diabetic recipients were transplanted with mixed islets of targets and bystanders (H-2^b or H-2^k). Mononuclear cell infiltrations and absence of insulin positive cells were observed in targets and H-2^b bystanders (rejected) of Marilyn recipients. Bystanders (H-2^b or H-2^k) of OT-1 recipients and (H-2^k) of Marilyn recipients had insulin positive cells (not rejected; 100 days post transplantation). **D:** Pre-sensitized diabetic recipients were transplanted with mixed islets of targets and bystanders (H-2^k). Absence of insulin positive cells and presence of mononuclear cell infiltration were observed in mixed grafts (rejected)

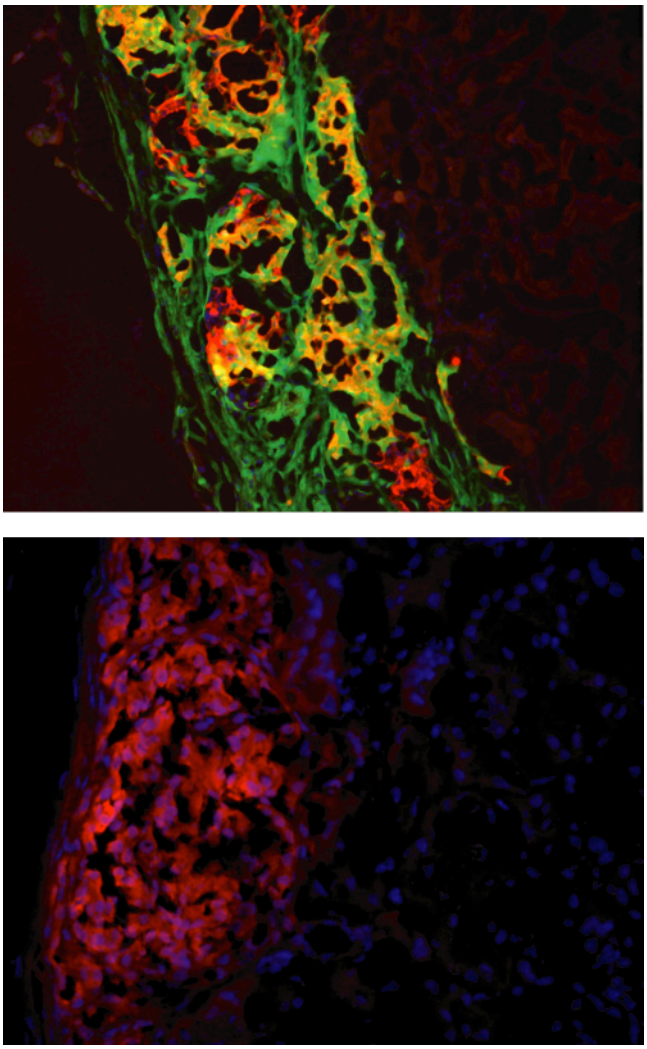


Figure 5-2: Absence of target islets (GFP+) in animals that did not exhibit bystander killing. Green- GFP, Red- Insulin, Blue- DAPI
Top: Presence of $GFP^{+/+}$ (orange) islets and $GFP^{-/-}$ islets from control $Rag^{-/-}$ recipients. ***Bottom:*** *Representative picture of islet grafts from Marilyn* recipients that were transplanted with $GFP^{+/+}$ targets and $GFP^{-/-}$ MHC mismatched bystander islets ($H\text{-}2^{K}$). Absence of target $GFP^{+/+}$ (yellow) islets, but presence of $GFP^{-/-}$ islets (red) indicated that the targets were killed.

5.3.5. Bystander killing by a polyclonal T cell repertoire

The above studies using TCR Tg mice, which have a high frequency of responding T cells, were useful to define the maximal potential for CD4 and CD8 T cells to generate bystander killing. However, it was important to test the occurrence of bystander killing in the setting of a normal polyclonal T cell repertoire of wild type mice (WT) and examine mechanisms that may normally limit bystander killing. Using diabetic C57BL/6 (B6) recipients we transplanted mixed islet grafts from allogeneic and syngeneic donors. There was little overt bystander killing in naïve or sensitized WT recipients **(Figure 5-3)**. However, the blood glucose levels in sensitized WT mice were always-higher than those of naïve WT mice, suggesting that there was some level of bystander killing in this group **(Figure 5-4)**. To begin to determine which mechanisms might be responsible for limiting bystander killing, we tested bystander killing in mice lacking the co-inhibitory receptor programmed death-1 (PD-1) or B and T lymphocyte attenuator (BTLA). We chose PD-1 because the ligands for PD-1 or BTLA are present in both lymphoid tissue and parenchymal tissue[14, 29, 30], including islets[31, 32] making them particularly good candidates for regulating bystander killing. Interestingly, bystander killing was only observed in a majority of the sensitized PD-$1^{-/-}$ mice but not in naïve PD-$1^{-/-}$ group **(Figure 5-3)**. However, the blood glucose levels were higher in naïve PD-$1^{-/-}$ than those of naïve WT mice **(Figure 5-4)**, indicating that there was some degree of bystander killing which occurred in the absence of PD-1, even in naïve mice. Histology of the rejected grafts revealed the absence of insulin positive cells **(Figure 5-3)**.

There was no bystander killing observed in sensitized BTLA$^{-/-}$ mice (>100 x4) suggesting that BTLA may not be required to limit bystander killing.

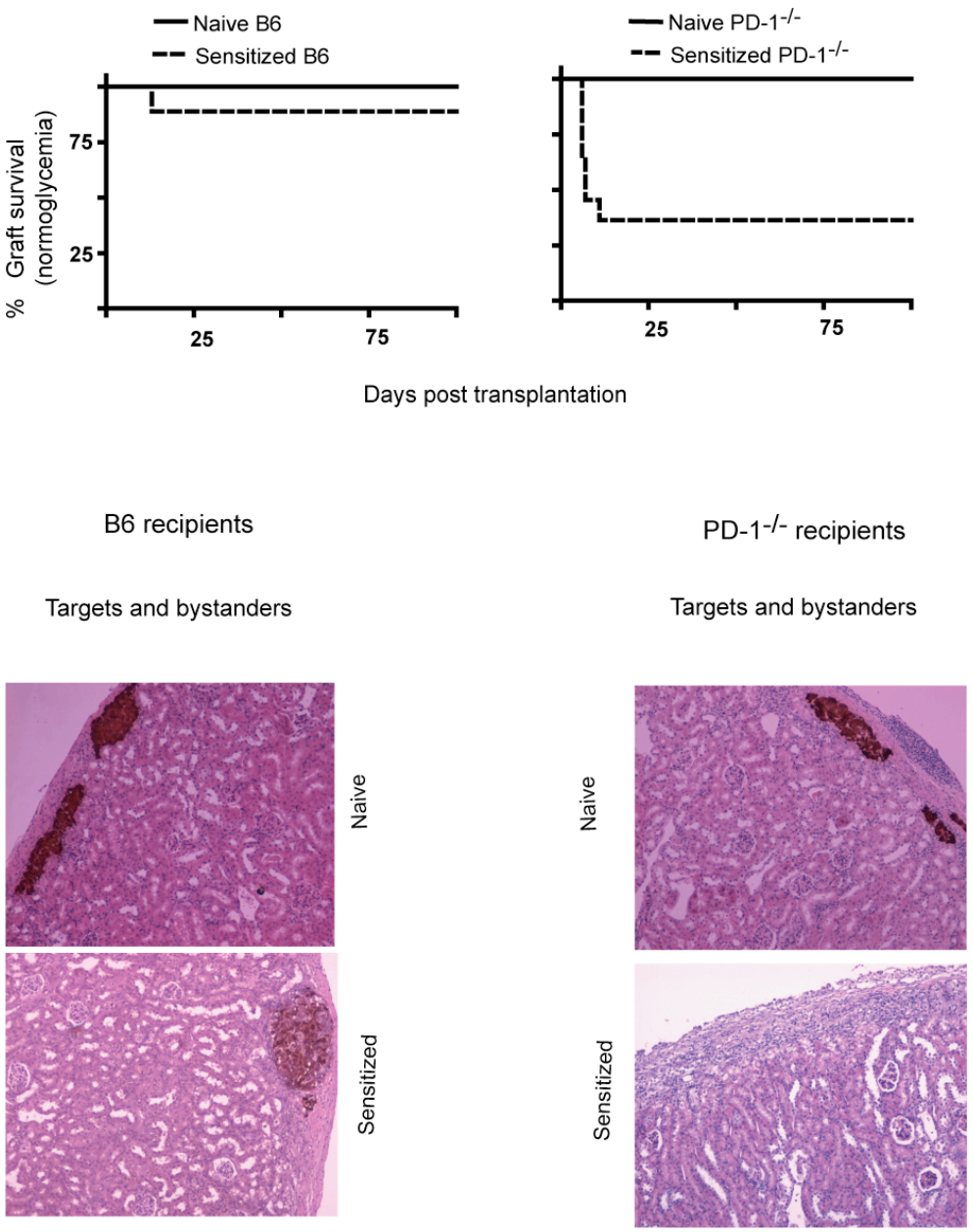


Figure 5-3: PD-1 can limit the bystander killing. *Top Left:* Diabetic naïve (black solid line; n=4) or sensitized (black dashed line; n=9) wild type recipients were transplanted with mixed islets of targets and bystanders. $p = 0.50$ between the two groups *Top Right:* Diabetic naïve (black solid line; n=5) or sensitized (black dashed line; n=11) PD-$1^{-/-}$ recipients were transplanted with mixed islets of targets and bystanders. $p = 0.02$ between the two groups. *Bottom:* Representative H&E-stained islet grafts from different groups of WT and PD-$1^{-/-}$ recipients (all stained with H&E and anti-insulin antibody; ×100). Presence of insulin positive cells (brown; not rejected) in mixed islet grafts from naïve, sensitized WT and naïve PD-$1^{-/-}$ recipients. Presence of mononuclear cell infiltrations and absence of insulin positive cells were observed in mixed islet grafts (rejected) of sensitized PD-$1^{-/-}$ recipients.

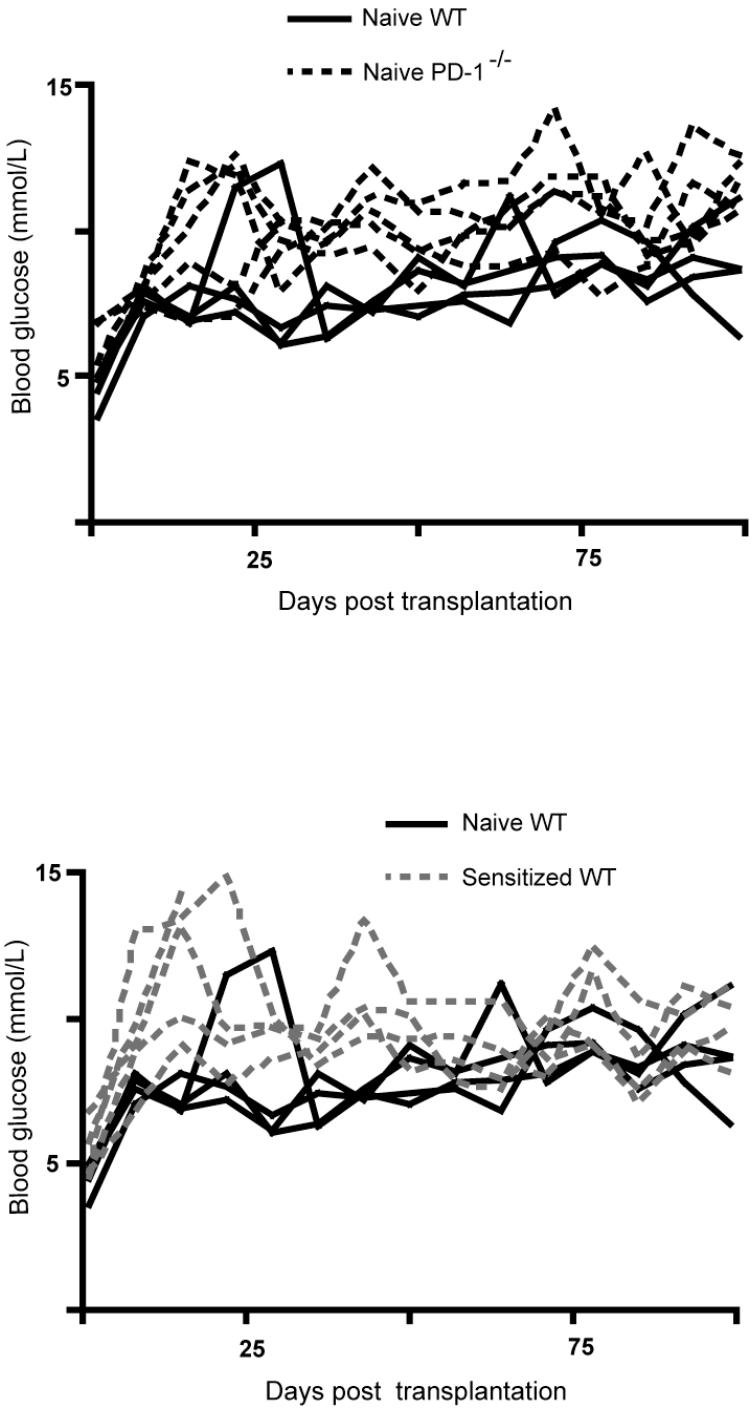


Figure 5-4: Increased blood glucose levels in sensitized WT and naïve PD-1$^{-/-}$ mice. *Top:* Blood glucose levels from naïve WT mice (black solid line; n=4) and naïve PD-1$^{-/-}$ recipients (black dashed line; n=5). *Bottom:* Blood glucose levels from naïve WT mice (black solid line; n=4) and sensitized WT recipients (grey dashed line; n=5). Data shown are blood glucose levels of individual mice.

