Mechanisms underlying lymphopenia-driven autoimmunity in the setting of co-inhibitory molecule deficiency

by

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Abstract:

T lymphocytes (T cells) are powerful directors and effectors of immunity. The system of pseudo-random rearrangements of the T cell receptor (TCR) loci that underlie their ability to recognize a vast universe of molecular patterns is at once useful and dangerous, because many T cells develop TCR that can recognize self, and while most developing T cells with high affinity for self are removed from the repertoire during thymic development, the process is not perfect and some dangerous clones escape. These newly generated T cells have only passed through one "filtration" process in the thymus, and therefore peripheral tolerance mechanisms are critical in order to avoid autoimmunity. Homeostasis of T cells in the periphery is tightly regulated by competition for a finite resource pool, including homeostatic cytokines and relevant peptide:MHC (pMHC) complexes with which a T cell can interact and receive at least a "tonic" or greater signal. In conditions of lymphopenia, which can arise as a consequence of viral infections, clinical interventions, and other stimuli, resources are in excess and T cells will undergo a process of lymphopenia-induced proliferation (LIP) to fill their available niche. Importantly, LIP is strongly associated with inflammatory disease.

The co-inhibitory receptor programmed death-1 (PD-1) is expressed on T cells where it provides inhibitory signals that help prevent inappropriate T cell activation or keep T cells in an unresponsive state. PD-1$^{-/-}$ mice are predisposed to infrequent and mild autoimmunity. In contrast, reconstitution of the lymphoid compartment of lymphopenic adult Rag$^{-/-}$ mice with PD-1$^{-/-}$ hematopoietic stem cells (HSC) gives rise to a rapid and severe systemic autoimmune disease shortly after the first newly generated T

cells emerge from the thymus. Thus a "three strikes" combination of newly generated T cells, deficiency of PD-1, and LIP synergizes to promote autoimmunity. Our goal herein was to explore the mechanisms and cells underlying the disease in this model. One of PD-1's ligands, PD-L1, has been associated with the generation of peripheral FoxP3+ regulatory T cells (pTreg), which are critical mediators of immune tolerance. Also, while PD-1 is known to be up-regulated following conventional T cell activation, we hypothesized that PD-1 might control the comparatively weak "tonic" signals T cells receive in response to self pMHC, and in the setting of LIP of newly generated T cells, this lack of restraint on normally tonic signals led to a widespread self pMHC-directed polyclonal response.

We examined the role of PD-1 in the control of pTreg generation and found that deficiency of PD-1 does not preclude pTreg generation or regulatory T cell function. Instead, PD-1 is important for restraining the expansion of both conventional and regulatory cells in a lymphopenic host, suggesting that clinical approaches to tumour immunotherapy mediated by PD-1 blockade are unlikely to work by inhibiting tumour-associated pTreg generation and may actually expand them. We also found that PD-1 indeed controls LIP of T cells in response to tonic pMHC signals, and thus PD-1$^{-/-}$ T cells out-expanded WT cells in competitive *in vivo* assay in an MHC-dependent manner but independent of signals from the IL-7 receptor. Using a model system where we could purify newly generated T cells from the periphery of an adult, lymphoreplete mouse, we found that these cells were not rapidly tolerized upon contact with the periphery but instead maintained their autoimmune-generating potential for a period of time. Mice lacking MHC Class II expression were spared from autoimmunity, suggesting that CD4+

T cells are the main effectors of disease in the PD-$1^{-/-}$ HSC model. Furthermore, disease was not dependent on either of two canonical T cell killing mechanisms, the Perforin-Granzyme B pathway or the Fas/FasL pathway. Together these data may suggest that disease in our model is driven by a cytokine storm, and beyond advancing our basic understanding of how co-inhibitors control homeostasis of T cells, this model may be useful for developing approaches to treat diseases associated with cytokinemias, such as graft-versus-host disease or immune reconstitution inflammatory syndrome in human immunodeficiency virus patients.

Preface

Chapter 2 of this thesis has been published as:
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The majority of the experiments reported within chapter 2 were performed by K. K. Ellestad. G. Thangavelu performed the antibiotic treatment studies reported in Figure 2-1 D, one *in vivo* Treg study reported in Figure 2-3 C, and the *in vivo* HSC transfer study reported in Appendix 1 that is referenced in this chapter. C. Ewen carried out a portion of the cell sorting for experiments in chapter 2. The original manuscript was written by K. K. Ellestad with major editorial contribution by C. C. Anderson.

For the experiment depicted in Appendix 4 (referenced within chapter 4), K. K. Ellestad sorted the cells and designed research, and J. Lin performed the injections, flow cytometry and designed research.

Dedication

For my grandfather, Roger De Roose, who always encouraged me, and the memory of whom gave me strength.

And for my daughter, Josephine, for whom I wish a life filled with curiosity and the freedom to chase it.

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List of abbreviations

7-AAD	7-Aminoactinomycin D
ACK	Ammonium-Chloride-Potassium
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIRE	Autoimmune regulator
ANOVA	Analysis of variance
APC	Antigen presenting cell *or* Allophycocyanin
B6	C57BL/6
BCR	B cell receptor
BTLA	B and T lymphocyte attenuator
CARD	Caspase recruitment domain
CCAC	Canadian Council on Animal Care
CFSE	Carboxyfluorescein succinimidyl ester
CIITA	MHC Class II transactivator
CNS	Central nervous system
cTEC	Cortical thymic epithelial cell
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CTV	Celltrace violet
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLC	Fetal liver cells
FoxP3	Forkhead-box P3
g	Gravity
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
HA	Haemagglutinin
HAART	Highly active antiretroviral therapy
HBSS	Hank's buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	High endothelial venules
HIV	Human immunodeficiency virus

HSC Hematopoietic stem cell
IEL Intraepithelial lymphocyte
IFN-γ Interferon gamma
Ig Immunoglobulin
IL-15 Interleukin-15
IL-7 Interleukin-7
ILC Innate lymphoid cell
iNKT Invariant natural killer T cell
IPEX Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRF Interferon regulatory factor
IRIS Immune reconstitution inflammatory syndrome
ITIM Immunoreceptor tyrosine-based inhibitory motifs
iTreg Induced regulatory T cell (*in vitro* derived)
JAK Janus Kinase
LCMV Lymphocytic choriomeningitis virus
LIP Lymphopenia-induced proliferation
LN Lymph node
LRR Leucine-rich repeat
LTi Lymphoid tissue inducer
MAIT Mucosal-associated invariant T cell
MALT Mucosa associated lymphoid tissue
MHC Major histocompatibility complex
MN Mature naïve
MOG Myelin oligodendrocyte glycoprotein
MPEC Memory precursor effector cell
MS Multiple Sclerosis
mTEC Medullary thymic epithelial cell
MyD88 Myeloid differentiation primary response gene 88
NBD Nucleotide binding domain
NF-κB Nuclear factor kappa-B
NK Natural Killer
NKT Natural killer T cell
NLR Nod-like receptor
OVA Ovalbumin
PAMP Pathogen associated molecular pattern
PBS Phosphate buffered saline
PCR Polymerase Chain Reaction
PD-1 Programmed Death 1
PD-L1 Programmed-death ligand 1
PD-L2 Programmed-death ligand 2
PE Phycoerythrin
PI Propidium Iodide

pMHC	Peptide:MHC complex
pMHC-I	Peptide:MHC-I complex
pMHC-II	Peptide:MHC-II complex
PRR	Pattern recognition receptor
pTreg	Peripherally-derived regulatory T cell
PYD	Pyrin domain
Rag	Recombination activating gene
RBC	Red blood cell
RFC	Relative fold change
RLR	RIG-I like receptors
RNA	Ribonucleic acid
RTE	Recent thymic emigrant
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
SH2	Src-homology 2 domain
SH3	Src-homology 3 domain
SLO	Secondary lymphoid organs
SP	Single positive
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
Tcon	Conventional T cell
TCR	T cell receptor
TEC	Thymic epithelial cell
Teff	Effector T cell
Tem	Effector-memory T cell
Tg	Transgenic
TGFβ	Transforming growth factor beta
Th	T helper cell
TIR	Toll-interleukin-1 receptor homology domain
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
TRA	Tissue-restricted antigen
Treg	Regulatory T cell
tTreg	Thymus-derived regulatory T cell
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VDJ	Variable-Diversity-Joining
WT	Wild type

Chapter 1

Introduction

1.1. A brief survey of innate and adaptive immunity and general introduction

For as long as life has existed, organisms have faced the challenge of acquiring and competing for resources and space, and protecting themselves from other organisms attempting to do the same at their expense. Selective pressure has thus favored the evolution[1] of mechanisms to promote the ability of an organism to recognize self vs. non-self and defend against foreign invaders and dangerous insults. This is the basis of the immune system, and over evolutionary time, it has radiated and elaborated and is found throughout the tree of life[2].

All multicellular organisms studied share a variety of evolutionarily ancient common mechanisms for dealing with the threat of external invasions of their niche or other insults[2]. These mechanisms are commonly referred to as "innate immunity". The most obvious such mechanism is compartmentalization via the use of anatomic barriers, such as skin or the intestinal mucosa, to separate the internal from the external environments. However, beyond such physical barriers more specific mechanisms exist that are geared toward recognizing common signals of "danger" or tissue damage, which function in part via a series of well-conserved, germline-encoded sensors. The majority of multicellular life on the planet uses strictly these mechanisms for their protection as they are rapidly orchestrated and provide good protection against foreign pathogens and insults. Interestingly and for unclear reasons, it is only in the vertebrates that an adaptive immune system is found, characterized by cells (B and T lymphocytes) armed with antigen receptors encoded by exon-fragments that are rearranged in the genome to potentially recognize a vast universe of molecular patterns. It is important to recognize that the vertebrate adaptive immune system evolved to take cues from innate immunity in order

to orchestrate an effective, appropriately directed response, and *vice versa*. Therefore innate and adaptive immunity are highly integrated.

A critical consequence of the evolution of somatically-recombined antigen receptors is the fact that many rearrangements will result in the potential to recognize self[3], and therefore many developing B and T lymphocytes will actually pose a threat to the host unless selection mechanisms are in place to eliminate self-reactive cells from the repertoire (negative selection, "central tolerance", section 1.2.1) or keep cells in the periphery with self-reactivity from becoming activated and attacking the host (peripheral tolerance, section 1.2.2). The latter mechanisms are crucial to immune homeostasis as self-reactive lymphocytes can be found in any healthy immune competent host and defective peripheral tolerance mechanisms can result in autoimmune disease. Importantly, certain conditions such as lymphopenia (low lymphocyte count) resulting from particular viral infections, environmental exposures, or clinical interventions can push peripheral tolerance to (and beyond) its limits.