5.4. Discussion

The concept of bystander killing is a controversial topic and has been in debate for many decades. Elegant studies conducted in 1960's[8] and 1980's[10] had provided the evidence for the highly specific nature of the effector mechanism by transplanting skin graft from allophenic mice (B6 and A/J) to either of its parental mice (B6 or A/J). The parental strain's immune system rejected the allogeneic part of the allophenic skin graft, but not the syngeneic component. In contrast to these studies, earlier studies reported that the effector mechanism could be non-specific[11, 12]. In addition, *in vitro* studies also reported that CD4 or CD8 CTL have the capacity to induce bystander killing[1, 2, 33, 34]. A recent study reported that there was a concurrent loss of alpha cells along with beta cells in the islets of newly diabetic NOD mice[35]. It was suggested that the loss was not due to the bystander injury, but based on the current study we think that the loss may be due to bystander killing. Although *in vitro* studies suggested that bystander killing can occur, it is not clear whether one would observe this phenomenon *in vivo.*

The current study hypothesized that bystander killing can occur *in vivo*. We tested this hypothesis using an islet transplant model in TCR transgenic (Tg) mice and demonstrated that bystander killing can occur *in vivo*. Anti-HY CD4 T cells from TCR transgenic (Tg) mice can kill female islets in the presence of male islets. The killing was reduced in recipients that were transplanted with MHC mismatched bystander islets. There are many possibilities that can explain the increased incidence of bystander killing of MHC matched bystander islets. One possibility

is due to the cross presentation of target antigen to Marilyn T cells by H-2^b bystander islet parenchymal cells or antigen presenting cells. The killing may also due to the expression of the positively selecting H-2^b allele by bystander cells. The affinity of the interaction between Marilyn's TCR and H-2^b on the bystander cells may influence the outcome of the bystander killing. It has been reported that in non-vascularized grafts such as skin grafts, host endothelial cells can pick up the target antigen from the graft and present it to T cells. The specific killing of endothelial cells can lead to ischemia and graft loss[17]. Like skin grafts, islet grafts are also classified under the non-vascularized category. There is also a possibility that bystander killing might have occurred due to specific killing of endothelial cells. However, in the current study, bystander killing was reduced using MHC mismatched bystander cells, which ruled out the above-mentioned possibility. An *in vitro* earlier study suggested that pre-activation of T cells by PMA allowed them to acquire the capacity of killing MHC mismatched bystander cells[2]. Similarly, we observed that activated CD4 T cells (sensitized mice) induced killing of MHC mismatched bystander cells *in vivo*.

Cell mediated cytotoxicity and cytokine production are some of the important mechanisms involved in the development of immunopathology induced by CD4 T cells[1]. There are two pathways that have been described in cytotoxic CD4 T cells namely, FasL-Fas[36] and perforin[37]. The FasL- Fas pathway has been shown to be involved in both antigen specific and non-specific killing[2, 3, 38]. Consistent with the results of previous *in vitro* studies[1, 39], we observed that *in vivo* bystander

killing was dependent on the Fas pathway. This result suggested that there is a high chance of occurrence of immunopathology during infections due to the bystander killing of Fas^{+} cells. However, CD8 T cells from the sensitized mice could kill the Fas mutant bystander cells, which suggest that Fas is not the primary pathway for CD8 T cells to induce bystander killing. CTL can release IFN-γ upon antigen dependent activation which can up regulate Fas expression on Fas negative bystander cells[40]. Although in the current model the bystander killing was inhibited by using IFN-γ neutralization treatment, the mechanism of bystander killing may not be due to IFN-γ dependent up-regulation of Fas. If bystander killing is dependent on up-regulation of Fas by IFN-γ, then the MHC mismatched bystanders would be expected to have been killed at a similar rate to the MHC matched bystanders in earlier experiments, as we used the same targets through out the experiments. Future studies involving use of $IFN\text{-}R^{-/-}$ mice as bystander islets will be helpful to explore the role of IFN-γ in the current model.

In contrast to naïve CD4 T cells (non-sensitized TCR transgenic recipients), naive CD8 T cells could not induce bystander killing regardless of the MHC of the bystander cells. A previous study suggested the requirement of a three-cell conjugate model for bystander killing, namely a specific target, bystander cells and CTL[41]. CD8 TCR Tg cells usually reject targets within two days and we speculate that the absence of persistent target cells prevented the rejection of bystander cells. However, we found that primed CD8 T cells (sensitized mice) can induce bystander killing and this result is consistent with those from previous

studies[42, 43], which demonstrated the occurrence of collateral damage to neurons using activated CD8 T cells. Although the results from TCR Tg mice are interesting, they may not recapitulate normal physiology due to the presence of a high frequency of monoclonal T cells instead of a polyclonal T cell repertoire. WT mice were used as recipients to assess the capacity of a polyclonal T cell repertoire in inducing bystander killing. In contrast to the results in TCR Tg animals, we did not observe bystander killing in naïve or sensitized WT mice. Similarly, studies in rats[7] and WT mice[6] also reported the absence of bystander killing using allogeneic and xenogenic targets, respectively. However, priming before transplantation killed syngeneic islets in the presence of xenogenic targets[13]. Discrepancies between these results may be due to the experimental model we used in the current study and also the methods used to declare the occurrence of bystander killing. We speculated that bystander killing can occur in WT mice, but it is limited by natural mechanisms such as co-inhibitory pathways. PD-1 is a co-inhibitory molecule expressed by activated T cells, B cells and other immune cells. It has been shown that PD-1 is required to establish the self-tolerance mechanism by knockout of the PD-1 gene in mice[15, 21]. PD-L1 is one of the known ligands of PD-1 and is expressed in islets[32], lymphoid tissues and other non-lymphoid tissues[29, 30]. Expression of PD-L1 in parenchymal tissue has a beneficial effect against autoimmune diabetes by controlling self reactive T cells in NOD mice[29].

In support of our speculation, bystander killing was observed in sensitized PD-1$^{-/-}$ mice. Even in non-sensitized mice, we observed increased blood glucose levels in the absence of PD-1 and this could be due to some degree of bystander injury.

The reasons for bystander killing in sensitized PD-1$^{-/-}$ mice may be due to the enhanced priming and cytolytic responses of T cells and also due to the possibility of increased cytokine responses such as, IFN-γ. The current data suggest that PD-1 is required to limit the bystander killing or collateral damage. This notion is also supported by those previous studies which used viral infection model and EAE[44]. In hepatic adenoviral infection, extensive hepatocellular damage was reported in PD-1$^{-/-}$ mice despite rapid clearance of the virus[45]. Similarly in EAE model, there was a loss of axon due to bystander damage in PD-1–deficient myelin mutant mice[46]. Furthermore, lack of PD-L1 induced bystander axonal damage due to increased pro-inflammatory cytokines[44]. Hence, the PD-1 pathway played an important role in the regulation of destructive immune responses generated against microbial infections as well as allograft rejections. Future studies focused on the role of other factors that can limit or prevent bystander killing are warranted. One of the possible factors could be Tregs due to their suppressive function on T cells and their role in the self-tolerance mechanism.

In summary, the current study demonstrated that the bystander killing can occur *in vivo* and also the mechanisms that can limit this process. The current findings may be limited to the transplant model we have used, but the rules might instead

be more generalizable and applicable to the other immune models as well. Bystander killing has a beneficial effect in controlling the spread of viral infection by killing the cells at the vicinity of the infected cells, before it presents the endogenous viral antigen. Elucidating the mechanisms that control bystander destruction will help in the development of more comprehensive approaches to prevent type-1 diabetes in disease prone individuals. Moreover, identification of the mechanisms involved in this understudied area of immune tolerance has important implications in designing tolerance strategies for islet transplantation.

Contribution

I performed all the experiments and analyzed the data

5.5. References

1. Gagnon SJ, Ennis FA, Rothman AL. Bystander target cell lysis and cytokine production by dengue virus-specific human CD4(+) cytotoxic T-lymphocyte clones. J Virol 1999; 73:3623-9.
2. Smyth MJ, Krasovskis E, Johnstone RW. Fas ligand-mediated lysis of self bystander targets by human papillomavirus-specific CD8+ cytotoxic T lymphocytes. J Virol 1998; 72:5948-54.
3. Smyth MJ. Fas ligand-mediated bystander lysis of syngeneic cells in response to an allogeneic stimulus. J Immunol 1997; 158:5765-72.
4. Smyth MJ, Sedgwick JD. Delayed kinetics of tumor necrosis factor-mediated bystander lysis by peptide-specific CD8+ cytotoxic T lymphocytes. Eur J Immunol 1998; 28:4162-9.
5. Burrows SR, Fernan A, Argaet V, Suhrbier A. Bystander apoptosis induced by CD8+ cytotoxic T cell (CTL) clones: implications for CTL lytic mechanisms. Int Immunol 1993; 5:1049-58.
6. Korsgren O, Jansson L. Characterization of mixed syngeneic-allogeneic and syngeneic-xenogeneic islet-graft rejections in mice. Evidence of functional impairment of the remaining syngeneic islets in xenograft rejections. J Clin Invest 1994; 93:1113-9.
7. Sutton R, Gray DW, McShane P, Dallman MJ, Morris PJ. The specificity of rejection and the absence of susceptibility of pancreatic islet beta cells to nonspecific immune destruction in mixed strain islets grafted beneath the renal capsule in the rat. J Exp Med 1989; 170:751-62.
8. Mintz B, Silvers WK. Histocompatibility antigens on melanoblasts and hair follicle cells. Cell-localized homograft rejection in allophenic skin grafts. Transplantation 1970; 9:497-505.
9. Rosenberg AS, Katz SI, Singer A. Rejection of skin allografts by CD4+ T cells is antigen-specific and requires expression of target alloantigen on Ia- epidermal cells. J Immunol 1989; 143:2452-6.
10. Rosenberg AS, Singer A. Evidence that the effector mechanism of skin allograft rejection is antigen-specific. Proc Natl Acad Sci U S A 1988; 85:7739-42.
11. Hasková V CJ, Biege G. The fate of long-term tolerated grafts after retransplantation in mice. Folia Biol (Praha) 1965; 11:446-51.
12. McCullagh P. Rejection of syngeneic skin grafts following retransplantation from histo-incompatible rats. Aust J Exp Biol Med Sci 1985; 63 (Pt 3):287-98.
13. Korsgren O, Wallgren AC, Satake M, Karlsson-Parra A. Xenograft rejection of fetal porcine islet-like cell clusters in the rat: effects of active and passive immunization. Xenotransplantation 1999; 6:271-80.
14. Murphy KM, Nelson CA, Sedy JR. Balancing co-stimulation and inhibition with BTLA and HVEM. Nat Rev Immunol 2006; 6:671-81.

15. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001; 291:319-22.
16. Lantz O, Grandjean I, Matzinger P, Di Santo JP. Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation. Nat Immunol 2000; 1:54-8.
17. Valujskikh A, Lantz O, Celli S, Matzinger P, Heeger PS. Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway. Nat Immunol 2002; 3:844-51.
18. Schaefer BC, Schaefer ML, Kappler JW, Marrack P, Kedl RM. Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions in vivo. Cell Immunol 2001; 214:110-22.
19. Ehst BD, Ingulli E, Jenkins MK. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. Am J Transplant 2003; 3:1355-62.
20. Silverman RH, Zhou A, Auerbach MB, Kish D, Gorbachev A, Fairchild RL. Skin allograft rejection is suppressed in mice lacking the antiviral enzyme, 2',5'-oligoadenylate-dependent RNase L. Viral Immunol 2002; 15:77-83.
21. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999; 11:141-51.
22. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 2003; 4:670-9.
23. Korbutt GS, Suarez-Pinzon WL, Power RF, Rajotte RV, Rabinovitch A. Testicular Sertoli cells exert both protective and destructive effects on syngeneic islet grafts in non-obese diabetic mice. Diabetologia 2000; 43:474-80.
24. Chan WF, Razavy H, Anderson CC. Differential susceptibility of allogeneic targets to indirect CD4 immunity generates split tolerance. J Immunol 2008; 181:4603-12.
25. Yamada K, Miyajima E, Nonaka K. Inhibition of cytokine-induced MHC class II but not class I molecule expression on mouse islet cells by niacinamide and 3-aminobenzamide. Diabetes 1990; 39:1125-30.
26. Giroux M, Schmidt M, Descoteaux A. IFN-gamma-induced MHC class II expression: transactivation of class II transactivator promoter IV by IFN regulatory factor-1 is regulated by protein kinase C-alpha. J Immunol 2003; 171:4187-94.
27. Kim KA, Kim S, Chang I, Kim GS, Min YK, Lee MK, et al. IFN gamma/TNF alpha synergism in MHC class II induction: effect of nicotinamide on MHC class II expression but not on islet-cell apoptosis. Diabetologia 2002; 45:385-93.
28. Nagata S, Golstein P. The Fas death factor. Science 1995; 267:1449-56.

29. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 2006; 203:883-95.
30. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol 2002; 169:5538-45.
31. Pakala SV, Ilic A, Chen L, Sarvetnick N. TNF-alpha receptor 1 (p55) on islets is necessary for the expression of LIGHT on diabetogenic T cells. Clin Immunol 2001; 100:198-207.
32. Wang J, Yoshida T, Nakaki F, Hiai H, Okazaki T, Honjo T. Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes. Proc Natl Acad Sci U S A 2005; 102:11823-8.
33. Duke RC. Self recognition by T cells. I. Bystander killing of target cells bearing syngeneic MHC antigens. J Exp Med 1989; 170:59-71.
34. Nikiforow S, Bottomly K, Miller G, Munz C. Cytolytic CD4(+)-T-cell clones reactive to EBNA1 inhibit Epstein-Barr virus-induced B-cell proliferation. J Virol 2003; 77:12088-104.
35. Pechhold K, Zhu X, Harrison VS, Lee J, Chakrabarty S, Koczwara K, et al. Dynamic changes in pancreatic endocrine cell abundance, distribution, and function in antigen-induced and spontaneous autoimmune diabetes. Diabetes 2009; 58:1175-84.
36. Ju ST, Cui H, Panka DJ, Ettinger R, Marshak-Rothstein A. Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. Proc Natl Acad Sci U S A 1994; 91:4185-9.
37. Blazar BR, Taylor PA, Vallera DA. CD4+ and CD8+ T cells each can utilize a perforin-dependent pathway to mediate lethal graft-versus-host disease in major histocompatibility complex-disparate recipients. Transplantation 1997; 64:571-6.
38. Lewinsohn DM, Bement TT, Xu J, Lynch DH, Grabstein KH, Reed SG, et al. Human purified protein derivative-specific CD4+ T cells use both CD95-dependent and CD95-independent cytolytic mechanisms. J Immunol 1998; 160:2374-9.
39. Wang R, Rogers AM, Ratliff TL, Russell JH. CD95-dependent bystander lysis caused by CD4+ T helper 1 effectors. J Immunol 1996; 157:2961-8.
40. Mullbacher A, Lobigs M, Hla RT, Tran T, Stehle T, Simon MM. Antigen-dependent release of IFN-gamma by cytotoxic T cells up-regulates Fas on target cells and facilitates exocytosis-independent specific target cell lysis. J Immunol 2002; 169:145-50.
41. Lanzavecchia A. Is the T-cell receptor involved in T-cell killing? Nature 1986; 319:778-80.
42. Gobel K, Melzer N, Herrmann AM, Schuhmann MK, Bittner S, Ip CW, et al. Collateral neuronal apoptosis in CNS gray matter during an oligodendrocyte-directed CD8(+) T cell attack. Glia 2010; 58:469-80.
43. McPherson SW, Heuss ND, Roehrich H, Gregerson DS. Bystander killing of neurons by cytotoxic T cells specific for a glial antigen. Glia 2006; 53:457-66.

44. Phares TW, Stohlman SA, Hinton DR, Atkinson R, Bergmann CC. Enhanced antiviral T cell function in the absence of B7-H1 is insufficient to prevent persistence but exacerbates axonal bystander damage during viral encephalomyelitis. J Immunol 2010; 185:5607-18.
45. Iwai Y, Terawaki S, Ikegawa M, Okazaki T, Honjo T. PD-1 inhibits antiviral immunity at the effector phase in the liver. J Exp Med 2003; 198:39-50.
46. Kroner A, Schwab N, Ip CW, Ortler S, Gobel K, Nave KA, et al. Accelerated course of experimental autoimmune encephalomyelitis in PD-1-deficient central nervous system myelin mutants. Am J Pathol 2009; 174:2290-9.

Chapter 6: Implications and future studies

A part of this chapter has been published. The citation is given below

Thangavelu and Anderson., 2011. Future Oncology. 7(8)929-932

An essential role for programmed death-1 in the control of autoimmunity: implications for the future of hematopoietic stem cell transplantation

6. Implications and future studies

The central function of co-inhibitory receptors is to control the activation of the lymphocyte responses by providing negative signals in conjunction with signals from lymphocyte antigen receptors ('co-signaling'). Recently, co-inhibitory molecules have been extensively studied due to the broad spectrum of their potential application in various novel immunotherapeutic approaches. The co-signaling aspect of these receptors is particularly appealing for clinical development, as non-depleting antibodies targeting these co-inhibitory receptors will only affect lymphocytes that are encountering (or have recently encountered) their cognate antigen; ligation of co-inhibitors alone, without antigen receptor signals, has no effect. Even though it is now forty years since the initial concept of co-inhibitory signaling was pioneered[1] (and reviewed in[2]), only recently have these signaling pathways been specifically targeted for therapy. Agonistic monoclonal antibodies to co-inhibitors are a promising new approach in the prevention of transplant rejection and graft versus host disease, and may also find application in treating autoimmune disease. Monoclonal antibodies that instead block these co-inhibitors are currently undergoing clinical trials in cancer treatment, and one such co-inhibitory blocker, specific to CTLA-4, has recently achieved FDA approval. Programmed death-1 (PD-1; CD 279) and B and T lymphocyte attenuator (BTLA; CD 272) are novel co-inhibitory molecules. They are involved in the regulation of immune responses and self-tolerance[3]. Autoimmunity only rarely can be attributed to variants of a single gene alone. Instead, discovery of the causes of most autoimmunity will be found in

synergisms between alleles of many different genes and their interactions with the environment.

PD-1 had previously been shown to have a relatively limited role in self-tolerance. Loss of PD-1 function led to autoimmunity directed at a single organ or a late life lupus/arthritis in only a fraction of mice. Like PD-1, loss of BTLA led to a narrow spectrum of autoimmunity and it was reported in only one strain of the mice. This raised the question of factors that might synergize with a deficiency in PD-1 or BTLA function to lead to a more severe autoimmunity with higher incidence. In the chapter 2nd and 3rd of my thesis, we demonstrated that PD-1 or BTLA function is essential in the newly generated T cells to prevent a lethal lymphopenia-induced multi-organ autoimmunity[4]. The results from those experiments suggested that PD-1's or BTLA's most critical function resides in controlling the first waves of T cells newly exported from the thymus as they respond to as yet unidentified lymphopenia potentiated signals. Like lymphopenia, PD-1 or BTLA deficiency by itself does not usually lead to such a severe multi-organ autoimmunity. Our data suggested that this may be due to the fact that the only stage where newly generated T cells naturally predominate is during the fetal/neonatal period, a time when lymphoid tissue is not yet fully developed and therefore has a reduced ability to support LIP of PD-1 or BTLA deficient T cells.

Co-inhibitory pathways have been exploited by tumors as an immune evasion mechanism[5]. There is a negative correlation between the expression of co-inhibitory molecules such as PD-L1 and prognosis in the cancer patients[6, 7]. Strategies have been designed to overcome this hurdle by blocking co-inhibitory pathways and are considered to have high therapeutic potential. For example, blockade of PD-1 inhibited the dissemination of CT26 colon carcinoma and B16 melanoma by increasing the priming and homing of effector T cells[8].

Dendritic cell vaccines have been used in animal models[9, 10] and as wells as in humans to induce anti-tumor immunity[11]. Although vaccines against cancer can induce a high number of anti-tumor T cells, it does not always lead to a protective immune response and therefore clinical efficacy is poor. Accumulating evidence suggests that dysfunction of these tumor-infiltrating lymphocytes is due to the upregulation of PD-1 expression[12, 13]. Studies from melanoma patients revealed that PD-1 is highly expressed by endogenous anti-melanoma specific T cells, melanoma vaccine induced specific T cells, as well as in tumor-infiltrating lymphocytes (TIL)[14, 15,16]. In the case of lymphoma, an increase in the absolute number of tumor cells was associated with increased PD-1 expression of CD4 T cells[17]. Interestingly, combining a tumor vaccine with anti-PD-1 augmented the anti-tumor responses and also vaccine efficacy[13, 18]. Furthermore, blocking BTLA-HVEM pathway also enhanced vaccine efficacy[19].

Similar to animal studies[13, 18], a preclinical study conducted ex-vivo reported that the combination of anti-PD-1 with dendritic cell myeloma fusions (DC/MF) enhanced anti-tumor responses[20]. Treatment of cancer patients with anti-PD-1 is an exciting new approach and the recent clinical trials[21, 22] have reported the beneficial effects of the antibody with the potential for less autoimmune side effects than anti-CTLA-4. Encouraged by these results, a clinical trial has already been started for multiple myeloma patients, which involves high dose chemotherapy, autologous HSC transplantation, followed by DC/MF with anti-PD-1 (http://clinicaltrials.gov/ct2/show/study/NCT01067287).

High dose chemotherapy is used to treat advanced cases of cancers to eradicate cancer cells more effectively. However, the downside of the high dose regimens is the killing of healthy cells, in particular hematopoietic cells, as this makes it necessary to give a HSC transplant to those patients. High dose chemotherapy followed by HSC transplantation has become a standard regimen to treat cancers like multiple myeloma and lymphoma[23-25]. Autologous stem cell transplantation **(Table 6-1; Figure 6-1)** has an advantage over allogeneic stem cells due to a lack of graft versus host disease[26], although the graft versus leukemia effect is lost with this approach.

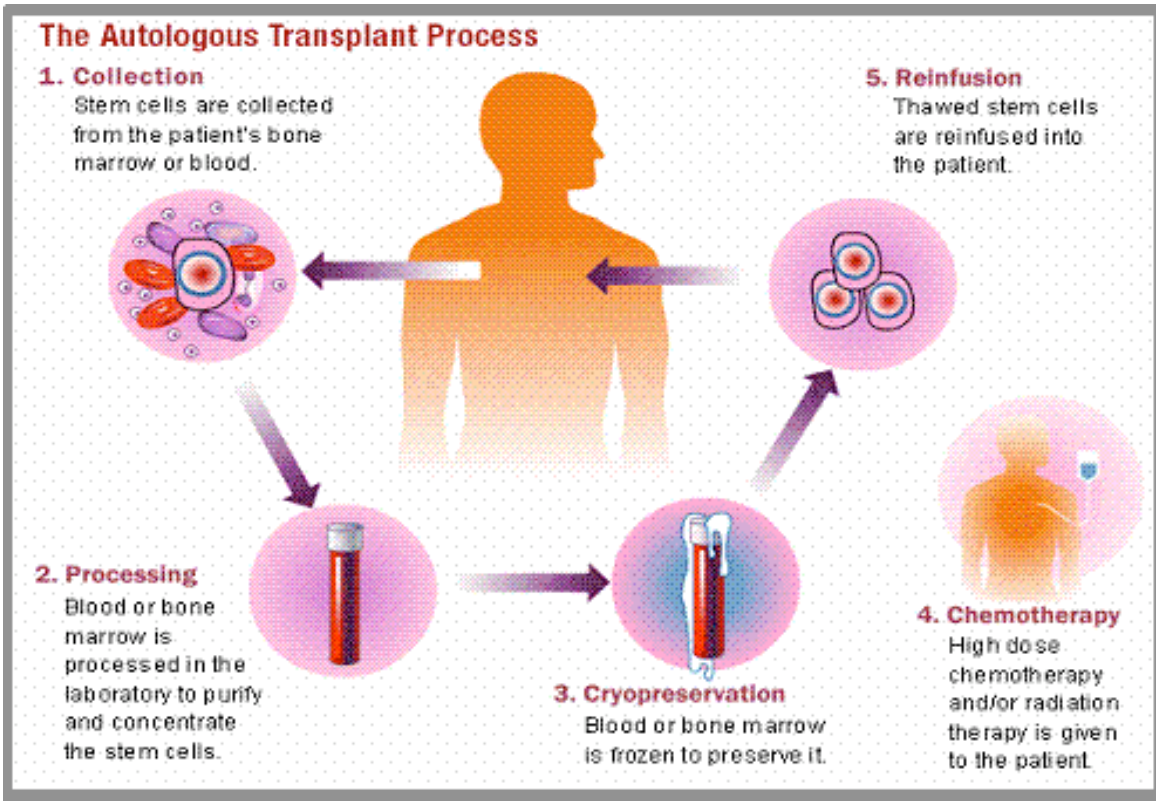
Our studies[4] suggest the potential for generation of significant autoimmune disease in patients where the immune system is 'restarted', such as patients undergoing therapies that are lymphoablative followed by autologous HSC with

anti-PD-1 treatment. Even without anti-PD-1 treatment, patients with alleles of PD-1 that demonstrate reduced function would be anticipated to be at higher risk of developing autoimmunity due to the newly generated T cells post HSC transplantation. In these patients, lymphopenia due to ablation will allow the newly generated T cells to expand and, together with reduced functioning of PD-1, these newly generated T cells can acquire the capacity to induce autoimmunity. Development of agonistic antibody to PD-1 or other co-inhibitors might be helpful to mitigate the autoimmunity in the aforementioned settings.

Table 6-1. Disease commonly treated with Hematopoietic Stem-Cell Transplantation.

Table 1. Diseases Commonly Treated with Hematopoietic Stem-Cell Transplantation.
Autologous transplantation*
Cancers
Multiple myeloma
Non-Hodgkin's lymphoma
Hodgkin's disease
Acute myeloid leukemia
Neuroblastoma
Ovarian cancer
Germ-cell tumors
Other diseases
Autoimmune disorders
Amyloidosis
Allogeneic transplantation†
Cancers
Acute myeloid leukemia
Acute lymphoblastic leukemia
Chronic myeloid leukemia
Myelodysplastic syndromes
Myeloproliferative disorders
Non-Hodgkin's lymphoma
Hodgkin's disease
Chronic lymphocytic leukemia
Multiple myeloma
Juvenile chronic myeloid leukemia
Other diseases
Aplastic anemia
Paroxysmal nocturnal hemoglobinuria
Fanconi's anemia
Blackfan–Diamond anemia
Thalassemia major
Sickle cell anemia
Severe combined immunodeficiency
Wiskott–Aldrich syndrome
Inborn errors of metabolism
* More than 30,000 autologous transplantations are performed annually worldwide, two thirds for multiple myeloma or non-Hodgkin's lymphoma.
† More than 15,000 allogeneic transplantations are performed annually worldwide, nearly half for acute leukemias. The vast majority are performed to treat lymphoid and hematologic cancers.