This dissertation explores the crucial role of a set of lymphocyte-expressed negative regulators of the immune response called "co-inhibitors" in the establishment of immune tolerance as cells of the adaptive immune system are first generated in a lymphopenic host. Via exploration of the mechanisms underlying failure to establish and/or maintain tolerance in situations where co-inhibition is deficient, we clarify the role of co-inhibition in controlling aspects of T cell biology such as the generation of regulatory T cells and the regulation of tonic TCR signaling.

1.1.1. Innate immunity – a blunt and effective first line of defense

A hallmark of innate immunity is the rapidity with which its effector mechanisms are activated upon detection of an insult[4]. For example, upon receiving a cut or abrasion to the skin, an inflammatory response occurs rapidly. Alterations in vascular permeability and chemokine cues promote the extravasation of leukocytes to the area of the injury. Both normally tissue-resident and trafficking phagocytes such as macrophages, dendritic cells (DC) and neutrophils can detect and engulf microbes or debris from damaged tissue at the site of injury, and produce toxic reactive oxygen and nitrogen species to eliminate foreign microbes.

1.1.1.1. Innate pattern recognition receptors

Several innate immune "sensor" mechanisms have been described[4], which allow the detection of invading pathogens via recognition of highly conserved pathogen associated molecular patterns (PAMPs), as well as endogenous signals of 'danger' released by damaged or dying tissue (damage associated molecular patterns (DAMPs)). Many cell types can express such sensors, known as pattern recognition receptors (PRR), although they are particularly highly expressed and important in phagocytic cells given their prominent role in immune surveillance. The toll-like receptors (TLR), named for their homology to an antifungal defense protein in *Drosophila* called Toll[5] are a family of PRR found on the cell surface or within endosomal compartments, comprised of leucine-rich repeat domains which interact with PAMPs/DAMPs, and a Toll-Interleukin-1 receptor signaling (TIR) domain. In humans and mice, there have been 13 TLRs described to date

with the majority present in both species[6]. The signaling pathways downstream of the TLRs are broadly characterized as MyD88-dependent and independent. All known TLRs with the exception of TLR3 can interact through their TIR domains directly or via additional adapter proteins with a signaling adapter known as MyD88, yielding activation of the NF-κB and MAP kinase pathways and generally resulting in the transcription of several genes encoding proinflammatory cytokines. TLR3, which localizes to endosomes and recognizes double-stranded RNA, signals exclusively through a MyD88-independent pathway involving the signaling adapter TRIF, driving the transcription of interferon genes via activation of interferon regulatory factor (IRF) 3 and IRF7. Some TLRs such as TLR4, which recognizes lipopolysaccharide (LPS), can signal via both pathways depending on subcellular localization.

Since the discovery of the TLRs, which are situated to detect extracellular and intra-endosomal PAMPs/DAMPs, the known repertoire of innate immune sensors has been expanded significantly to include those poised to detect intracellular signals of danger. RIG-I like receptors (RLR) are a group of cytoplasmic RNA helicases which sense the presence of viral RNA and induce interferon production in response to its presence[7,8]. Intracellular sensor complexes known as inflammasomes serve to couple recognition of DAMPs and PAMPs to the autocatalytic activation of caspase-1[9-11]. Activation of caspase-1 can result in the maturation and secretion of cytokines such as IL-1β and IL-18, pools of which are maintained in an inactive precursor form. Depending on additional regulatory mechanisms it can also result in cellular apoptosis, or the induction of pyroptosis (“fiery death”), a process that releases intracellular DAMPs and may fuel ongoing inflammation, including during chronic inflammatory diseases. Formation of

inflammasome complexes is catalyzed by ligand recognition through their sensor components, which include members of the NOD-like receptor (NLR) family that bind diverse PAMP or DAMP ligands, as well as intracellular DNA sensors belonging to the PYHIN (pyrin and HIN200 domain-containing protein) family. Like TLRs, NLR family members contain leucine-rich repeat (LRR) domains involved in ligand binding. They also contain a nucleotide-binding domain (NBD or NACHT) involved in oligomerization, and either a caspase-recruitment domain (CARD), a pyrin domain (PYD) or both. ASC, a PYD- and CARD-containing adapter protein, can interact via its PYD with PYD-containing NLRs to facilitate recruitment of inactive caspase-1 to the inflammasome. It should be noted that the NLR family is diverse and also includes members not involved in inflammasome formation such as the class II transactivator (CIITA) which controls the expression of MHC class II [12,13].

1.1.1.2. Innate lymphoid cells and unconventional T cells

In addition to phagocytes, the innate immune system contains populations of lymphoid cells that, unlike their adaptive counterparts, lack antigen receptors with high diversity and yet perform a crucial role monitoring for markers of cellular stress or infection. Innate lymphoid cells (ILC) have been classified into three groups according to their functional characteristics and effector cytokine production[14]. Natural killer (NK) cells, members of the group I ILC, are perhaps the best studied ILC. They lack TCR, and play a critical role in immune surveillance. NK cells express a variety of activating receptors that have evolved to recognize molecules associated with cellular stress and viral infection, as well as inhibitory receptors that interact specifically with molecules

involved in antigen presentation such as MHC class I and II [15]. The balance between activating and inhibitory signals that an NK cell receives dictates the outcome of NK interaction with a potential target. For example, a common viral strategy for immune evasion is to cause an infected cell to down-regulate MHC expression to inhibit presentation of viral antigens to T cells. In this case, via reduced signals through inhibitory receptors, NK cells can identify a deficiency of MHC molecules on a cell and thus target that cell for killing. NK cells also mediate antibody-dependent cell-mediated cytotoxicity (ADCC) [16], where antibodies bound to their target crosslink Fc receptors on NK cells, activating NK cells to kill the target. Other ILC including ILC1, ILC2, and ILC3 in many ways parallel the Th1, Th2, and Th17 subsets of CD4+ T cells, respectively, in terms of their cytokine production following activation and the transcription factors that promote their lineage commitment[14,17]. Precursors of these ILC can infiltrate damaged or infected tissues and depending on the local cytokine, DAMP, and PAMP milieu undergo maturation to acquire the various phenotypes. The cytokines they in turn produce can locally modulate the innate immune functions of DC and other cells and promote antigen-bearing DC migration to lymph nodes. Furthermore ILC can process and present antigen on MHC-II and thus modulate the function of CD4+ T cells. Many of the details of the biology of ILC remain unclear, and reagents to specifically manipulate them experimentally are still scarce[14]. However their position at the interface of innate and adaptive immunity makes them attractive therapeutic targets for a variety of diseases.

Finally, blurring the definition between innate and adaptive immunity are populations of unconventional T cell subsets with rearranged but low diversity T cell receptors

(TCR). Recognized members of this group continue to expand and include natural killer T (NKT) cells, intraepithelial lymphocytes (IEL), and mucosal associated invariant T (MAIT) cells among others[18].

1.1.2. The vertebrate adaptive immune system - extreme versatility comes with a price

The rearranged, high diversity receptors characteristic of cells of the adaptive immune system facilitate the ability for T and B cells to recognize essentially any potential molecular pattern. While this ability is clearly an advantage in terms of protecting the host from threats such as pathogens, it introduces significant challenges for maintaining self-tolerance (discussed in detail in section 1.2). Extreme diversity in T and B cell antigen receptors also leads to one of the hallmarks of the adaptive immune response – a significant lag time between the initial encounter with foreign antigen, for example due to an infection, and the generation of a response of significant magnitude to clear the infection. VDJ recombination of the TCR loci can theoretically give rise to more than 10^{15} different TCR sequences[19], while a mouse and a human have approximately 10^{8} and 10^{11} total peripheral naïve T cells, respectively. In reality, the actual diversity realized in the T cell repertoire is much smaller – in mice the diversity has been estimated to be around 2×10^{6} individual clones[20] while in humans it has been estimated at about 2.5×10^{7} clones[21] although it has been suggested that these are lower bounds and true diversity may actually be higher[22]. Nevertheless, as a result, the naïve T cell repertoire contains a relatively low frequency of T cells with a TCR capable of recognizing epitopes associated with a given infectious agent. Upon encounter with their cognate antigen and

activation, T cells must undergo clonal expansion in order to reach numbers capable of clearing the infection, and this can take 1-2 weeks. The concept of low naïve precursor frequency and a requirement for clonal expansion in order to mediate a useful magnitude of immune effector function applies to B cells as well.

Clonal expansion gives rise to the second hallmark of adaptive immunity – memory. Following expansion of antigen specific B and T cells and clearance of an infection, the population of responding antigen specific lymphocytes contracts, but a proportion becomes long-lived memory cells that reside within the lymphoid organs. Because these antigen specific cells remain present at a frequency much higher than their initial frequency in the naïve lymphocyte pool, the lag associated with population expansion upon repeated encounter with the same antigen is dramatically reduced. Furthermore, these memory cells can have different functional characteristics from naïve cells such as a lower requirement for stimulation before engagement of effector functions such as cytokine production, up-regulation of Fas ligand (FasL), or up-regulation of perforin and granzyme B[23-25]. Therefore, the adaptive immune response to a pathogen previously encountered can be relatively rapid, limiting the ability of that pathogen to productively infect the host. This is the principle behind vaccination against disease.

1.1.2.1. Lymphoid organs – a brief introduction

The sites of lymphocyte development are referred to as the primary lymphoid organs. Bone marrow is a primary lymphoid organ and is the site where hematopoietic stem cells (HSC) reside and give rise to the precursors of B and T lymphocytes. Stromal cells in the

bone marrow niche provide trophic support for HSC and facilitate their long-term survival. B cells develop and undergo negative selection processes in the bone marrow, whereas T cell precursors develop in the bone marrow and then migrate to another primary lymphoid organ, the thymus, where they undergo T cell developmental processes including positive and negative selection (section 1.2) as well as lineage commitment.

Continuous trafficking of lymphocytes through secondary lymphoid organs and blood facilitates efficient antigen surveillance given the large diversity within lymphocyte populations and low clonal precursor frequency. Secondary lymphoid organs (SLO) include the spleen, as well as lymph nodes (LN) and mucosa associated lymphoid tissue (MALT). Overall spleen, LN, and MALT have a tissue architecture that maintains B and T cells in separate regions with their localization maintained and controlled by chemokine gradients (reviewed in[26-28]). In LN, T cells are enriched in the paracortex due to the presence of the chemokines CCL19 and CCL21 (produced by fibroblastic reticular cells), via CCR7. B cells localize to the B cell follicles via CXCR5 sensing of CXCL13 (produced by follicular dendritic cells). Antigen presenting cells (APC) like DC populate LN in the steady state and also traffic/drain in through afferent lymphatic vessels bearing antigens for presentation to T cells. Incoming lymph (carrying antigens) from afferent lymphatics enters the subcapsular sinus area and then makes its way through conduits, into the paracortex[29]. In sum, the arrangement facilitates optimal encounter of T cells in SLO with APC bearing antigen and the coordinated orchestration of subsequent immune responses involving other cell types such as B cells. Entry of lymphocytes into lymph nodes from blood is governed by migration through high endothelial venules (HEV), which is regulated by expression of L-selectin (CD62L) on T cells that binds to ligands

expressed only on HEV. Generally, naïve T cells and central memory T cells express CD62L and home to the lymph nodes, whereas activated effector T cells down-regulate CD62L to facilitate their exclusion from LN and maintenance in the circulation. Specialized cells known as fibroblastic reticular cells lining conduits within the paracortex (T cell area) of lymph nodes also produce the cytokine IL-7[30], which is an important resource governing the survival of memory and naïve T cells in the periphery as will be discussed in section 1.3.