Reproduced with permission from Edward A. Copelan. N Engl J Med. 2006; 354(17):1813-26.



Adapted from MAKNA (2008) [http://www.makna.org.my/bonemarrow.asp]

Figure 6-1. Autologous stem cell transplantation procedure.

In the case of clinical trials with anti-PD-1 treatment, even the patients with fully functional alleles of PD-1 would be at risk of developing autoimmunity, due to the disruption of PD-1 function. However there are a few caveats to be considered in interpreting the murine data in relation to the approaches undergoing clinical trials. Firstly, the lymphopenia in most of the murine studies was absolute, employing recipient mice that completely lacked both T and B cells due to the absence of a *Rag* gene. In the subset of experiments where recipients were instead lymphoreplete (immunocompetent), conditioning with a supralethal dose

of irradiation was required to observe the severe disease. The presence of a 'competitor' population of T cells substantially blocked disease, perhaps explaining why recipients with lower doses of irradiation did not succumb to severe disease (our unpublished data). Lymphopenia induced in the clinical setting is not absolute, and the remaining T cells resistant to chemotherapy or other depletional regimens may inhibit autoimmunity caused by newly generated T cells.

From this viewpoint, any efforts to develop protocols that more fully ablate the patients hematopoietic system would be ill advised if combined with dysfunction of co-inhibitors (due to patient specific alleles or blocking antibody treatment). A second caveat is that the pattern of immune reconstitution differs substantially between mouse models and the human setting. Immune reconstitution post HSC transplantation in humans is notoriously slow and incomplete. Any efforts to augment immune reconstitution, such as through the development of methods that increase the patient's thymic function, would increase the output of newly generated T cells and the potential for autoimmunity. Despite the caveats discussed above, future clinical trials involving high dose chemotherapy or lymphoablation with stem cell transplantation may need to consider polymorphisms regulating the PD-1 or BTLA pathway in protocol development. Given the central role of these co-inhibitory signals upon immune system start up, any attempts to 'perfect' ablation and immune reconstitution protocols will need to be carefully planned if severe autoimmunity is to be avoided.

6.1. Future studies in the HSC model

In the future, studies will be designed to control the lethal disease induced by PD-$1^{-/-}$ or BTLA $^{-/-}$ HSC. In particular, by harnessing the signals of other co-inhibitory molecules in the immune system. The effect of CTLA-4 Ig fusion protein or anti-BTLA-4 antibody, or a combination of both, in controlling the PD-1-/- HSC disease can be tested. The benefits of these agonistic antibodies in controlling graft versus host disease and also the induction of allograft tolerance have been well documented[27-29]. The other approach would be the forced expression of CTLA-4 in all the conventional naïve T cells from PD-$1^{-/-}$ or BTLA $^{-/-}$ HSC.

It is unknown whether the disease induced by PD-$1^{-/-}$ or BTLA $^{-/-}$ HSC is dependent on either CD4+ T cells or CD8+ T cells or both. This can be tested by using the corresponding depleting antibodies in HSC recipients. Can the forced expression of these co-inhibitory molecules in either CD4 + T cells or CD8+ T cells rescue the breakdown of tolerance? This can be done by using either CD4+ or CD8+ co-receptor as a promoter. Another interesting question to be tested is whether the forced expression of these co-inhibitory molecules in either Tregs or conventional T cells can prevent the disease in $Rag^{-/-}$ recipients.

Blockade of co-stimulatory molecules can attenuate T cell responses. CD40-CD40L plays a key role in the pathogenesis of autoimmune diseases[30] and also anti-CD40L treatment inhibited the rapid lymphopenia induced proliferation of

CD4+ T cells[31]. A study involving anti-CD40L to control the disease in the current coinhibitor$^{-/-}$ HSC model would be worth considering.

Characterization of the effector mechanisms of tissue destruction by T cells would help to tailor the strategies that control the autoimmunity. Previous studies reported the involvement of perforin/granzyme and Fas/Fas-L pathways in autoimmunity[32-35]. The role of perforin or Fas/Fas-L pathway can be tested by using fetal liver cells from perforin $^{-/-}$ or PD-1$^{-/-}$/BTLA$^{-/-}$*gld* mice, respectively. Moreover, Rag$^{-/-}$ *lpr* can also be used as HSC recipients to test Fas/Fas-L pathway. Cytokine secretion is one of the effector pathways that can also mediate target cell destruction and has been shown to be involved in various autoimmune diseases. In my study, it was found that there was an increase in number of Th1/Th2 cytokines in sera of PD-1$^{-/-}$ HSC recipients. The role of cytokines such as IFN-γ, IL-6 and TNF-∝ in PD-1$^{-/-}$ HSC recipients can be tested by using neutralizing antibodies or cytokine receptor deficient recipients, for example, IFN-γR$^{-/-}$ mice.

Recent studies have divulged the importance of microRNAs (miRNAs), small noncoding ribonucleic acids (RNAs), in immune homeostasis and self-tolerance[36, 37]. It should be investigated whether miRNAs are dysregulated in the current disease model and might provide necessary cues to better understand the mechanism of the induction of the disease. The identification of these bio-markers

may lead to novel therapeutic strategies namely miRNA-based gene therapies to control disease.

6.1.1. Innate immunity

Dendritic cells (DCs) are professional antigen presenting cells responsible for the priming of naïve T cell[38]. It should be determined whether there is any difference in the subset of DCs between sick (PD-$1^{-/-}$ or $BTLA^{-/-}$ HSC) and control (WT HSC) mice. There may be expansion of a specific subset of DCs in the recipients of PD-$1^{-/-}$ or $BTLA^{-/-}$ HSC that is not expanded in recipients of WT HSC. Identification of those cells may provide insights for designing therapies to control the severity of the disease either by depletion of those particular DCs or blocking the function of those specific cells using monoclonal antibodies. On the other hand, approaches for the transfer of tolerogenic DCs to PD-$1^{-/-}$ or $BTLA^{-/-}$ HSC recipients may help in the restoration of tolerance.

6.2. Implications of bystander killing studies

Type-1 diabetes is an autoimmune disease characterized by a lack of tolerance to insulin producing β cells of the pancreas. Although islet transplantation is an exciting treatment option for type-1 diabetes, the success is hampered by various technical and biological barriers[39]. Among the biological barriers, recurrent autoimmunity or allograft rejection of islet grafts by the immune system is a major one. Moreover, our research finding also reported the possibility of bystander killing of islet cells in islet transplantation.

Knowledge of the mechanisms controlling bystander destruction will allow the development of more comprehensive approaches to prevent type-1 diabetes in disease prone individuals. Identification of the mechanisms involved in the bystander killing has important implications for the future design of tolerance strategies in islet transplantation by targeting cytokines or using Fas mutant islets.

Recent studies have demonstrated the involvement of co-inhibitory molecules in the dysfunction of T cells during chronic viral infections[40, 41]. Blockade of these co-inhibitory molecules induced potent anti-viral immune responses and cleared the infection. However, our findings divulged the importance of PD-1 in limiting bystander killing. Future clinical trials involving co-inhibitory blockade treatment to treat chronic viral infections will have to be properly designed to avoid inadvertent killing of healthy bystander cells. However, in tumor immunology, the application of the bystander killing concept to eradicate cancer will be a useful strategy for consideration.

6.2.1. Future studies in bystander killing

Bystander killing in Marilyn mic was dependent on MHC alleles of the bystander cells. For example, MHC matched (I-A^b) bystander cells were more susceptible to killing than those of MHC mismatched (I-A^k) or class-II mutant (I-A^{bm12}) bystander cells. The increased susceptibility to bystander killing may be due to two reasons. Firstly, there may be presence of more I-A^b donor antigen presenting

cells at the graft site of MHC bystander cells. This possibility can be tested by using bystander cells from bone marrow chimera. Bone marrow chimeras can be developed by lethally irradiating C57BL/6 (B6) I-A^{bm12} mice followed by reconstitution with B6 $Rag^{-/-}$ bone marrow and *vice versa*. Secondly, it is also possible that female bystander cells could have picked up male antigen from target cells and cross presented those to Marilyn T cells. The role of cross-presentation can be tested using bystander cells that have been engineered to present a single peptide in I-A^{b42}.

6.2.2. Role of Tregs and co-inhibitory molecules in preventing bystander killing

Future research aimed to test the role of T regulatory cells (Tregs) in controlling bystander killing would be highly valuable and could be tested in both TCR (T cell receptor) Tg (transgenic) and WT mice by adoptive transfer experiments.

6.2.2.1. TCR Tg mice

Unlike WT mice, Marilyn mice lack Tregs and this may be the reason why anti-HY CD4+ T cells have the ability to induce bystander killing. This idea can be tested by transferring FACS sorted Tregs (CD4+ CD25+) from WT mice into Marilyn recipients with mixed islets targets and MHC matched bystanders. CD4+ CD25- T cells can be used as control for Tregs in a separate cohort of recipients with mixed islets. Furthermore, if Tregs have the capacity to inhibit bystander killing, then the mechanism of controlling has to be elucidated. The mechanism

may be mainly mediated through cytokine or co-inhibitory pathways. Involvement of suppressive cytokines namely, TGF-β and IL-10 can be tested by using neutralizing anti- TGF-β antibody or Tregs from IL-$10^{-/-}$ mice, respectively. The role of co-inhibitory function in Tregs can be tested by using Tregs from co-inhibitory molecule deficient mice.

6.2.2.2. Wild type mice

Adoptive transfer can be used to test the role of Tregs in the prevention of bystander killing in WT mice. B6-*Rag*$^{-/-}$ recipients transplanted with mixed BALB/c and syngeneic B6 bystander islets will receive FACS sorted $CD3^{+}CD25^{-}$ cells from naïve B6 mice, with or without the addition of FACS sorted Tregs. Ratios of effectors ($CD3^{+}CD25^{-}$) to regulators ($CD4^{+}CD25^{+}$) will be optimized to determine the particular ratio of these two cell types that will prevent the bystander rejection (B6 islets) without preventing the rejection of the target BALB/c islets. In addition, whether Tregs ($CD4^{+}$Foxp-3^{+}) are present within the non-rejected bystander islets can be detected by FACS and also by assessing the expression of Foxp3 mRNA and protein using Q-PCR and immunohistochemistry, respectively. Our study demonstrated that the T cells from sensitized PD-$1^{-/-}$ mice have the ability to induce bystander killing. However, it is unclear whether co-inhibitory molecules are required in Teff or Tregs to control bystander killing. This can be tested by adoptive transfer of PD-$1^{+/+}$ Tregs from WT mice into sensitized PD-$1^{-/-}$ recipients with mixed islets. If there is no evidence for a role of Tregs in the prevention of bystander rejection, it

would suggest that co-inhibitors act directly on effector cells. The role of other co-inhibitory molecule namely CTLA-4 in preventing bystander killing can also be tested using monoclonal antibodies (anti-CTLA-4) in WT mice.

6.3. Conclusions

My thesis work uncovered the most important previously unknown roles of co-inhibitory molecules in the regulation of immune tolerance. In particular, their role in controlling recent thymic emigrants to prevent lymphopenia induced autoimmunity. Furthermore, PD-1 is required to limit bystander killing or collateral damage.

I believe that co-inhibitory molecules maintain tolerance by regulating T effector class switch responses (Th1 or Th17 or Th2). Experiments are warranted to test the above hypothesis. PD-1 plays an important role in mediating spontaneous tolerance to a single minor antigen mismatched allograft. The "Holy Grail" of transplant immunology is the achievement of immunological tolerance towards the donor allografts without immunosuppression. In other words, the requirement of toxic immunosuppressants can be avoided by inducing immunological tolerance. Attempts have been made to induce donor specific tolerance by mimicking natural tolerance pathways namely, central and peripheral tolerance. Mixed haematopoietic chimerism is a central tolerance strategy that has been used in the clinical setting since 1970[43]. Although mixed haematopoietic chimerism

can induce tolerance, the toxicity related to transplantation protocols, risk of GVHD and existence of split tolerance[44] have limited its wide application.

On the other hand, peripheral tolerance strategies such as co-stimulation blockade[28] or ex-vivo expanded antigen specific Tregs[45, 46] are promising in inducing long term acceptance of allografts. Results from clinical trials[47] involving the CTLA-4 Ig fusion protein, Belatacept (LEA29Y), in diminishing alloimmunity were promising. Co-inhibitory signals play a key role in mediating peripheral tolerance. Future studies should focus more on the unexplored roles played by the co-inhibitory molecules in the regulation of immune responses. Of-note, it will be helpful to develop new generation therapies in order to prevent autoimmunity, graft versus host disease and transplant rejection.