The formation of lymph nodes is initiated by the actions of innate lymphoid cells known as lymphoid tissue inducer (LTi) cells, during development[31]. Mice lacking the common gamma chain (γc), a receptor subunit common to the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 cytokine receptors, do not develop lymph nodes[32] and thus can be a useful model for studying the importance of lymph nodes in immune function. Similarly mice deficient in another molecule, lymphotoxin-α (LT-α), also lack normal lymph node development[33] and are useful as a lymph node deficient model.

1.2. T cell tolerance mechanisms

The ability of B and T cells to generate an enormous diversity of somatically recombined antigen receptors is clearly an advantage in terms of being able to detect and strongly respond to essentially any non-self or altered-self antigen the host might encounter during its lifespan. However, such a system introduces the formidable challenge of discriminating self from non-self and avoiding autoimmune pathology

mediated by these potent immune effectors, and the field of immunology has a long history of efforts to explain how immune tolerance is established and maintained.

Some of the best early evidence that 'acquired tolerance' was possible came from early studies by Peter Medawar and colleagues. Skin grafts are among the most difficult tissues to successfully transplant across histocompatibility barriers – normally grafts between non-identical siblings will undergo rapid rejection. However, in 1951 while attempting to apply this technique as a way to distinguish between monozygotic and dizygotic cattle twins, Medawar's group noticed that skin grafts between dizygotic cattle twins would often, unexpectedly, be accepted[34]. A few years previously, Owen had described the ability to detect a high frequency of blood group compatibility between dizygotic bovine twins which was linked to the exchange of blood cells between the siblings through anastomoses formed in the womb[35]. Importantly, Owen could detect erythrocytes of both "donor" and "recipient" type in these animals even as adults, suggesting that hematopoietic precursor cells were exchanged and successfully engrafted in the bone marrow to generate hematopoietic chimerism, and this led to robust long-term tolerance to donor antigens. Subsequently Billingham, Brent, and Medawar carried out the pivotal experiment of injecting allogeneic blood cells from one mouse strain to embryonic mice of another, yielding long-term engraftment and tolerance to skin grafts from the donor[36]. Contemporaneously, Milan Hašek published a model whereby vascular anastomoses between chick embryos led to a subsequent failure to produce antibody against red blood cells of the partner, but not to a third party chick, upon immunization with blood after hatching[37].

While multiple tolerance mechanisms have since been elucidated, our understanding of immune tolerance is still incomplete – evidenced by the continued failure to control autoimmunity in a relatively large proportion of the population and the frequent need for lifelong immune suppression in transplant recipients. However, the known tolerance mechanisms can be grouped into two categories based on their location and timing of occurrence during the ontogeny of T cells: thymic central tolerance and peripheral tolerance.

1.2.1. Thymic central tolerance

Following rearrangement of their TCR loci developing T cells in the thymus undergo processes of positive and negative selection based on the affinity of their newly developed TCR. Positive selection ensures that the developing T cells have generated a TCR that is able to interact at least weakly with self peptide:MHC complexes (pMHC) displayed on the surface of thymic epithelial cells (TEC) – cells unable to interact with self pMHC do not receive a required survival signal and undergo death by neglect. It is currently accepted that selection for TCR able to weakly interact with self pMHC enriches the repertoire of developing T cells for those that will be "useful" – that is, able to bind foreign peptides presented in the context of self MHC (foreign pMHC). Although it remains unclear how, biophysically, self pMHC binding affinity could be predictive of the affinity of foreign pMHC binding, studies examining this relationship using the expression level of CD5 as a marker of self pMHC affinity during positive selection[38] have suggested that this is the case[39], whereas other studies have been unable to

demonstrate this relationship[40,41]. Regardless, alternate interpretations of the evolutionary impetus for and purpose of the process of positive selection to self pMHC, such as for tumor surveillance or T cell repertoire diversity management, will be discussed later. It should be highlighted at this point, however, that selection for the ability of T cells to even weakly interact with self pMHC raises the spectre of potential self-reactivity should conditions conspire in the post-thymic environment to push T cells toward activation despite a weak TCR signal. This highlights the importance of peripheral tolerance mechanisms.

Developing T cells next undergo negative selection. During this process T cells with rearranged TCR that strongly recognize self pMHC undergo death by apoptosis, removing them from the repertoire. The most definitive early evidence for clonal deletion in the thymus came from studies of TCR transgenic mice in the late 1980s[42-44] where T cells would be deleted in mice bearing the cognate antigen (or an endogenous retrovirus-derived "superantigen", Mls which could bind strongly to the transgenic TCR via a non-classical mechanism). Mechanisms to ensure negative selection in the thymus to tissue-restricted antigens also exist. The transcription factor AIRE (autoimmune regulator) is a promiscuous transcription factor expressed within the thymus in medullary TEC and DC[45-48]. AIRE drives the expression of numerous genes encoding normally tissue restricted antigens (TRA) [48-50], ensuring that developing T cells have the chance to be interrogated for high affinity interactions with these TRA. Recently multiple studies have shown that besides mediating negative selection of T cells to TRA as has been generally accepted, AIRE plays a key role in mediating the generation of thymic regulatory T cells (Treg, discussed below) specific for TRA[51-54]. The importance of

AIRE in mediating tolerance is illustrated by the development of systemic autoimmune disease in mice lacking its expression[55,56]. At the same time, the relatively mild severity of the autoimmunity in AIRE$^{-/-}$ animals could be viewed as a testament to the power of peripheral tolerance mechanisms, given that AIRE controls the expression of nearly 4000 tissue-restricted genes[50].

In retrospect, negative selection in the thymus was probably responsible for the majority of tolerance in Medawar's early experiments[57] - donor-derived MHC-expressing cells were probably in many cases able to gain access to the thymus of the recipient and mediate the negative selection of strongly donor-reactive T cells. Clinically, the strategy of generating hematopoietic chimerism to promote tolerance has also been applied in the contemporary organ transplant setting with combined bone marrow/solid organ transplantation, with promising results[58-63].

As brought up in the discussion of AIRE, an additional fate for positively selected T cells in the thymus is to become a Treg, which are important for mediating tolerance in the periphery as will be discussed in the next section. Initially described in mice as a population of CD4+CD25+ cells[64], Treg were later found to express a transcription factor FoxP3 which was necessary for their development and function [65]. The first evidence that high-affinity interactions with a self pMHC in the thymus promote development of thymic Treg (tTreg) as an alternate fate to negative selection came from studies using mice with transgenic TCR recognizing an epitope from influenza hemagglutinin (HA). It was found that crossing these TCR transgenics with HA transgenic mice did not yield deletion of the HA reactive cells in the thymus but instead resulted in their development into CD4+CD25+ Treg with suppressive activity[66]. However, some studies using other

TCR transgenic systems have suggested that apparent diversion of T cells toward the Treg lineage by high affinity/avidity agonist pMHC interactions may stem instead from increased survival of CD4+CD25+ cells compared to CD4+CD25- cells in the presence of agonist, and thus the actual decision of a T cell to deviate toward becoming a Treg may be mediated by other cues[67]. Further evidence that tTreg have higher affinity for self pMHC came from studies where endogenously rearranged TCRα chains from a transgenic mouse with a fixed TCRβ chain were cloned from CD4+CD25+ and CD25- cells. Expression of the cloned TCRα from CD25+ cells conferred enhanced ability of T cells to undergo rapid homeostatic proliferation (see section 1.3.2) upon transfer into a lymphopenic host, compared to those cloned from CD25- cells[68]. An additional approach using a Nur77-GFP reporter system which uses GFP expression as a marker of TCR signal strength found that tTreg had about twice the mean fluorescence intensity for GFP compared to conventional T cells (Tcon) [69].

1.2.2. Control of T cell activation in the periphery and peripheral tolerance

Control of T cell responses in the periphery is critical for the long-term survival of the host. Activation of anti-self responses must be avoided while preserving the ability to mount strong immune responses against foreign entities. Although thymic selection removes the majority of strongly self-reactive T cells from the developing repertoire, one can still detect such cells in any healthy immune competent animal, and indeed, expand them[70,71]. Such is the basis for models of autoimmunity such as experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS), where

inoculation of animals with a peptide derived from a component of myelin (myelin oligodendrocyte glycoprotein, MOG_{35-55}) emulsified in Freund's complete adjuvant yields expansion of a population of MOG_{35-55} reactive cells that cause central nervous system (CNS) lesions and paralysis. Clearly these MOG reactive T cells were initially present in the post-thymic T cell repertoire. So what keeps such self-reactive cells in check?

1.2.2.1. Activation of T cells requires co-stimulatory signals

Lederberg's 1959 model of clonal deletion postulated that the outcome of antigen receptor signaling depended on the timing of exposure to antigen during the ontogeny of the individual lymphocyte[72]. If an immature lymphocyte encountered antigen, it would be deleted, whereas upon maturity such a stimulus would promote activation. However, it was recognized that B cells could undergo a form of receptor diversification now known as somatic hypermutation, and Lederberg's model could not address the problem of self-reactivity arising *de novo* among mature cells, which would pose a risk to the host. Early observations that injection of purified proteins in the absence of adjuvants could induce tolerance (inhibit antibody production) to those proteins gave genesis to the idea that generation of an immune response to an antigen required multiple signals: one antigen-specific signal (ie: through the B cell receptor (BCR, membrane bound immunoglobulin)) as well as one "nonspecific" signal mediated by protein aggregates or bacterial components typically found in an adjuvant[73,74]. In 1970 Bretscher and Cohn proposed a more stringent two-signal model to explain B cell tolerance[75], suggesting that

B cell responses required the cells to receive two antigen specific signals – one through its antigen receptor, and the other via a helper T cell (Th cell) which recognized an alternate determinant on the antigen. The model invoked another ‘layer’ of antigenic specificity screening into the lymphocyte activation decision, but did not address an issue it raised: what regulated or “helped” the first Th cell and thereby ultimately determined whether to respond or be tolerant to a given antigen? Lafferty and Cunningham made a significant advance toward our current understanding of peripheral tolerance with their publication of a revised two signal model[76] which extended the model of Bretscher and Cohn to T cells and proposed that T cell activation required recognition of antigen (in the context of MHC[77]) as well as a co-stimulatory signal from a specialized APC. Dendritic cells (DC), the most potent APC known; critical for the activation of naïve T cells, were described by Steinman around the same time[78-81]. However Lafferty and Cunningham’s model raised the question of what regulated the extent of co-stimulation provided by the APC?

Charles Janeway postulated that PAMPs provided the critical accessory signals for generation of productive immune responses[82] and later predicted the existence of specific receptors involved in sensing these microbial patterns and causing up-regulation of co-stimulatory molecules on APC[83]. This was borne out by discovery of the first TLR[5]. Polly Matzinger instead took the view that signals of “danger”, for example from damaged tissue, were responsible for stimulating APC co-stimulatory molecule expression[84] – an attractive theory as it more readily accounts for allogeneic graft rejection and anti-tumoural immune responses which occur, at least in most cases, in the absence of infection.

It is now clear that numerous PAMPs and DAMPs can signal through various PRR (see section 1.1.1.1) to "mature" or up-regulate co-stimulatory molecule expression on APCs. Mature DC can present pMHC-II to naïve CD4+ T helper (Th) cells and provide co-stimulation to the T cells, resulting in their activation. In the absence of "maturation" DC cannot provide sufficient co-stimulatory signals to CD4+ T cells, causing them to become anergic[85,86], die, or become peripheral Treg (pTreg) [87]. Upon activation, CD4+ Th cells can provide help to B cells through CD40/CD40L interactions. They can also 'license' DC via CD40/40L interactions to present antigen to and activate CD8+ T cells (CTL) [88]. While not all CD8+ T cell responses require CD4+ T cell help to be initiated, in many such cases Th help is required for the formation of a strong memory population[89]. Thus CD4+ Th cells are situated as key orchestrators of the immune response, and they represent a key control point for the activation of the key effector mechanisms of the adaptive immune system.

Several co-stimulatory molecules are now known to participate in mediating optimal effector and memory B and T cell responses, although CD28 on T cells and its APC-expressed ligands B7-1 and B7-2 (also known as CD80 and CD86) are the best characterized[90-93]. The *CD28*$^{-/-}$ mouse described by Shahinian and colleagues[94] has significantly reduced steady-state levels of immunoglobulin (~20% of normal). While IgM levels are normal, IgG1, IgG2b, and IgG3 subclasses are reduced, indicative of a lack of Th help to B cells for activation and induction of class switching. Likewise T cells from *CD28*$^{-/-}$ mice show reduced proliferation and IL-2 production in response to mitogens *in vitro*. On the other hand the mice mounted a normal primary anti-LCMV CD8+ T cell response[94]. In terms of signaling, the intracellular portion of CD28 contains

motifs for the docking of src homology 2 and 3 (SH2 and SH3) domain-containing proteins upon phosphorylation including phosphoinositide-3 kinase (PI3K), growth factor receptor-bound protein 2 (Grb2), and filamin A (FLNa) [92]. Recruitment of these and other factors upon receptor engagement provides for activation of numerous downstream signaling pathways including those shared with the TCR/CD3 complex, yielding enhanced TCR signals with lipid raft recruitment to the immunological synapse[95] and NF-κB activation/nuclear translocation[96,97].

In addition to CD28, other co-stimulatory receptors including but not limited to ICOS, OX40 and 4-1BB[93,98,99] contribute to mediating the second signal for robust T cell activation. Moreover, beyond tethered ligands on APC, cytokines also play a crucial accessory role for T cell stimulation, and are also important for determining which of several potential phenotypes[100-104] the Th cell will acquire following encounter with the APC (Figure 1-1). IL-1 was one of the earliest described soluble factors, produced by LPS-treated macrophages, which could significantly enhance T cell proliferation and survival[105-108]. More recently IL-1 has been shown to act directly on T cells to promote escape from regulatory T cell suppression, aid in the formation of memory cells, and generally act as a co-stimulator for T cell activation and expansion[109,110]. Importantly, the IL-1 receptor (IL-1R) contains an intracellular TIR domain and the response to IL-1 triggers nuclear translocation of activated NF-κB[111], similar to CD28. Furthermore a CpG DNA-triggered MyD88-dependent signaling pathway yielding activation of PI-3 kinase/Akt has been reported in CD4+ T cells[112]. Given that release of active IL-1 family members is a key outcome of innate immune PRR stimulation (section 1.1.1.1), these

findings further highlight the critical linkage between innate recognition of diverse PAMPs and DAMPs and the provision of co-stimulation for T cell responses.

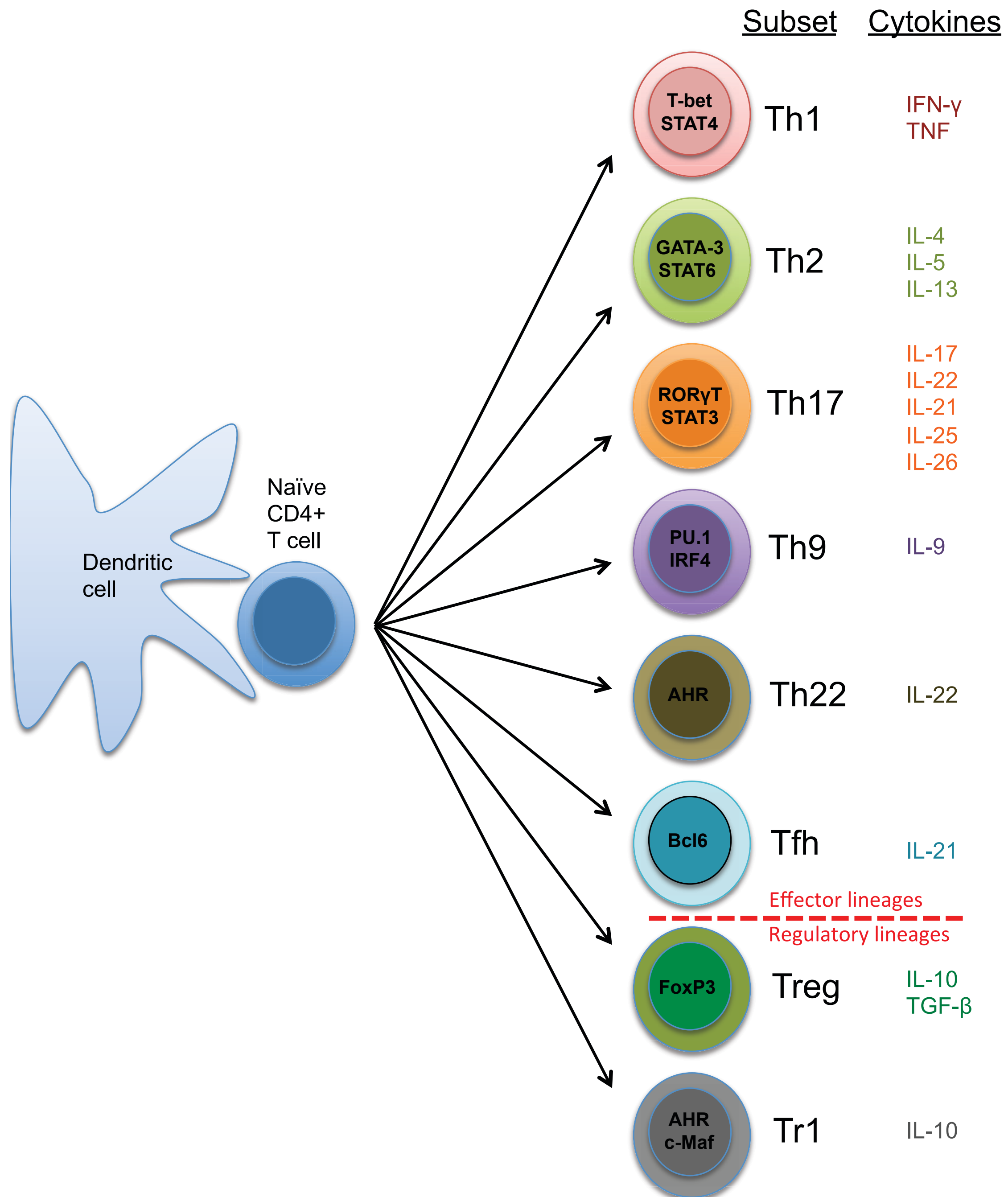

Figure 1-1. Naïve CD4+ T cells can differentiate into numerous potential effector Th or regulatory subsets.

Depending on signals received during / following activation, a naïve CD4+ T cell can acquire one of a variety of Th or regulatory phenotypes. Some evidence also suggests there is plasticity between the phenotypes. Transcription factors associated with the

various phenotypes are indicated within the cell diagrams. Key cytokines produced by the subsets are indicated to the right of the subset labels. STAT – signal transducer and activator of transcription. RORγ – RAR related orphan receptor gamma, IRF – interferon regulatory factor. AHR – Aryl hydrocarbon receptor. FoxP3 – Forkhead box P3. Adapted from O'Shea, 2010 (Ref. 102) and Raphael, 2015 (Ref. 100).

1.2.2.2. Co-inhibitory signals negatively regulate T cells

In the models of antigen-driven immune activation discussed, the decision to be tolerant to an antigen depends on the lack of co-stimulatory signals received by the T cell in addition to the antigen receptor signal. However, the basic idea that co-inhibitory signals might control adaptive immune responses (antibody production) was proposed by Nicholas Sinclair even before Lafferty and Cunningham's two signal model[113]. Sinclair also proposed later that these co-inhibitory (a term he coined) signals also applied to the regulation of T cell responses[114,115]. It is now well recognized that co-inhibitory molecules that provide a negative signal to T cells exist and are important for regulating peripheral immune responses[115,116]. Indeed, B7-1 and B7-2, the ligands for CD28 (which is expressed on resting T cells), can also bind with higher avidity to the co-inhibitory

receptor CTLA-4 which is up-regulated upon T cell activation[117]. Co-inhibitory receptors on T cells, upon interaction with their ligands on APC or other cell types including T cells themselves, generally function by recruiting phosphatases via intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) to the immune synapse to inhibit TCR signaling cascades[116] (Section 1.2.2.3). Although many co-inhibitory receptors like CTLA-4 are up-regulated upon T cell activation, even low levels induced during the naïve T-cell activation process may play a role by counterbalancing co-stimulatory signals and/or shaping the phenotypic outcome (Figure 1-1) of T cell activation. Taking the molecular mechanisms of TCR signaling, co-stimulation, and co-inhibition into account yields a more quantitative view of the nature of the TCR signal leading to activation. Rather than a binary decision between T cell activation and tolerance based on the presence or absence of co-stimulation, respectively, the balance of positive and negative influences on TCR signaling will dictate the apparent "strength" of the TCR signal and its ability to mobilize the complex transcriptional program associated with T cell activation[115,118].