6.4. References

1. Sinclair NRS CP. Regulation of the immune response. IV. The role of the Fc-fragment in feedback inhibition by antibody. Adv Exp Med Biol 1971; 12:609-15.
2. Thangavelu G, Smolarchuk C, Anderson CC. Co-inhibitory molecules: Controlling the effectors or controlling the controllers? Self Nonself 2011; 1:77-88.
3. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999; 11:141-51.
4. Thangavelu G, Parkman JC, Ewen CL, Uwiera RR, Baldwin TA, Anderson CC. Programmed death-1 is required for systemic self-tolerance in newly generated T cells during the establishment of immune homeostasis. J Autoimmun 2011; 36:301-12.
5. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med 2002; 8:793-800.
6. Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, et al. Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 2006; 66:3381-5.
7. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. Proc Natl Acad Sci U S A 2007; 104:3360-5.
8. Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. Int Immunol 2005; 17:133-44.
9. Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, et al. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. Nat Med 1995; 1:1297-302.
10. Celluzzi CM, Mayordomo JI, Storkus WJ, Lotze MT, Falo LD, Jr. Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. J Exp Med 1996; 183:283-7.
11. Rosenblatt J, Vasir B, Uhl L, Blotta S, Macnamara C, Somaiya P, et al. Vaccination with dendritic cell/tumor fusion cells results in cellular and humoral antitumor immune responses in patients with multiple myeloma. Blood 2011; 117:393-402.
12. Mumprecht S, Schurch C, Schwaller J, Solenthaler M, Ochsenbein AF. Programmed death 1 signaling on chronic myeloid leukemia-specific T cells results in T-cell exhaustion and disease progression. Blood 2009; 114:1528-36.
13. Sierro SR, Donda A, Perret R, Guillaume P, Yagita H, Levy F, et al. Combination of lentivector immunization and low dose chemotherapy or PD-

1/PD-L1 blocking primes self-reactive T cells and induces anti-tumor immunity. Eur J Immunol 2011.
14. Wong RM, Scotland RR, Lau RL, Wang C, Korman AJ, Kast WM, et al. Programmed death-1 blockade enhances expansion and functional capacity of human melanoma antigen-specific CTLs. Int Immunol 2007; 19:1223-34.
15. Fourcade J, Kudela P, Sun Z, Shen H, Land SR, Lenzner D, et al. PD-1 is a regulator of NY-ESO-1-specific CD8+ T cell expansion in melanoma patients. J Immunol 2009; 182:5240-9.
16. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood 2009; 114:1537-44.
17. Shimauchi T, Kabashima K, Nakashima D, Sugita K, Yamada Y, Hino R, et al. Augmented expression of programmed death-1 in both neoplastic and non-neoplastic CD4+ T-cells in adult T-cell leukemia/lymphoma. Int J Cancer 2007; 121:2585-90.
18. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci U S A 2010; 107:4275-80.
19. Han L, Wang W, Fang Y, Feng Z, Liao S, Li W, et al. Soluble B and T lymphocyte attenuator possesses antitumor effects and facilitates heat shock protein 70 vaccine-triggered antitumor immunity against a murine TC-1 cervical cancer model in vivo. J Immunol 2009; 183:7842-50.
20. Rosenblatt J, Glotzbecker B, Mills H, Vasir B, Tzachanis D, Levine JD, et al. PD-1 Blockade by CT-011, Anti-PD-1 Antibody, Enhances Ex Vivo T-cell Responses to Autologous Dendritic Cell/Myeloma Fusion Vaccine. J Immunother 2011; 34:409-18.
21. Berger R, Rotem-Yehudar R, Slama G, Landes S, Kneller A, Leiba M, et al. Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. Clin Cancer Res 2008; 14:3044-51.
22. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol 2010; 28:3167-75.
23. Fermand JP, Ravaud P, Chevret S, Divine M, Leblond V, Belanger C, et al. High-dose therapy and autologous peripheral blood stem cell transplantation in multiple myeloma: up-front or rescue treatment? Results of a multicenter sequential randomized clinical trial. Blood 1998; 92:3131-6.
24. Rodriguez J, Caballero MD, Gutierrez A, Marin J, Lahuerta JJ, Sureda A, et al. High-dose chemotherapy and autologous stem cell transplantation in peripheral T-cell lymphoma: the GEL-TAMO experience. Ann Oncol 2003; 14:1768-75.

25. Campen CJ, Tombleson RL, Green MR. High-dose chemotherapy with hematopoietic stem cell transplantation for the treatment of primary central nervous system lymphoma. J Neurooncol 2011; 101:345-55.
26. Joseph RW, Couriel DR, Komanduri KV. Chronic graft-versus-host disease after allogeneic stem cell transplantation: challenges in prevention, science, and supportive care. J Support Oncol 2008; 6:361-72.
27. Albring JC, Sandau MM, Rapaport AS, Edelson BT, Satpathy A, Mashayekhi M, et al. Targeting of B and T lymphocyte associated (BTLA) prevents graft-versus-host disease without global immunosuppression. J Exp Med 2010; 207:2551-9.
28. Truong W, Plester JC, Hancock WW, Merani S, Murphy TL, Murphy KM, et al. Combined coinhibitory and costimulatory modulation with anti-BTLA and CTLA4Ig facilitates tolerance in murine islet allografts. Am J Transplant 2007; 7:2663-74.
29. Wallace PM, Johnson JS, MacMaster JF, Kennedy KA, Gladstone P, Linsley PS. CTLA4Ig treatment ameliorates the lethality of murine graft-versus-host disease across major histocompatibility complex barriers. Transplantation 1994; 58:602-10.
30. Peters AL, Stunz LL, Bishop GA. CD40 and autoimmunity: the dark side of a great activator. Semin Immunol 2009; 21:293-300.
31. Le Saout C, Villard M, Cabasse C, Jacquet C, Taylor N, Hernandez J. IL-2 mediates CD4+ T cell help in the breakdown of memory-like CD8+ T cell tolerance under lymphopenic conditions. PLoS One 2010; 5:e12659.
32. Chervonsky AV, Wang Y, Wong FS, Visintin I, Flavell RA, Janeway CA, Jr., et al. The role of Fas in autoimmune diabetes. Cell 1997; 89:17-24.
33. Kagi D, Odermatt B, Seiler P, Zinkernagel RM, Mak TW, Hengartner H. Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. J Exp Med 1997; 186:989-97.
34. Blanco P, Pitard V, Viallard JF, Taupin JL, Pellegrin JL, Moreau JF. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus. Arthritis Rheum 2005; 52:201-11.
35. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002; 2:735-47.
36. Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, et al. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. J Exp Med 2008; 205:1983-91.
37. Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. J Exp Med 2008; 205:1993-2004.
38. Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature 2007; 449:419-26.
39. Huang X, Moore DJ, Ketchum RJ, Nunemaker CS, Kovatchev B, McCall AL, et al. Resolving the conundrum of islet transplantation by linking metabolic dysregulation, inflammation, and immune regulation. Endocr Rev 2008; 29:603-30.

40. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 2006; 439:682-7.
41. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 2009; 10:29-37.
42. Ignatowicz L, Kappler J, Marrack P. The repertoire of T cells shaped by a single MHC/peptide ligand. Cell 1996; 84:521-9.
43. Bach FH, Albertini RJ, Joo P, Anderson JL, Bortin MM. Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome. Lancet 1968; 2:1364-6.
44. 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.
45. Issa F, Hester J, Goto R, Nadig SN, Goodacre TE, Wood K. Ex vivo-expanded human regulatory T cells prevent the rejection of skin allografts in a humanized mouse model. Transplantation 2010; 90:1321-7.
46. Nadig SN, Wieckiewicz J, Wu DC, Warnecke G, Zhang W, Luo S, et al. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. Nat Med 2010; 16:809-13.
47. Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blancho G, et al. Costimulation blockade with belatacept in renal transplantation. N Engl J Med 2005; 353:770-81.

Appendix

A.1. Reagents and protocols

A.1.1. Acetate buffer (to dissolve streptozotocin)

- Solution A: 0.7775mL glacial acetic acid + 49.2225 mL ddH_2O
- Solution B: 1.36 g sodium acetate trihydrate added to ddH_2O to make the total volume up to 50 mL
- Solution C: 15.25 mL solution A + 9.75 mL solution B + 25 mL ddH_2O
- Add 0.45 g of sodium chloride to solution C. Mix well and correct the pH to 4.5
- Sterilization of the pH corrected solution was done by vacuum filtration and the buffer was stored at 4ºC

A.1.2. Streptozotocin injection

- Always dissolve streptozotocin (STZ) in acetate buffer freshly before injection.
- The dose of STZ is 200mg/kg body weight and the route of inj is i.p.

A.1.3. Phosphate-buffered saline (PBS) stock solution

- To make 10X PBS, dissolve 2 g of potassium chloride, 2 g of potassium dihydrogen phosphate, 80 g of sodium chloride and 9.2 g of disodium hydrogen phosphate in 1 L of ddH_2O
- Correct the pH to 7.2-7.4
- Sterilization of the pH corrected solution was done by vacuum filtration and stored at room temperature (RT)

A.1.4. Antibody diluent in flow cytometry

- To 45 mL of 1X PBS, add 5 mL of dialyzed 10% bovine serum albumin (BSA)
- Sterilization was done by vacuum filtration and the diluent was stored at 4ºC

A.1.5. Fc receptor blocking solution

- Mix 3.333 mL each of mouse, rat and hamster sera and add 300 μg of anti-CD16/32 antibody to the mixture of sera
- Sterilization was done by vacuum filtration and the solution was stored in small aliquots at 4ºC

A.1.6. Red blood cell (RBC) lysis buffer

- To 1 L of ddH_2O, add 8.29 g of ammonium chloride, 1 g of potassium bicarbonate and 37.2 mg of disodium ethylenediamine tetra acetic acid and stir well
- Adjust pH to 7.2-7.4
- Sterilization was done by vacuum filtration and the buffer was stored at 4ºC

A.1.7. Immunolabeling of cells for flowcytometry

<u>I. For conjugated primary antibodies:</u>

1. Stain 25 µL of cells (heparinized blood, spleen, lymph node, thymus, BM, peritoneum, *etc*.)
2. Add 25 µL of Fc receptor blocing solution and incubate for 5 mins at RT
3. Add antibody cocktail at the ratio of 1:1 to cells and incubate at 4ºC for 15-30 minutes after mixing it well
4. After incubation with primary antibody, wash cells with 3 mL of cold RBC lysis buffer in case of blood sample; 3 mL of cold 1X PBS in case of other cell types by centrifugation at 4ºC @ 1200 rpm for 10 minutes
5. After discarding the supernatant the cell pellet was resuspended in 200-300 ml of cold 1X PBS and analyzed on flow cytometer

II. For combination of biotinylated and conjugated primary antibodies:

1. Follow the steps in the above-mentioned protocol in the same order up to step 3.
2. Wash the cells using cold 1X PBS by following the centrifugation requirements mentioned in step 4
3. Upon removal of supernatant, add streptavidin conjugated to Tricolor or allohycocyanin at the ratio of 1:1 to cells and incubate at 4ºC for atleast 15 minutes
4. Again wash the cells using RBC lysis buffer incase of blood sample or cold 1X PBS in case of other cell types as mentioned in step 4 and prepare the cells for flow cytometry anlysis as mentioned in step 5 in the above-mentioned protocol.

A.1.8. Hematoxylin and eosin staining

1. Deparaffinize the tissue sections by dipping in histoclear or xylene (3 times, 5 minutes each)
2. Rehydrate the sections by dipping in descending grades of ethanol
    a) 100% ethanol - 3 times, 2 minutes each
    b) 95% ethanol - 1 minute
    c) 70% ethanol – 1 minute
3. Wash in ddH_2O for 5 minutes
4. Dip the tissue sections in Haris hematoxylin for 1 to 1.5 minutes
5. Rinse the slides in running tap water for 1-2 minutes or until clear
6. Dip the slides in 0.25% acid alcohol (3 dips) and in water for 30 seconds
7. Dip in lithium carbonate for 30 seconds and wash in water for 30 seconds
8. Dip the tissue sections in 95% ethanol for 30 seconds
9. Stain with alcoholic eosin for 35 seconds
10. Dehydrate the tissue sections by washing in ascending grades of alcohol
    a) 95% ethanol - 30 seconds
    b) 100% ethanol - 4 times; 30 seconds each
    c) Xylene - 3 times; 5 minutes each
11. Dry the slides and apply coverslip on the tissue sections using permanent mounting medium.