Why develop a system of co-inhibitory receptors when T cell activation could presumably be controlled simply through modulation of co-stimulators? Up-regulation of co-inhibitory molecules on activated T cells suggests a key regulatory role for these molecules in the period after T cell activation. One can easily envisage situations where a mechanism to temper the activity of activated T cells in order to avoid immune pathology would be advantageous, particularly in certain sensitive tissues such as those found in the central nervous system (CNS). Moreover, T cells activated and induced to clonally expand by foreign pMHC for which they have high affinity will naturally have at

least a low affinity for self pMHC by virtue of having gone through the positive selection process. In this case, in the context of a strong response against the foreign antigen in a target tissue, cells displaying self pMHC may, due to stochastic factors, be subject to off-target effects known as bystander killing or collateral damage. Thus, co-inhibitory ligand expression in such tissues may be important for ensuring that only high-affinity interactions with activated T cells will invoke T cell effector mechanisms, avoiding such off-target effects[119]. Indeed, since weak TCR signals from self pMHC can provide a tonic survival signal to T cells (see Section 1.3), co-inhibitory molecules may be important for hastening the contraction of the effector T cell (Teff) population once the response is completed and foreign antigen is limiting. One could speculate that these basic problems may have provided the selective pressure for the evolution of a system of co-inhibitory ligands and receptors in the face of a repertoire of T cells positively selected against self pMHC. On the other hand, the ability to defuse activated T cells via co-inhibitory ligand expression represents a point of vulnerability prone to exploitation by pathogens and tumours. Accordingly, chronic antigen exposure in the context of viral infections and cancer can induce a state of unresponsiveness to antigen commonly referred to as T cell "exhaustion" [120], characterized by high expression of co-inhibitory molecules. It could be argued, however, that "exhaustion" in this case is a misnomer given that the T cells retain the potential to make effector responses upon co-inhibitory molecule blockade. Nevertheless, the use of the term in this context pervades the literature.

1.2.2.3. Properties of selected co-inhibitors

1.2.2.3.1. Programmed death-1 (PD-1)

Programmed death-1 (PD-1) is an immunoglobulin superfamily (IgSF) member related to CD28. It is a type I transmembrane protein of 50-55 kDa and exists on the cell surface as a monomer[121]. The intracellular domain of PD-1 contains two tyrosine-containing motifs – an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) [122,123], the latter of which is critical for its inhibitory function[124,125]. PD-1 is expressed on activated T and B cells, NK cells, NKT cells, and myeloid cells (APC) [126]. Expression is up-regulated on "exhausted" T cells and is associated with their dysfunction[127]. PD-1 has two known ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L1 is widely expressed on hematopoietic and non-hematopoietic cells whereas PD-L2 expression is restricted to hematopoietic cells[128]. On T cells, PD-1 is recruited to the immune synapse in TCR signaling microclusters upon T cell interaction with APC[129,130]. The tyrosine residue within the ITSM becomes phosphorylated upon engagement of PD-1 by its ligands and recruits SHP-2, a protein tyrosine phosphatase which can then dephosphorylate proximal signaling molecules associated with TCR (CD3ζ, Zap70, PKC-θ) [131] and B cell receptor (Igβ, Syk, PLCγ2, Erk) [124] signaling. In T cells this results in inhibition of CD28-mediated PI3K and Akt activation[125,128].

Consistent with its inhibitory role in controlling immune responses, PD-1 deficiency in mice leads to enhanced graft rejection and increased incidence of autoimmunity, although the outward phenotype of PD-1$^{-/-}$ mice depends greatly upon their genetic background. On the BALB/c background, mice develop autoimmune dilated

cardiomyopathy[132], whereas on the C57BL/6 background the mice develop a late-onset, lupus-like autoimmune disease with glomerulonephritis and autoimmune arthritis[133]. Blockade of PD-1 or PD-L1 in non-obese diabetic (NOD) mice hastens the onset of diabetes[134]. PD-1 also appears to play a role in preventing collateral damage or bystander killing of non-target grafts in an islet transplantation model[119]. In humans, therapeutic blockade of PD-1 with monoclonal antibodies has in recent years proven to be an extremely successful strategy for enhancing anti-tumoural immune responses in a variety of cancers[135].

As mentioned earlier and discussed in section 1.2.2.4, conventional T cells can become pTreg depending on signals they receive on encounter with pMHC, although the precise nature of these signals *in vivo* remains unknown. Some studies of the ligands for PD-1 have suggested that PD-L1 interactions with PD-1 can control the conversion of Tcon to pTreg[136,137], which could thus be an important mechanism of tumour immune escape that therapeutic PD-1 blockade inhibits. However, PD-L1 can also bind to B7-1[128,138-142], and whether a cell intrinsic role of PD-1 exists for pTreg generation has not been appropriately examined. Other studies have also suggested that PD-L1/PD-1 interaction might skew T cell development away from the Th17 lineage after activation[143,144].

1.2.2.3.2. B and T lymphocyte attenuator (BTLA)

The co-inhibitory receptor B and T lymphocyte attenuator (BTLA) is a ~70 kDa type I transmembrane glycoprotein which, like PD-1, is a member of the IgSF related to

CD28[145]. Its intracellular domain contains two ITIMs and upon engagement by its ligand herpesvirus entry mediator (HVEM) [146,147] can recruit the phosphatases SHP-1 and SHP-2[145,148]. BTLA is expressed on B and T lymphocytes (higher on Th1 cells), as well as DC and myeloid cells[145,149] and can be up-regulated upon T cell activation (transiently) or DC maturation. BTLA's ligand HVEM is a tumour-necrosis factor/tumour-necrosis factor receptor (TNF/TNFR) superfamily member, which is expressed by T cells, B cells, NK cells, DC, and myeloid cells and its expression can be induced in other tissues[149]. HVEM expression is high on naïve T cells and decreases upon activation. While triggering of BTLA delivers an inhibitory signal via phosphatase recruitment, BTLA engagement of HVEM can direct a reverse stimulatory signal that promotes NF-κB activation[150]. HVEM can also engage the inhibitory receptor CD160 on T cells, the stimulatory receptor LIGHT on B and T cells (both of these interactions can also stimulate the HVEM-bearing cell), or interact with and be engaged by the soluble protein lymphotoxin α (LT-α) [151]. Thus the signaling arrangement among BTLA, HVEM, and HVEM's other binding partners is complex.

BTLA$^{-/-}$ DO11.10 T cells polarized to Th1 *in vitro* showed an increased proliferative response to OVA peptide, markedly increased antibody responses, and BTLA$^{-/-}$ mice on the 129SvEv background had a higher susceptibility to EAE induced by a suboptimal dose of myelin oligodendrocyte glycoprotein (MOG) peptide emulsified in complete Freund's adjuvant[145]. Upon aging, 129SvEv BTLA$^{-/-}$ mice developed hypergammaglobulinemia, anti-DNA antibodies, spontaneous autoimmune hepatitis, and multi-organ inflammatory infiltrates, accompanied by enhanced peripheral T cell activation and reduced long term survival compared to WT controls[152]. The latter study

found no reduction in total CD4+ FoxP3+ cells associated with BTLA deficiency and no defect in suppressive function in BTLA$^{-/-}$ Treg. To our knowledge no specific examination of whether BTLA can control the generation of potentially smaller subsets of Treg such as pTreg has been carried out. Indeed, BTLA deficient mice were shown to be resistant to the induction of oral tolerance or tolerance driven by high-dose peptide[153]. While HVEM is down-regulated on activated T cells, it is up-regulated on FoxP3+ Treg[154]. One study has suggested that a mechanism of Treg suppression may be mediated via HVEM interacting with BTLA on conventional T cells[154].

BTLA expression is, like PD-1, associated with T cell dysfunction in tumour-antigen specific T cells in human cancer, and blockade of BTLA can help to enhance their function[155,156]. Given the recent successes of therapeutic blockade of co-inhibitory molecules in tumour immunotherapy, BTLA is among a growing list of targets for therapeutic intervention[157].

1.2.2.4. Regulatory T cells are critical for self-tolerance

Regulatory T cells (Treg) are a group of T cell subsets that mediate tolerance largely via the suppression of activation of conventional T cells (Tcon). The best-studied subsets of Treg are CD4+ and express the transcription factor FoxP3 which is necessary for their function – ectopic expression of FoxP3 in conventional T cells can confer upon them a suppressive phenotype[158]. The importance of these FoxP3+ Treg cells in tolerance is clearly demonstrated by the development of a fatal lymphoproliferative and autoimmune

disorder in mice (scurfy) and humans (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX)) with loss of function mutations in the *Foxp3* gene[65,159]. The importance of FoxP3 in T cells specifically was reinforced by studies in mice with a conditional deletion of *FoxP3* in T cells, which recapitulated the scurfy phenotype[160]. As discussed in section 1.2.1, natural (thymic) tTreg are selected based on high affinity in the thymus and multiple lines of evidence suggest they have TCR repertoires skewed toward self reactivity[66,68,69]. Peripheral Treg (pTreg) also express FoxP3 and have suppressive activity, and are converted from Tcon in the periphery[161,162]. Induced Treg (iTreg) can be generated *in vitro* by TCR stimulation in the presence of TGFβ[163,164], although these may not recapitulate all of the functional properties of naturally derived pTreg. The factors directing natural (*in vivo*) conversion of Tcon to pTreg are not completely understood, although TGFβ and retinoic acid (RA) appear to be important[165-167]. Some evidence suggests that co-inhibitory signaling through PD-L1/PD-1 may also be involved[136,137] and like tTreg, high affinity interaction with pMHC appears be a contributing factor to promoting Tcon to pTreg conversion[168,169]. The lack of reliable specific markers differentiating pTreg from tTreg complicates research on this cell subset. Both tTreg and pTreg are critical for the maintenance of systemic self-tolerance as demonstrated by development of autoimmunity in mice with specific deficiencies in one or the other[170-172], although pTreg are enriched at mucosal sites and may be particularly important for tolerance to exogenously derived antigens from food or commensal microbiota[173,174].

Functional mechanisms of Treg are incompletely characterized but can be generalized into two categories – cell contact independent and cell contact dependent.