A.1.8.1. Immunohistochemistry for insulin

Tissue fixation

- Overnight incubation of islet grafts with buffered zinc formalin fixative (Z-Fix)

Tissue processing

- Fixed tissues were embedded in paraffin and those paraffin embedded tissues were cut using microtome at the desired thickness of the tissue section (3 μm) and placed on charged histobond slides

Immunohistochemical staining for insulin

1. Tissue sections were deparaffinized using xylene and rehydrated using descending grades of ethanol
2. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide solution in methanol for 10 minutes and washed using 1X PBS
3. To avoid non-specific binding, tissue sections were incubated with 10% of normal goat serum for 15 minutes at RT
4. Tissue sections were incubated with guinea pig anti-insulin antibody (1:1000; Dako Cytomation, Missisangua, ON) for 30 minutes at RT and then washed thrice using 1X PBS
5. After washing, tissue sections were incubated with biotinylated goat anti-guinea pig IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) for 20 minutes at RT and then washed thrice using 1X PBS

6. Tissue sections were then incubated with avidin-biotinylated enzyme complex (Vector Laboratories, Burlingame, CA) for 40 minutes at RT and washed thrice using 1X PBS
7. Diaminobenzidine (DAB) was used as chromogen, which produces a brown color end product for immunopositivity
8. Tissue sections were then washed to stop the chromogen development, counterstained, washed and coverslipped using mounting medium

A.1.9. Mouse islet isolation

Preparation of solution

1) Supplement HBSS with 50% Dextrose, Penn/Strep, and HEPES (5mL each per 1L of HBSS) then place on ice
2) Supplement 500mL of M199 with 55mL newborn calf serum (heat inactivated) and 2.5 mL Penn/Strep, sterile filter and leave at room temperature
3) Prepare 0.1% to 0.2% collagenase-TypeV using HBSS (5 mL required per mouse), sterile filter and place on ice
4) Add 15mL supplemented HBSS to a 50mL centrifuge tube and place on ice
5) Set out 4 tubes for ficoll gradients, 1 for the cannula, 1 for a vacuum line, 4/10 mice for the first ficoll, 2/10 mice for the second ficoll, and 1/10 mice for the final wash
6) Place the glass funnel with 500 um screen in one of the first 4 tubes

7) Place the 5mL pipette in the vacuum line tube and attach to the vacuum line
8) Place the cannula in the cannula tube and attach one of the 10mL syringes
9) Pour ficoll gradients into labelled tubes (10mL/tube for the bottom, 6mL/tube for the other gradients)

Excision of pancreas

1) Immobilize the anaesthetized animal on mouse board with skin anchors
2) Clip the heart
3) Using 3mL syringe, draw up 2.5mL collagenase solution
4) Attach 27G needle and bend to a 45^0 angle, bevel inside
5) Attach mosquito clamps to the common bile duct where it joins the small intestine and expose the common bile duct.
6) Cannulate bile duct at the bifurcation with the syringe and needle, slowly injecting all of the collagenase solution
7) Remove the syringe and needle, then carefully cut the pancreas away from all connective tissue
8) Place perfused pancreas in the tube with 15mL HBSS on ice

Islet isolation

1) Transfer pancreases to the tube with collagenase
2) Cap tightly and place in shaking water bath (digestion time can vary from 9 to 14 minutes)

3) Remove tube from the bath and add COLD HBSS to stop the digestion
4) Centrifuge at 1500rpm for 30 seconds
5) Remove supernatant and resuspend the pellet in 15mL HBSS with a vortex
6) Pour suspended tissue through the filter funnel into a 50ml tube
7) Rinse the digestion tube and pour the rinse through the filter funnel
8) Draw up 10mL cold HBSS in a syringe attached to 23G needle
9) Spray the HBSS onto the filter to wash any islets into the tube
10) Remove filter funnel from the tube and split the tissue into the other first ficoll tubes evenly
11) Fill the tubes with HBSS and cap
12) Centrifuge at 1500rpm for 30 seconds
13) Remove all the supernatant and add 10mL of the ficoll bottom layer to each of the tubes
14) Cap and vortex the tubes until all the tissue is suspended
15) Slowly add 6mL of each of the remaining ficoll gradients
16) Centrifuge the tubes at 2000rpm for 10 minutes, NO BRAKE
17) Use the 10mL syringe and cannula to remove the islets from the first ficoll interface and place in the tubes for second ficoll
18) Fill the tubes with HBSS and centrifuge at 2000rpm for 1 minute
19) Remove supernatant with vacuum and repeat wash
20) Remove all the supernatant and add 10mL of the bottom ficoll layer
21) Repeat ficoll layers and centrifuge at 2000rpm for 2 minutes

22) Using the same cannula, remove the islets from the ficoll interface and place in last wash tubes
23) Add HBSS to the top and centrifuge at 2000rpm for 1 minute
24) Remove supernatant and repeat wash
25) Remove all the supernatant and resuspend islets in 10mL Medium199
26) Pour islets into petri dish
27) Rinse tube with Medium199 and add to petri dish
28) Using Pasteur pipette connected to the hand pipettor, pick out all the islets and transfer to another dish
29) Culture islets in CMRL or Ham's F-10 medium

Islet transplantation

1) Make a small incision through skin and muscle of the left back side of the anaesthetized mouse (halothene machine with a nose cone is used)
2) Carefully lever the kidney outside of the body using a wet swab and the kidney should be maintained in moist state throughout the procedure
3) Make a small nick in the anterior/dorsal portion of the kidney capsule using a 27G needle
4) Make an open pocket in that hole with the aid of a siliconized glass probe
5) Place a piece of PE-50 tubing approximately 10" long onto a 23G needle and attach to the micromanipulator
6) Collect the islets from the microcentrifuge tube by placing the tubing in the pellet of cells and turning the manipulator handle AWAY from you

7) Seal the end of the tubing with a ligaclip
8) Spin the manipulator and tubing until the islets collect into a pellet at the end of the tubing
9) Remove the ligaclip and trim the tubing so there are no sharp edges
10) Insert the tubing under the kidney capsule and slowly inject the islet pellet by turning the manipulator handle TOWARDS you
11) Close the capsule with the cautery
12) Sew the muscle layer and close the skin with staples
13) Remove the inhalant anaesthetic, which will allow to wake up the mouse

A.2 Tregulatory cells can control the lethal disease induced by PD-1$^{-/-}$ HSC

Having demonstrated that co-transfer of WT HSC with PD-1$^{-/-}$ HSC can prevent the disease (Chapter 2), we further tested whether monoclonal T cells can prevent the disease. Regulatory T cells (Tregs) are required for the immunological tolerance[1]. Hence, we tested the frequency of Tregs in the PD-1 $^{-/-}$ HSC recipients and also tested whether Tregs can prevent the disease.

A.2.1. Materials and Methods

A.2.1.1. Mice

C57BL/6 (WT), C57BL/6-*Pdcd1-/-* (PD-1$^{-/-}$), B6.129S7-Rag1^{tm1mom}/J (abbreviated as Rag$^{-/-}$), C57BL/6-TgN (OT-I)-RAG1^{tm1Mom}, C57BL/6-TgN (OT-II.2a)-RAG1^{tm1Mom} mice were used in the experiments.

A.2.1.2. Stem cell transplantation

Recipients were injected with 15 x 10^6 fetal liver cells from PD-$1^{-/-}$ mice alone or with OT-I or OT-II mature T cells. In other experiments 15 x 10^6 female (sex determined by PCR) fetal liver cells from PD-$1^{-/-}$ mice were given to female Marilyn mice.

A.2.1.3. FACS sorting

Spleens from WT mice were collected aseptically in PBS with 5% FBS. TCR+ CD4+ CD25+ or CD25- cells were sorted aseptically from those splencoytes by using FACS BD influx™ cell sorter (BD Biosciences). The purity of the sorted cell population was >96%.

A.2.2. Results

A.2.2.1. Tregs can prevent the disease

We tested whether co-transfer of monoclonal CD4 or CD8 TCR transgenic cells with PD-$1^{-/-}$ HSC can prevent the disease. Interestingly, monoclonal T cells failed to prevent the disease **(Figure A.2 A)**. Moreover, monoclonal CD4 or CD8 TCR transgenic mice were also susceptible to the disease **(Figure A.2 A)**. Disease in TCR$^{-/-}$ mice (B cell sufficient) suggested that B cells are also unable to act as competitors for the prevention of the disease **(Figure A.2 B)**.

There was a significant decrease in the frequency of Foxp3+ CD4 T cells in PD-$1^{-/-}$ HSC recipients **(Figure A.2 B)** indicating that PD-1 is required to establish the

ratio between Tregs and effector cells in a newly generated T cell repertoire. We therefore tested whether co-transfer of CD4+ CD25+ T cells with PD-1$^{-/-}$ HSC could prevent disease development. Interestingly, FACS sorted CD4+ CD25+ T cells from WT B6 or even PD-1$^{-/-}$ whole splenocytes had the capacity to prevent the disease while CD4+ CD25- T cells could only delay the disease onset **(Figure A.2 C)**.

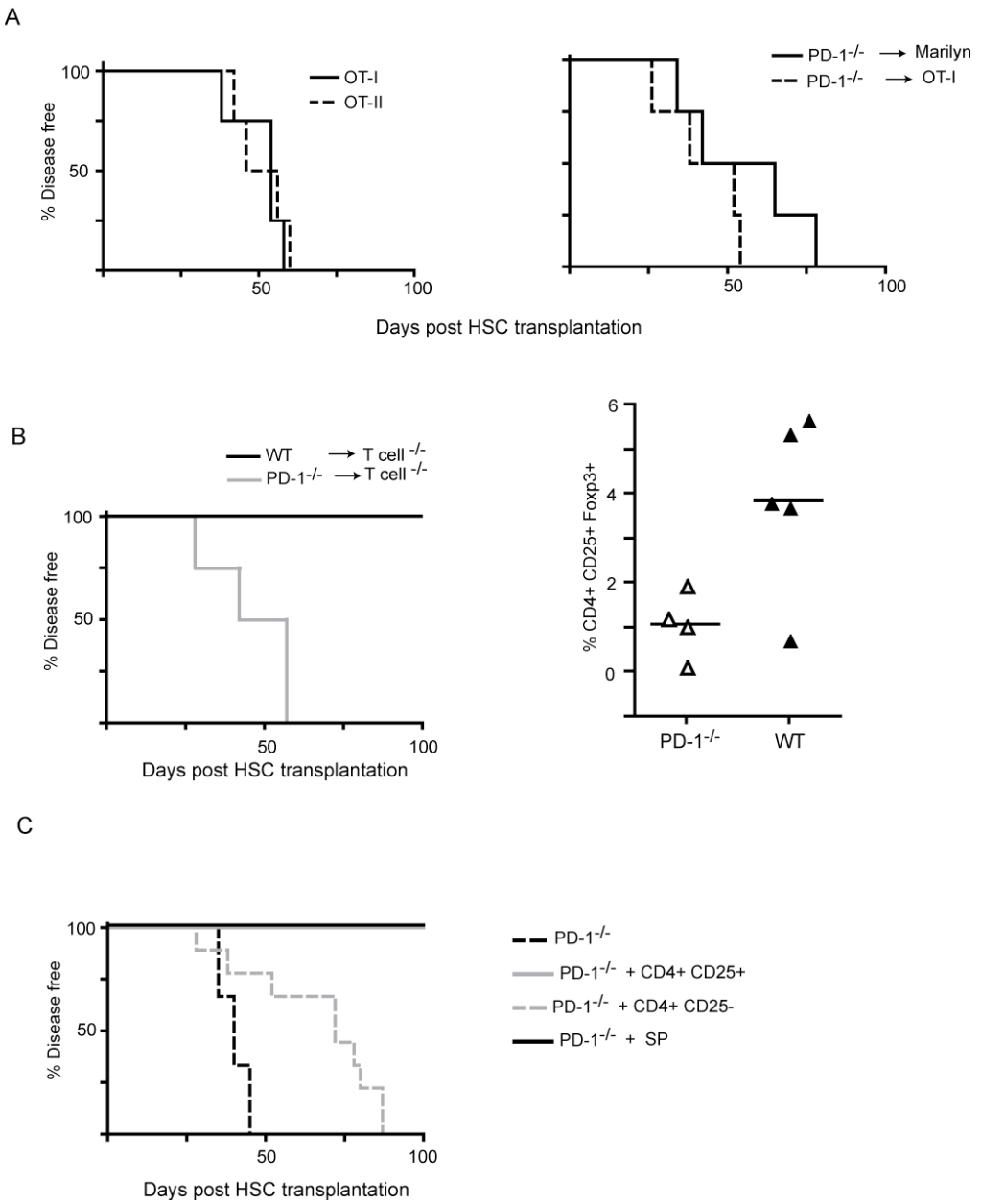


Figure A.2. Lethal inflammatory disease caused by PD-1$^{-/-}$ HSC can be controlled by Tregs. (*A*) *Left:* Adult Rag$^{-/-}$ mice (n=4 per group) were given PD-1$^{-/-}$ HSC together with 3.5 x 10^{6} OT-I or OT-II mature T cells. *Right:* In another set of experiment, female Marilyn mice (n=4) were given female PD-1$^{-/-}$ HSC. (B) *Left:* Adult TCR$^{-/-}$ mice were given either WT HSC or PD-1$^{-/-}$ HSC (n=4). *Right:* Adult Rag $^{-/-}$ were given PD-1 $^{-/-}$ HSC together with either 1 x 10^{6} TCR+ CD4+CD25+(n=3) or TCR+ CD4+CD25- (n=9) or whole PD-1$^{-/-}$ splenocytes (SP) (n=5). Controls were given PD-1 $^{-/-}$ HSC alone. (C) *Left:* Frequency of CD25^{+} Foxp3^{+} cells within CD4^{+} cells in spleens of individual adult Rag$^{-/-}$ mice given PD-1$^{-/-}$ or WT HSC and assessed 40 days post HSC (n=4-5; P< 0.05).

A.3. Depletion of detectable gut microflora did not prevent the disease

It was reported that the homeostatic proliferation of T cells was controlled by the host gut flora[2]. Furthermore, acute GVHD can be blocked by eliminating host flora with multiple antibiotics[3, 4] and we tested whether elimination of gut flora can prevent the disease.

A.3.1. Materials and Methods

A.3.1.1. Antibiotic treatment

Mice were given 0.5 grams per litre of Vancomycin hydrocholoride (Pharmaceutical partners of Canada), 1 gram per litre of metronidazole (Alberta health services), 1 gram per litre of ampicillin (Novopharm), 1 gram per litre of neomycin sulfate (Sigma Aldrich) in drinking water[5]. After four to seven weeks of medication, colonic microflora were determined by removing fecal material from colons aseptically. Homogenized contents were diluted and plated on different media for the growth of anaerobes and aerobes.

A.3.1.2. Fecal culture

Fresh mice feces were collected aseptically and homogenized at 300 x g (very slow) for 3 minutes to settle the cell debri. The supernatant layer was used to plate for culturing in MRS agar plates under anaerobic and aerobic conditions at 37C for 24 to 48hrs. Anaerobic GasPaks and anaerobic jars were used to create anaerobic condition.

A.3.2. Results

A.3.2.1. Elimination of gut flora did not prevent the disease

We found elimination of detectable (culturable) commensal flora from recipients that were given medicated water **(Figure A-3; A – D)**. However, medicated water treatment did not protect recipients from disease **(Figure A-3.1)**.

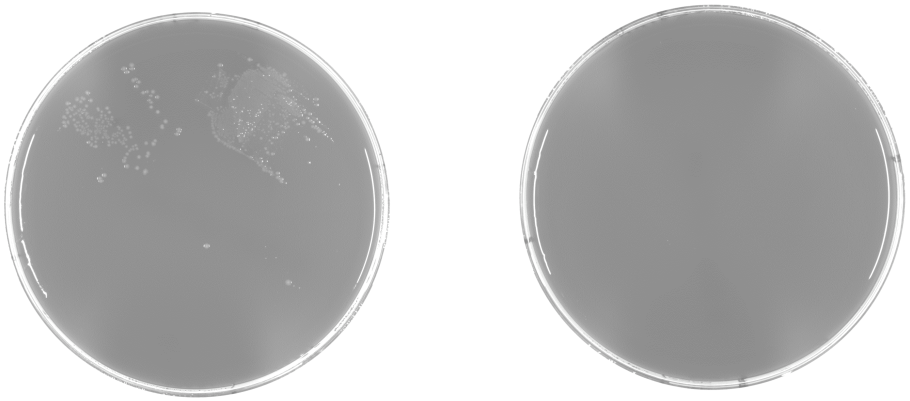


Figure A-3A. Bacterial culture of feces from control (***left***; non-medicated water) and test mice (***right;*** medicated water) under anerobic condition after 24 hrs

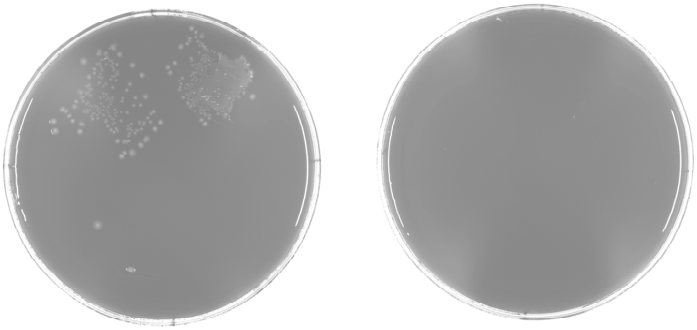


Figure A-3B. Bacterial culture of feces from control (***left***; non-medicated water) and test mice (***right;*** medicated water) under anerobic condition after 48 hrs

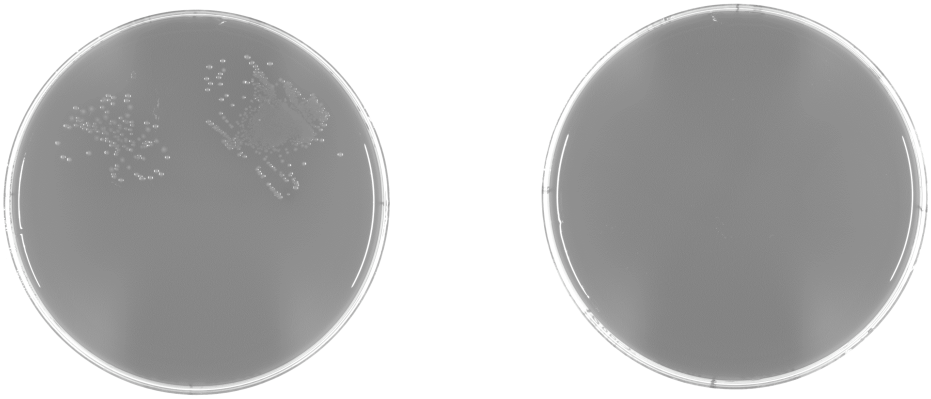


Figure A-3C. Bacterial culture of feces from control (***left***; non-medicated water) and test mice (***right;*** medicated water) under aerobic condition after 24 hrs

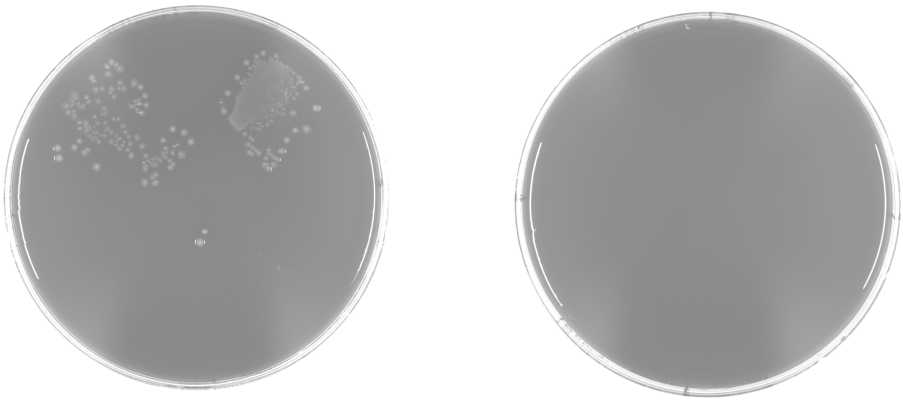


Figure A-3D. Bacterial culture of feces from control (***left***; non-medicated water) and test mice (***right***; medicated water) under aerobic condition after 48 hrs

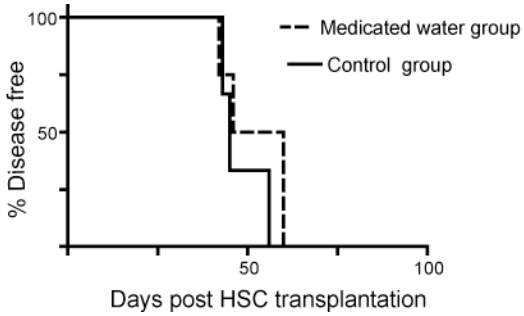


Figure A-3.1. Lack of disease in antibiotic treated recipients. Adult $Rag^{-/-}$ mice (n=4) were given medicated water and after 4 weeks recipients were given $PD\text{-}1^{-/-}$ HSC. Medicated water was given until the end of the experiment. In another set of experiment, recipients (n=3) were given regular water (non-medicated water; control) followed by $PD\text{-}1^{-/-}$ HSC transplantation.

A.4. Antigen presenting cells in stem cell recipients are not completely replaced by donor antigen presenting cells

The objective of this experiment was to examine the source of antigen presenting cells in stem cell (HSC) transplant recipients after transplantation.

A.4.1. Results

The percentage of CD11b+ GFP+ cells (n=3; 49.27 ± 1.61) was significantly higher ($p < 0.0001$) than those of CD11b+ GFP- (n=3; 1.34± 0.11). Similarly, the percentage of CD11c+ GFP+ cells (n=3; 8.4 ± 1.93) was significantly higher ($p < 0.05$) than those of CD11c+ GFP- (n=3; 2.26 ± 0.73). These preliminary data suggested that the majority of the antigen presenting cells are host derived rather than of donor origin.

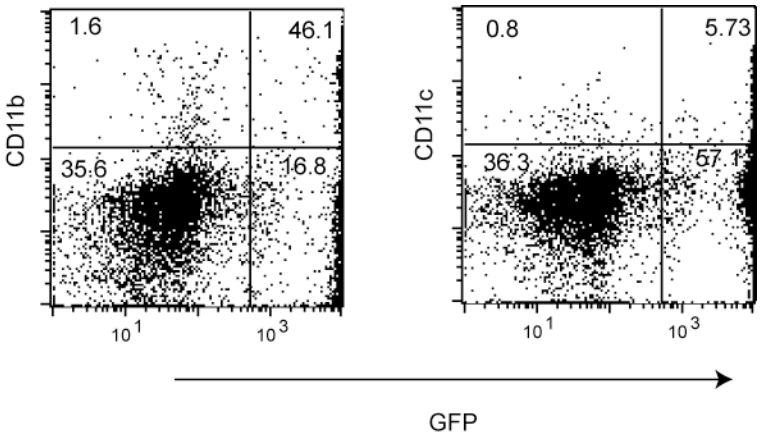


Figure A-4.1. Majority of antigen presenting cells is of host origin. $Rag^{-/-}$ GFP mice were used as recipients for WT HSC transplantation. Forty days after HSC

transplantation, the percentages of CD11b+ CD11c+ GFP+ or GFP- cells in the spleens of HSC recipient were determined.

A.5. Peripheral tolerance of CD4+ T cells to a localized antigen

The objective of this experiment is to dissect the mechanism(s) involved in CD4+ T cell peripheral tolerance.

A.5.1. Methodology

Chemically induced female diabetic B6 $Rag^{-/-}$ recipients were transplanted with either male or female islet grafts from B6 $Rag^{-/-}$ donors. After 3-6 months post islet transplantation, recipients were reconstituted with female fetal liver cells from B6 WT (CD45.1) and Marilyn (anti-HY CD4+ T cells; CD45.2) in a ratio of 4:1.

A.5.2. Results

Although a small percent (< 30%) of recipients that were transplanted with male islet grafts rejected their transplants due to the high frequency of Marilyn T cells in the periphery, majority of the recipients tolerated the peripheral male islet transplants. The frequency of Marilyn T cells ($CD45.2^+$, $CD45.1^-$, $CD4^+Vb6^+$) in the peripheral blood of those tolerant recipients was lower than that of control recipients of a female islet graft (Fig A-5.1). This data suggested the antigen specific deletion of Marilyn T cells in the periphery. Moreover, recipients that were tolerant to male islet grafts were also tolerant to male skin grafts (Fig A-5.2).

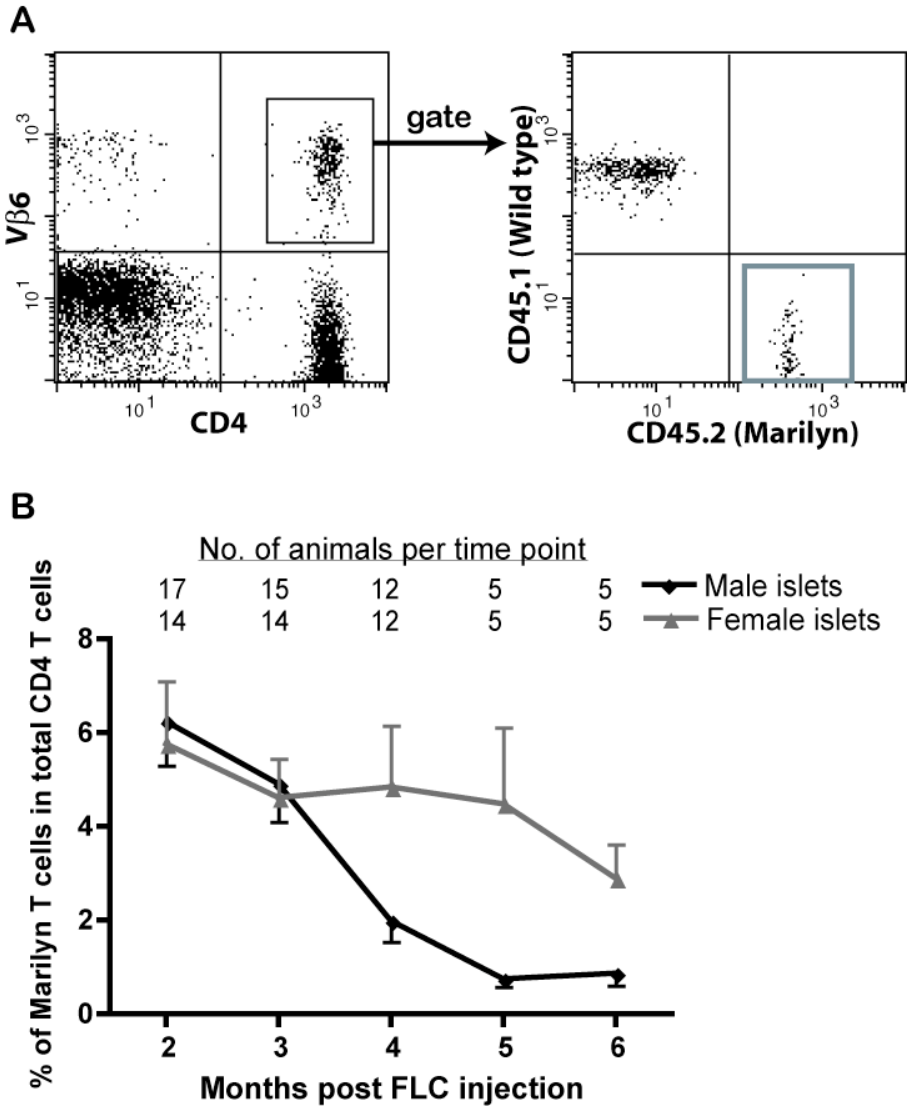


Figure A-5.1. Antigen specific progressive deletion of Marilyn T cells. (A) Identification of Marilyn T cells (grey box). (B) Frequency of Marilyn T cells, Mean and SE.