CD25, one of the original markers used to identify Treg, is the high affinity IL-2 receptor α chain. IL-2 serves as an important survival factor for Treg cells, which express high levels of CD25, but effector T cells (Teff) also up-regulate CD25 and IL-2 promotes their survival and activity. Sequestration of IL-2 from Teff has thus been suggested as a mechanism of Treg function, although a large proportion of CD4+FoxP3+ cells with suppressive activity do not express CD25[174]. Numerous secreted factors have been associated with Treg suppression including IL-10[175], IL-35[176], and TGFβ[177]. Using an *in vitro* suppression assay system in which responder T cells were segregated from Treg with a porous membrane, it has been demonstrated that soluble factors cannot account for Treg suppression of responder cells and that cell contact dependent mechanisms were required[178]. However, *in vivo* the situation is more complicated, as mice with conditional deletion of IL-10 in FoxP3+ cells displayed enhanced inflammatory responses at environmental interfaces, namely colitis, exaggerated immune-mediated airway hyperreactivity, and skin hypersensitivity[179]. Thus, the extent to which soluble factors produced by Treg participate in immune suppression seems to vary with the tissue. Another presumably non-specific but cell contact-dependent mechanism of suppression involves the CD4 homologue LAG3 that is expressed on Treg cells[180]. LAG3 has a higher affinity for MHC-II than CD4[181] and could theoretically compete for MHC-II binding with Tcon-expressed CD4, perhaps interfering with CD4-mediated recruitment of lck to the TCR signaling complex. However, one study demonstrated that LAG3 on Treg could function via cross-linking MHC-II and inducing an inhibitory signaling cascade that inhibits DC maturation[182]. Indeed multiple studies have indicated that Treg can modulate the expression of co-stimulatory molecules on APC[183-186].

If nonspecific soluble-factor / ligand mediated inhibitory mechanisms are the major mode of Treg function, it raises the important question of how the maintenance of self-tolerance by Treg does not yield widespread immune suppression. Treg TCR specificity is likely to play a key role beyond the initial Treg-activating event in guiding ongoing Treg suppressive mechanisms. Though several reported mechanisms for Treg activity function through modulation of the DC's ability to initiate activation of Tcon, how is specificity achieved in this context? One study examined interaction of Treg, Tcon, and DC in explanted lymph nodes by using two-photon laser scanning microscopy, and found that Treg could make persistent antigen-specific contacts with DC, correlating with the inhibition of Tcon activation[187]. This suggests that Treg can compete with Tcon for pMHC-binding through the formation of stable interactions with DC presenting their cognate pMHC. Notably the authors did not detect stable Tcon:Treg interactions associated with suppression. Furthermore, neuropilin-1, expressed by many (but not all) thymic Treg[188,189], has been shown to contribute to prolonging interactions between Treg and DC, facilitating increased Treg antigen sensitivity and suppression[190]. Finally, another intravital lymph node imaging study demonstrated that Treg could significantly decrease the formation of stable contacts between Tcon and DC in the presence of antigen[191]. CTLA-4 is an inhibitory receptor up-regulated on activated T cells that is also expressed constitutively by Treg. Multiple studies have demonstrated that CTLA-4 is required for Treg suppressive function[192,193]. Importantly, mice in which CTLA-4 deficiency is restricted to the FoxP3+ lineage develop severe systemic autoimmunity similar to the scurfy mouse[194], demonstrating the critical role of CTLA-4 in systemic tolerance mediated by Treg. Indeed, CTLA-4 has been demonstrated to 'strip' molecules

of B7-1 and B7-2 directly off of the surface of DC by a process of trans-endocytosis[195]. However, modulation of the levels of co-stimulatory molecules by Treg in this way again raises the issue of the specificity of regulation by Treg. APC presenting pMHC derived from a foreign entity that demands a productive T cell response will invariably also be presenting many self pMHC against which Treg are specific and mediate suppression. Thus removal of co-stimulatory molecules from the APC by Treg would seem to preclude a productive response to the foreign pMHC, unless some mechanism exists to regulate a response to pMHC from different sources separately. This remains one of the more important dilemmas in immunology. A potential solution to this problem is proposed and discussed in Chapter 5.

1.2.2.5. Recent thymic emigrants have altered functional properties that may promote tolerance

The continuous output of newly generated T cells from the thymus plays an important role in maintaining the diversity of the peripheral T cell repertoire[196-201]. However, these newly minted T cells emerging into the post-thymic environment, termed recent thymic emigrants (RTE), have essentially only passed through the first "filtration" process against self-reactivity and thus scrutiny by peripheral tolerance mechanisms is crucial at this point in their ontogeny. In addition to the previously described requirement for multiple signals to induce T cell activation and the importance of suppression by Treg, studies of RTE generally indicate that a defined time window after emergence from the thymus exists during which these cells display altered functional properties and phenotype compared to the mature naïve (MN) T cell population.

RTE have been studied using several different experimental systems of widely varying reliability. Although most single-positive (SP) thymocytes are newly generated T cells, peripheral T cells can recirculate back to the thymus potentially complicating interpretation of experiments simply using thymic CD4 or CD8 SP as RTE[202]. A reporter strain of mice known as Rag2pGFP[203,204] have become a popular tool for investigating the characteristics of RTE. These mice bear a reporter transgene containing the Rag2 promoter upstream of an expression cassette for green fluorescent protein (GFP). The Rag2 promoter is active during early VDJ recombination and inactive after successful TCR rearrangement. Thus in Rag2pGFP transgenic mice, GFP is expressed in T cells during TCR rearrangement, and ceases when TCR rearrangement is complete. GFP remains detectable at variable intensities in the resulting T cells for approximately 3 weeks depending on the number of division cycles the T cells go through, permitting identification and purification of recent thymic emigrants in the periphery. In characterizing the model Boursalian et al. reported that GFP^{hi} cells had left the thymus within the last week, and GFP^{low} cells were 2-3 weeks old[204].

In terms of cell surface phenotype examined using the Rag2pGFP system, the transition from GFP^{hi} RTE to GFP- MN cells is associated with age-dependent reduction in CD24 and CD3 expression among both the CD4 and CD8 SP populations, though the change in CD3 expression was more pronounced in the CD8 SP population[204]. Expression of CD28 and IL-7Rα gradually increased during RTE maturation but the magnitude of the change was small. Functionally, Boursalian et al. found that purified CD4+GFP+ RTE proliferated less *in vitro* in response to anti-CD3 + anti-CD28 than non-RTE, up-regulated CD25 to a lower extent, and produced less IL-2[204]. IL-2

supplementation was unable to rescue the proliferative defect in CD4+ RTE. CD8+ RTE also proliferated less than non-RTE in response to anti-CD3 although the defect could be rescued by IL-2 supplementation. A recent study suggested that RTE are more sensitive to IL-7 than MN T cells, but are predisposed to survival rather than proliferation in response to signals through the IL-7R and undergo less lymphopenia-induced proliferation (section 1.3.2) compared to MN T cells upon transfer to a lymphopenic host[205]. RTE generated *in vitro* via fetal mouse thymic organ culture were found to express CTLA-4, and unlike mature cells were unable to induce graft-versus host disease in NK-depleted and irradiated allogeneic SCID recipient animals[206]. Furthermore defective CTL generation[204], *in vitro* TNF-α production[207], and *in vivo* memory precursor effector cell (MPEC) generation and IFNγ production in response to infection[208] have been reported in CD8+ RTE. Interestingly, CD4+ RTE have been shown to be resistant to polarization toward the Th1, Th17, and iTreg lineages via *in vitro* culture with polarizing cytokine and blocking antibody cocktails, but biased toward the Th2 lineage[209]. On the other hand, some studies have reported that CD4+ RTE are more predisposed to become iTreg due to enhanced retinoic acid sensitivity[210], have very similar capacity for proliferation[211] or even enhanced IFNγ and IL-4 production in response to TCR stimulation[212] compared to resident peripheral naïve cells. Complicating matters, differences between RTE purified from neonates vs. adult animals have been reported, with neonatal RTE producing more IFNγ and IL-4 in response to polyclonal stimulation *in vitro*, and neonatal cells able proliferate in response to only IL-7 whereas adult RTE cannot[213]. Beyond CTLA-4 expression in RTE[206], the extent of co-inhibitory molecule expression among RTE vs. MN cells has not been well studied,

although PD-1 appears to be functionally important[214]. Overall, the bulk of the data suggest that the functional properties of RTE are altered compared to MN T cells and this may be geared toward promoting tolerance or preventing self-directed immune pathology during the first weeks of life in the periphery for newly generated cells. However, given the caveats associated with various methods for identifying, tracking, and purifying RTE, along with differences in RTE from animals of different ages, the variability in experimental design in the RTE literature renders the interpretation somewhat unclear.

1.3. Peripheral homeostasis of T cells

While newly generated T cells are continuously emerging from the thymus, the total size of the T cell pool remains relatively constant in an adult animal. This is due to mechanisms of peripheral T cell homeostasis that serve to maintain the number of T cells within a normal range. Because ongoing immune responses and tolerance mechanisms can potentially skew the dynamics of the T cell repertoire, for example by expanding particular clones, or causing particular specificities to become anergic or deleted due to chronic antigen exposure, a constant influx of RTE into the periphery serves an important role in maintaining a diverse T cell repertoire that is ready to respond to a given threat[196-201]. However, in order to accommodate these new cells and maintain a constant total number of T cells in the host, older cells must be removed. But how does the system determine which cells should be removed, if the goal is to maintain a diverse repertoire?

1.3.1. Competition for resources determines survival and expansion of T cells

In general, T cells compete for available “niche” or “space”, which is defined by the resources and cellular interactions they require for survival [215,216]. Thus, the levels of resources available, number of competitors for those resources, and trafficking ability can greatly influence T cell homeostasis.

1.3.1.1. pMHC as a resource

Long term survival of naïve but not memory T cells in the periphery depends in part on their receipt of “tonic” stimulatory signals through the TCR that are, in the steady state, provided by interaction with self pMHC[217-222]. While some of the same self pMHC complexes that drive positive selection in the thymus may provide the tonic TCR stimulus in the periphery[222], recent evidence suggests that thymic and peripheral pMHC repertoire are significantly different. Cortical thymic epithelial cells (cTEC), have been shown to express an alternate proteasomal subunit, β5t, and the resulting “thymoproteasome” generates at least some unique peptides for loading into MHC that are specific to the thymus[223-226]. These peptides are important for CD8 positive selection as $\beta 5t^{-/-}$ mice have severely reduced CD8 SP development. Similarly, expression of thymus-specific serine protease (TSSP) [227,228] and cathepsin L[229,230] in cTEC yields thymus specific peptides that drive positive selection of CD4+ T cells. The absence of thymus-specific pMHC in the periphery may have important consequences for the regulation of T cell survival and maintenance of repertoire diversity.