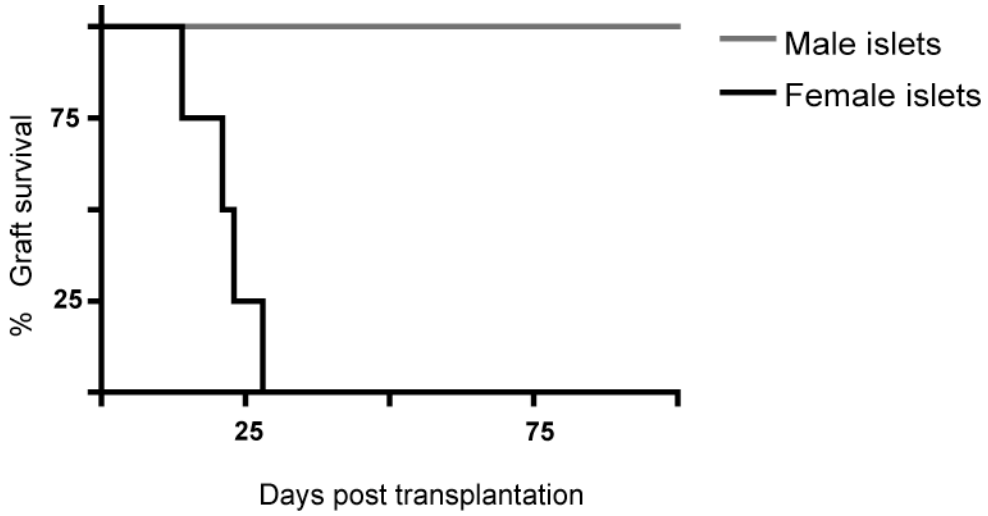


Figure A-5.2. Male skin grafts were accepted by recipients that were tolerant to male islet grafts. Male skin grafts were transplanted to male islet tolerant Rag$^{-/-}$ recipients (n=5) 6 months post fetal liver cell injections. Two recipients were found dead on day 50 and 79 post skin transplantation for unknown reasons with male skin grafts intact. On the other hand, all the recipients (n=4) with female islet grafts rejected the male skin grafts.

A.6. Neutralizing antibodies to IL-6 or IFN-γ or TNF-α failed to control the disease induced by PD-1-/- HSC

The objective of this experiment is to test whether neutralization of IL-6 or IFN-γ or TNF-α cytokine can prevent the disease induced by PD-$1^{-/-}$ HSC in $Rag^{-/-}$ recipients.

A.6.1. Methodology

Adult $Rag^{-/-}$ recipients were given PD-$1^{-/-}$ HSC. After 20 days post HSC, recipients were treated, weekly once, with 250μg of anti-IL-6 (clone 20F3) or anti-IFN-γ (XMG 1.2) or anti-TNF-α (XT22) or isotype control (mouse IgG1) antibody.

A.6.2. Results

None of the neutralizing antibodies prevented the disease. However, recipients that were treated with anti-IFN-γ did not develop dermatitis. Future experiments involving increased dosage of neutralizing antibodies may be warranted.

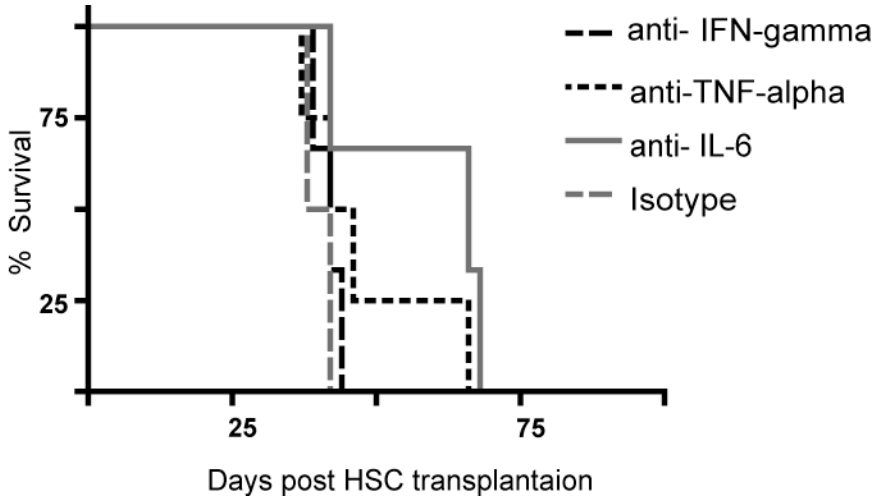


Figure A-6.1. Neutralizing antibodies to IL-6, IFN-γ or TNF-α failed to control the disease induced by PD-1$^{-/-}$ HSC. Adult Rag$^{-/-}$ recipients were transplanted with PD-1$^{-/-}$ HSC. After few weeks, recipients were treated with 250μg of anti-IL-6 (n=3) or anti-IFN-γ (n=3) or anti-TNF-α (n=4) or isotype control antibody (n=2).

A.7. Agonistic anti-BTLA (6A6) failed to control the disease induced by PD-1$^{-/-}$ HSC

The objective of this experiment is to test whether agonistic anti-BTLA (6A6) can prevent the disease induced by PD-1$^{-/-}$ HSC in Rag$^{-/-}$ recipients.

A.7.1. Methodology

Adult Rag$^{-/-}$ recipients were given PD-1$^{-/-}$ HSC. After 18 days post HSC, recipients were treated with anti-BTLA (6A6; 10μg/g body weight) or isotype control (mouse IgG1) antibody once a week till the onset of the disease.

A.7.2. Results

Anti-BTLA treatment failed to prevent the disease induced by PD-1$^{-/-}$ HSC.

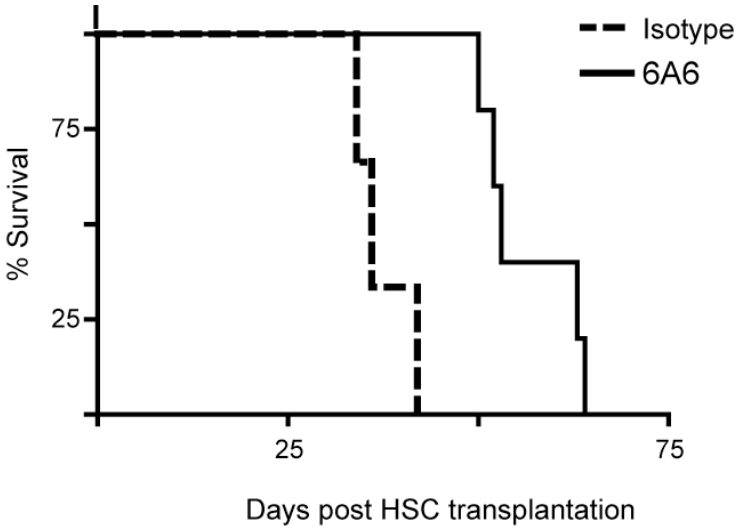


Figure A-7.1. Agonistic anti-BTLA treatment failed to control the PD-1$^{-/-}$ HSC induced disease. Adult Rag$^{-/-}$ recipients were given PD-1$^{-/-}$ HSC followed by treatment with anti-BTLA (6A6; 10μg/g body weight). There was a significant difference in survival between (*p <0.005)* the control and anti-BTLA group.

A.8. PD-1$^{-/-}$ CD24^{hi} or CD24 ^{low} splenic T cells failed to induce disease in immunodeficient recipients

The objective of this experiment is to investigate the role of PD-1$^{-/-}$ CD24^{hi} (recent thymic emigrants) or CD24^{low} T cells (mature T cells) in lymphopenia induced autoimmunity.

A.8.1. Methodology

TCR+ CD24^{hi} or CD24^{low} cells were sorted from pooled spleen cells (4-6 weeks) of C57BL/6 PD-1$^{-/-}$ mice by using CD24 , TCR fluorochrome conjugated antibodies (eBioscience) and FACS BD influx™ cell sorter (BD Biosciences). The purity of the sorted cell populations was 92%. Adult Rag$^{-/-}$ recipients (n=3/group) were injected either with 1X10^{6} CD24^{hi} or CD24^{low} cells.

A.8.2. Results

The disease was not observed in recipients of either groups TCR+ CD24^{hi} or CD24^{low} cells. One of the mice in each group had anal prolapse along with weight loss after 55-60 days post HSC. However, the disease (2 or more symptoms) was not observed in either of the Rag$^{-/-}$ recipients.

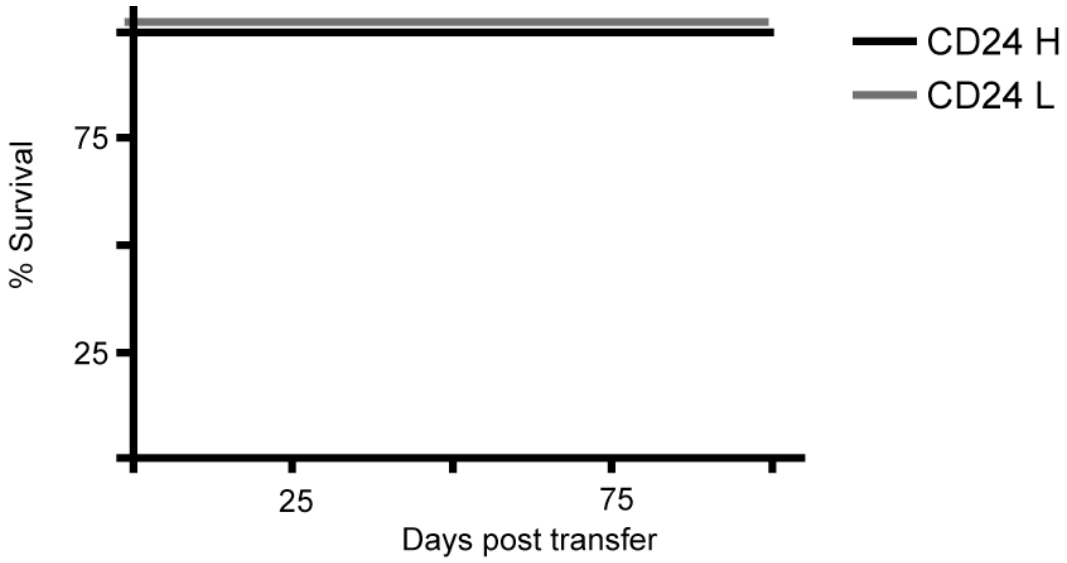


Figure A-8.1. Disease was not observed in Rag$^{-/-}$ recipients of either CD24^{hi} or CD24^{low} T cells. Adult Rag$^{-/-}$ recipients were given either TCR+ CD24^{hi} (CD24 H) or CD24^{low} (CD24 L) cells. There was no significant difference (*p > 0.05)* between the two groups.

A.9. PD-1$^{-/-}$ CD4 or CD8 single positive thymocytes alone failed to induce disease in immunodeficient recipients

The objective of this experiment is to test the role of PD-1$^{-/-}$ CD4+ or CD8+ single positive thymocytes in lymphopenia induced autoimmunity.

A.9.1. Methodology

TCR+ CD4 or CD8 cells were sorted from pooled thymocytes (3-5 weeks) of C57L/6 PD-1$^{-/-}$ mice by using fluorochrome conjugated antibodies (eBioscience) and FACS BD influx™ cell sorter (BD Biosciences). The purity of the sorted cell populations was > 90%. Adult Rag$^{-/-}$ recipients were adoptively transferred either with 1X10^{6} TCR+ CD4+ (n=3) or TCR+ CD8+ (n=2).

A.9.2. Results

The disease (2 or more symptoms) was not observed in either of the $Rag^{-/-}$ groups.

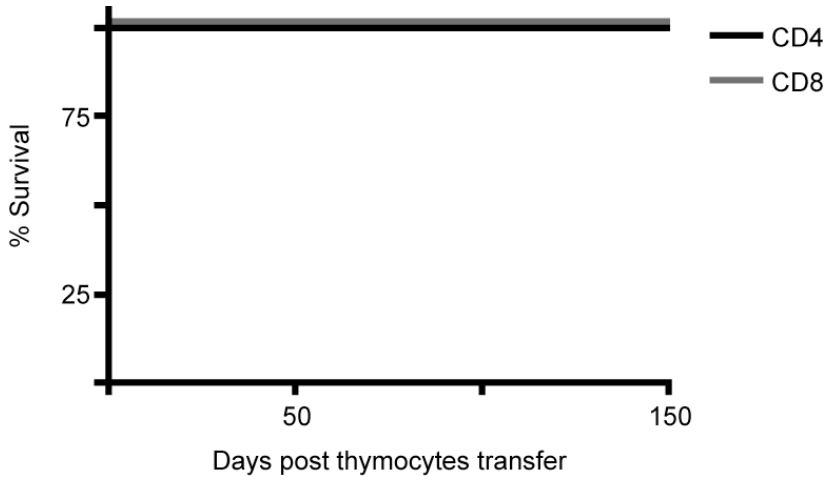


Figure A-9.1. Disease was not observed in $Rag^{-/-}$ recipients of either $PD\text{-}1^{-/-}$ CD4+ or CD8+ thymocytes. Adult $Rag^{-/-}$ recipients were given either TCR+ CD4+ or CD8+ single positive thymocytes. There was no significant difference (*$p > 0.05$)* between the two groups.

A.10. References

1. Piccirillo CA, Thornton AM. Cornerstone of peripheral tolerance: naturally occurring CD4+CD25+ regulatory T cells. Trends Immunol 2004; 25:374-80.
2. Kieper WC, Troy A, Burghardt JT, Ramsey C, Lee JY, Jiang HQ, et al. Recent immune status determines the source of antigens that drive homeostatic T cell expansion. J Immunol 2005; 174:3158-63.
3. Heidt PJ, Vossen JM. Experimental and clinical gnotobiotics: influence of the microflora on graft-versus-host disease after allogeneic bone marrow transplantation. J Med 1992; 23:161-73.
4. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut 2010; 59:1079-87.
5. Turer EE, Tavares RM, Mortier E, Hitotsumatsu O, Advincula R, Lee B, et al. Homeostatic MyD88-dependent signals cause lethal inflamMation in the absence of A20. J Exp Med 2008; 205:451-64.