Despite these peripheral vs. thymic pMHC repertoire differences, the same basic criteria that developing thymocytes must meet in order to pass positive selection and exit the thymus – the ability to at least weakly interact with available self pMHC - also determine their survival in the periphery. Moreover, for a given T cell, the set of pMHC with which it can interact to receive a tonic survival signal comprises a resource for which it must compete in order to survive with other T cells of the same clone (intraclonal competition) [231-235], and also with different T cell clones with which it shares overlap in pMHC specificity (interclonal competition) [236-238]. Indeed, T cells with high promiscuity in their TCR:pMHC interactions and/or with higher affinity for self pMHC may have a significant survival or proliferative advantage versus less promiscuous or high affinity cells. Furthermore, as discussed in section 1.2.2.4, one mode of Treg suppression might be competition with Tcon for access to pMHC, and in this regard Treg could be viewed as "professional competitors" in terms of the battle for the pMHC resource during establishment of homeostasis. Thus a complex and dynamic system of intra- and interclonal competition for self pMHC likely contributes to controlling the peripheral diversity of the T cell repertoire.

1.3.1.2. Homeostatic cytokines as a resource

Beyond exposure to pMHC, cytokine stimulation is also required for the survival and homeostasis of both naïve and memory CD4+ and CD8+ T cells *in vivo*. Indeed, CD4+ and CD8+ memory cells have been shown to survive independently of TCR:pMHC interactions[239-241] and depend primarily on cytokines in this regard. While multiple

cytokines can play a role in promoting T cell survival and proliferation, depending on the circumstance, IL-7 is the most important[216,242-244]. A critical role for IL-7 was suggested by the demonstration of impaired survival and proliferation of naïve and memory T cells following transfer to IL-7$^{-/-}$ hosts or neutralization/blockade with anti-IL-7/anti-IL-7R[245-248]. However, it should be noted that studies that employ IL-7$^{-/-}$ hosts and outline a critical role for IL-7 in facilitating proliferation in lymphopenic hosts should be interpreted with caution given that IL-7 is important for lymph node development[31] which may have influenced the proliferation seen in those studies[245,246]. A more recent study generated mice in which IL-7R expression could be conditionally ablated in peripheral T cells via tamoxifen administration. This demonstrated a cell-intrinsic role for IL-7R signaling for peripheral T cell survival and also implicated IL-7R signals in control of glucose metabolism in T cells[249]. Furthermore, provision of supraphysiological levels of IL-7 via transgenic overexpression[250,251], or administration of IL-7[252-255] significantly boosts T cell numbers, consistent with the notion that levels of this resource contribute greatly to dictating T cell space and thus the size of the T cell pool. IL-15, another homeostatic cytokine the receptor for which shares a subunit with the IL-7R (discussed below), also contributes to the persistence of CD8+ memory T cells *in vivo*[256-258] but IL-7 plays a non-redundant role in their survival[259].

The IL-7 receptor (IL-7R) is a heterodimeric complex of the IL-7 receptor α chain (CD127) as well as the common γ chain (CD132), a subunit also shared with the receptors for IL-2, IL-4, IL-9, IL-15 and IL-21[260]. Briefly, two key signaling pathways downstream of the IL-7R are known to be important in the survival and proliferation of T cells - the phosphoinositide 3-kinase (PI3K) signaling pathway, which, notably, is also

downstream of CD28 (see section 1.2.2.1), and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, via JAK1/3, and the transcription factor STAT5 [244,261-263]. Although many details of the signaling pathways downstream of IL-7R remain to be worked out, some data suggest that the JAK/STAT and PI3K pathways may control the survival and proliferation of T cells in reponse to varying doses of IL-7 separately. Low levels of IL-7 (0.1 ng/mL) were shown to promote the survival but not proliferation of human RTE *in vitro*, which was independent of PI3K activity, whereas long term culture (>72 h) with high IL-7 (10 ng/mL) promoted their efficient proliferation in a PI3K-dependent manner[262]. Proliferation of T cells is associated with up-regulation of the glucose transporter Glut-1[262,263], which could be blocked with the PI3K/AKT inhibitor LY294002[262]. IL-7 is associated with T cell survival through up-regulation of anti-apoptotic factors such as Bcl-2, Bcl-Xl, and Mcl-1, which can be blocked by the JAK/STAT pathway inhibitor Pan-Jak but not LY294002[264]. Another study suggested that in mice, TCR ligation can directly inhibit IL-7-mediated Bcl-2 expression and promote survival by driving anti-apoptotic Bcl-Xl and A1 expression[265]. Thus, two mutually exclusive pathways leading to T cell survival exist in mice, with the TCR signal-mediated pathway dominant when sufficient TCR triggering occurs. Overall, variable responses (survival vs. proliferation) to increasing availability of IL-7 and pMHC resources is important for the regulation of the size of the peripheral T cell compartment, and gives rise to a phenomenon known as lymphopenia-induced proliferation.

1.3.2. Lymphopenia and lymphopenia-induced proliferation (LIP)

Lymphopenia (or lymphocytopenia) is the condition where the number of lymphocytes in a host is low compared to the normal range. Lymphopenia can result from viral infections, radiation exposure, drugs (often chemotherapeutic drugs), or other factors. As discussed in section 1.3.1, competition for resources like pMHC and cytokines, particularly IL-7, is responsible for maintaining the relatively constant size of the T cell pool. Remaining T cells in an otherwise normal host that has been rendered lymphopenic, or T cells transferred to a fully lymphopenic host such as a $RAG1^{-/-}$ or $RAG2^{-/-}$, will thus "sense" the increase in T cell space (availability of resources) and expand to fill the niche[216,266,267]. This process is termed lymphopenia-induced proliferation (LIP), and beyond simply repopulating the T cell compartment, LIP is associated with spontaneous accumulation of a memory phenotype in many of the dividing cells[268-270] and it can promote the development of autoimmune disease (section 1.3.3). If one considers a host that supports LIP of transferred T cells to be lymphopenic, then neonatal mice in which the periphery is first being seeded with lymphocytes would also fall into this category[271-273]. Thus the process of LIP and its regulation is important not only for recovery from lymphocyte-depleting insults, but for the establishment from the very start of a functional and diverse peripheral immune system.

1.3.2.1. Resources, competition, and basic requirements for LIP

The factors that drive LIP are the same as those required for T cell survival (section 1.3.1). Therefore naïve CD4+ and CD8+ T cells expand in response to TCR/pMHC

interaction and IL-7 and fail to expand significantly in MHC-deficient lymphopenic hosts[219,274-279] whereas central memory T cells require only cytokines (IL-7 or IL-7+IL-15) [280] (although pMHC can still influence their LIP[281]). Efficient LIP requires lymphocytes to gain entrance to secondary lymphoid organs; particularly lymph nodes[30,282,283], where specialized IL-7 producing cells known as fibroblastic reticular cells are situated in the T cell zone (paracortex, see section 1.1.2.1), along with DC presenting self pMHC. The concept of competition between T cells for "space" in the SLO was demonstrated by Dummer et al. [283] by taking advantage of the ability of pertussis toxin (PTX) to block entry into the T cell zones of SLO by interfering with the chemokine receptor CCR7. Thy1.2+ Polyclonal competitor CD4+ T cells pre-treated with or without pertussis toxin (PTX) were co-injected with carboxyfluorescein succinimidyl ester (CFSE)-labeled responder cells, and competitors which could gain access to the SLO (not PTX treated) were able to block proliferation. Thus, lymph nodes represent finite reservoirs of resources for T cell survival and LIP.

Given that the pool of resources dictates the T cell niche, variances in the ability to compete for those resources between T cell clones would be expected to influence their rate of expansion in a lymphopenic host. Accordingly, the intensity of the signal resulting from TCR interacting with pMHC is considered to be an important factor in the extent of LIP that a T cell undergoes[270,284]. T cells from various TCR transgenic models have been shown to undergo differential LIP after transfer to lymphopenic hosts[222,281,285,286]. The level of CD5 expression on T cells has been reported to directly correlate with the intensity of the positively-selecting TCR/self pMHC interaction they experienced in the thymus[38]. Several studies have demonstrated that CD5 expression

also correlates with the extent to which various TCR transgenic T cells undergo LIP post-transfer to a lymphopenic host lacking the cognate antigen[236,286-288] although others have found that CD5 was not a good predictor of LIP potential[281]. Studies of LIP in polyclonal populations gave rise to the observation that some cells would undergo very rapid proliferation upon transfer into a lymphopenic host and lose all of their CFSE proliferation dye label, in contrast to a varying proportion of the transferred population which remained CFSE positive (had undergone generally < ~7 divisions). This gave rise to the idea that two forms of LIP existed: "spontaneous" or "endogenous" proliferation (CFSE- cells), and "slow" or "homeostatic" proliferation. The fast, spontaneous form of LIP in polyclonal T cells was shown to depend largely, but not entirely, on commensal gut microbiota as it is decreased significantly in germ-free mice[289,290]. The effect is not entirely dependent on a specific T cell response to microbial antigens, however, as TCR transgenic $Rag^{-/-}$ OT-II CD4+ T cells also underwent spontaneous proliferation in the absence of OVA that was abrogated in germ-free hosts[291]. Importantly, spontaneous proliferation of OT-II cells could be rescued in germ-free hosts by provision of cecal bacterial lysate-pulsed DCs, which depended on MyD88 and IL-6 expression in the DCs[291]. Thus innate immune stimuli can promote nonspecific stimulation of T cells and enhance their LIP. The division of LIP into two categories based on CFSE dilution is highly artificial as it depends on the length of transferred cell residency in the recipient (which differs between studies, clouding interpretation of the literature) and there is no evidence that any meaningful qualitative difference exists between cells that have undergone 7 vs. 8 divisions, for example, after a certain period of time. Indeed, transfer of a small number of CFSE-labeled OT-I $Rag^{-/-}$ TCR transgenic T cells (1×10^6) into

Rag$^{-/-}$ hosts gave rise to what would be considered both “spontaneous” and “homeostatic” proliferation via this scheme, whereas transfer of 1 x 10^7 of the same cells gave rise to only the slow “homeostatic” version[292]. Therefore even within a population of T cells with identical specificity for non-microbial antigens some cells will undergo significantly more divisions than others and competition plays a large role in shaping this outcome. Taking the aforementioned observations on differential LIP based on TCR signal intensity and level of competition into account, the dichotomy between spontaneous and homeostatic proliferation can likely be resolved simply by recognizing that T cell interactions with self pMHC would be generally expected to be weaker than the interactions with microbial antigen-derived foreign-pMHC, and thus TCR/foreign-pMHC interactions can more strongly promote LIP in the subset of microbial antigen-responsive T cells within a polyclonal population. Indeed transfer of TCR transgenic T cells into a host bearing the cognate antigen yields a potent proliferative response compared to a non-cognate antigen bearing host, and this can also result in autoimmunity[293] (section 1.3.3). Although some evidence has suggested that fast spontaneous and slow homeostatic proliferation have differential requirements for IL-7[294], the inability of anti-IL-7Rα blockade to inhibit spontaneous proliferation may result from strong signals through the TCR compensating for the loss of the IL-7R-mediated signal as might be expected if the overall strength of various integrated signals (TCR, cytokines, and other) dictate the efficiency of competition and LIP. Furthermore, as TCR ligation has been demonstrated to result in a block of IL-7/IL-7R mediated survival signals in an affinity-dependent manner[265], particularly strong agonist pMHC might block responsiveness to IL-7 entirely.

1.3.2.2. Co-stimulation and co-inhibition affect LIP

Co-stimulation is important for the productive activation of T cells during normal immune responses in lymphoreplete hosts (as discussed in section 1.2.2.1) and likewise co-inhibition is an important mechanism of restraint. While it is clear that signals through the TCR and cytokines can promote LIP, additional signals such as those mediated by co-stimulatory and co-inhibitory molecules can also play a role. However, the role of these co-signaling molecules during LIP has received far less study. Prlic et al. initially reported that polyclonal CD28$^{-/-}$ T cells could undergo relatively normal LIP compared to WT cells upon transfer into irradiated hosts, including those lacking expression of the additional co-stimulatory molecules CD40 or 4-1BBL[295]. This suggested that co-stimulation (at least via these well-characterized co-stimulatory mechanisms) is not a requirement for LIP. Further comparison of equally mixed WT and CD28$^{-/-}$ polyclonal naïve T cells co-transferred into Rag$^{-/-}$ lymphopenic recipients demonstrated that co-stimulation through CD28 played a minimal role in LIP of CD8+ T cells, but the CD4+ compartment became dominated by WT cells which were ~10 fold more numerous than CD28$^{-/-}$ cells by 8 days post-transfer[296]. Importantly, treatment with CTLA-4-Ig or transfer into B7-deficient hosts could completely block this competitive advantage conferred by co-stimulation. Likewise CTLA-4-Ig treatment could inhibit LIP of DO11.10[296,297] and HA TCR transgenic CD4+ cells[296]. Interestingly CTLA-4-Ig also appeared to have a B7-dependent inhibitory effect on LIP of CD28$^{-/-}$ DO11.10 and polyclonal CD4+ T cells[296], suggesting that B7 on APCs could promote LIP via an additional interaction. B7-1 is known to be capable of interactions with PD-L1, a ligand for the co-inhibitor PD-1 on T cells which itself is expressed on T cells[128,138-142]. Butte et

al. determined that PD-L1 and B7-1 interaction provided bidirectional negative signals[138], and thus increased LIP would instead be expected from blocking their interaction. However one could speculate that the higher affinity CTLA-4-Ig interaction with B7-1 relieves its sequestration (*in trans* or potentially *in cis*) of PD-L1, facilitating PD-L1's interaction with PD-1 on the T cell and thus inhibition of LIP. The interplay of these molecules is complicated even further by recent reports that PD-L1 can interact with B7-1 expressed by T cells to augment their proliferation[140]. Further experimental exploration of the dynamics of the interactions between B7 family members in LIP is certainly warranted.

Receptors with known co-inhibitory function have also been demonstrated to control LIP. TGF β is an immunoregulatory cytokine important for the generation of Treg cells (section 1.2.2.4) but was also recently shown to act directly on conventional T cells to restrain their lymphopenia-driven expansion[298]. Notably, by 7 days after a transfer of equally mixed polyclonal CFSE-labeled WT and TGF β receptor II (TGFβRII) deficient cells to Rag1$^{-/-}$ mice, both CD4+ and CD8+ TGFβRII$^{-/-}$ cells predominated by a factor of 5. Using the "fast" vs. "slow" LIP paradigm discussed above; whereas only a subset of the WT control cells underwent fast LIP after transfer, all of the TGFβRII$^{-/-}$ cells did. Moreover while antibiotic treatment of the recipients could block fast LIP of the WT cells to a large extent, it did not significantly affect LIP of the TGFβRII$^{-/-}$ cells[298]. The same study also demonstrated the restraint of TGFβRII-mediated signals on LIP of OT-I TCR transgenic T cells. It remains unclear whether the rapid LIP of these TGFBRII$^{-/-}$ cells was due to an exaggerated response to self pMHC or other resources or factors present during lymphopenia.

Similar results have been found in studies of T cells deficient in expression of the co-inhibitory receptor BTLA. 1 month following transfer of 1:1 mixed polyclonal WT and BTLA$^{-/-}$ T cells to lymphopenic recipients, BTLA$^{-/-}$ T cells outnumbered WT T cells by ~3-4 fold in the lymph nodes and ~20-26 fold in the spleen[299]. Likewise, transfer of CFSE-labeled WT or BTLA$^{-/-}$ OT-I T cells resulted in greater proliferation of BTLA deficient cells examined one week post-transfer[299]. Additional co-inhibitory molecules that have been shown to inhibit LIP include LAG-3[300] and the transmembrane adapter protein SIT[301].

It is interesting to note that transfer of TGFβRII-deficient T cells into lymphopenic recipient mice was associated with the development of autoimmune disease[298]. Another co-inhibitory molecule, PD-1, was recently reported by our group to be critically important for the establishment of immune homeostasis after reconstitution of the lymphoid compartment of adult Rag1$^{-/-}$ mice with hematopoietic stem cells (HSC). Mice reconstituted with PD-1$^{-/-}$ HSC developed severe systemic autoimmunity[214] characterized by infiltration of multiple tissues with T cells, weight loss, skin and ocular lesions, diarrhea, and death. While this latter study did not directly study the cell-intrinsic effects of PD-1 on control of LIP using a competitive assay (i.e. a mixture of WT and PD-1$^{-/-}$ T cells to test which proliferated more), elevated splenic T cell numbers, increased BrdU incorporation, and enhanced effector-memory phenotype acquisition in the T cell compartment were seen in PD-1$^{-/-}$ compared to WT HSC recipients, strongly suggesting that PD-1 also plays an important role in the control of LIP. This is supported by the finding that PD-1 was a valuable marker for a population of LCMV-specific memory T cells that expanded poorly after transfer to lymphopenic mice[302]. It is particularly

interesting to note that neonatal Rag1$^{-/-}$ mice that received PD-1$^{-/-}$ HSC were relatively spared from autoimmunity and had markedly reduced skewing of peripheral T cell populations toward the T effector memory (Tem) phenotype[214]. Such a finding may be a strong reflection of reduced T cell "space" in a Rag1$^{-/-}$ neonate, despite being lymphopenic, simply by virtue of anatomic size.

1.3.2.3. Regulatory T cells control LIP

Available pMHC is a critical resource for T cell homeostasis and LIP. The level of competition for this resource between conventional T cells, as has been described, helps to define the available T cell space. The mechanisms of regulatory T cell function described in section 1.2.2.4 generally involve regulating the access of Tcon to pMHC presented by APC by virtue of acting as a "super competitor", or regulating the level of co-stimulation provided by APC. As both of these factors (pMHC availability and co-stimulation) are regulators of LIP, it is not surprising that regulatory T cells have been demonstrated to play a role in its control[186,292,296,303-308], although mixed results in terms of their ability to do so[309] and the mechanisms responsible have been reported by various groups likely owing to the variety of experimental approaches (particularly the use of polyclonal vs. monoclonal Treg and "responder" populations).

Using a purely polyclonal system, an early study determined that equal co-injection of CFSE-labeled polyclonal CD45RB^{high} (naïve) CD4+ T cells and CD45RB^{low} CD25+ Treg cells into C57BL/6J Rag2$^{-/-}$ mice resulted in increased maintenance of the CFSE^{high} population (22%) compared to transfer of only the CD45RB^{high} responders (3%)[304].

Importantly, a 10-fold decrease in recovery of peripheral T cells from the mice receiving the Treg co-injection was noted, highlighting the importance of examining total T cell counts after LIP rather than relying on proliferation-dye dilution plots for interpretation. Later studies also demonstrated that polyclonal CD4+CD25+ cells could inhibit the accumulation of responding CD4+CD25- T cells more efficiently than an equal number of CD4+CD25- cells[296,303], suggesting that polyclonal Treg might be pragmatically viewed as more efficient competitors for resources than conventional T cells. It should be noted that CD4+ Treg also directly suppress LIP of CD8+ T cells[292], in which case they are clearly not directly competing for pMHC-I. CD4+ conventional T cells can also "help" LIP of CD8+ T cells[292] and therefore the extent that Treg suppression affects LIP of individual populations directly or indirectly in an experimental system containing a mixture of these cells can be difficult to decipher.

The ability of Treg to suppress LIP *in vivo* has been reported to depend in part on the affinity of the responding cells' TCR, as measured by CD5 expression. While sorted $CD5^{high}$ and $CD5^{low}$ polyclonal CD4+ T cells underwent similar LIP in MBP TCR Tg $Rag^{-/-}$ hosts which lack Treg, in the presence of Treg (MBP TCR Tg $Rag^{+/+}$ hosts), expansion of the $CD5^{low}$ cells was much more efficiently suppressed[305]. The same study also noted an increase in TUNEL positive and Annexin V positive cells in the lymph nodes of polyclonal T cell recipients bearing Treg as well as a decrease in their IFNγ and IL-2 production. This result may stem from a reduced ability of conventional T cells with lower affinity for self pMHC to compete with Treg for those pMHC. This would suggest that T cells on the higher end of the spectrum of self pMHC affinity might have an even greater advantage over low affinity T cells during LIP in the presence of Treg.

However, one study using Vβ/CDR3 spectratyping has suggested that the presence of Treg during immune reconstitution via LIP preserves the overall diversity of the T cell repertoire[308].

In terms of the mechanism of Treg modulation of LIP, $CD45RB^{low}$ CD25+ cells from $IL\text{-}10^{-/-}$ animals were reported to be unable to reduce the accumulation of responding $CD45RB^{high}$ cells, suggesting that IL-10 was critical for Treg control of LIP[304] although this requirement has been disputed by other studies which instead found IL-10 production by Treg was only important for the control of colitis generated by CD4+CD25- T cell transfer[292]. CTLA-4, a Treg-specific knockout of which leads to scurfy-like autoimmune disease[194], was shown to be critical for the *in vivo* control of LIP[186,307]. CTLA-4 mediated TGF-β[307] or indoleamine 2,3-dioxygenase (IDO) [186,307] production were also ruled out as functional mechanisms of Treg suppression of LIP. Furthermore, consistent with the earlier discussion of the role of CTLA-4 on Treg function (section 1.2.2.4), Bolton et al. very recently demonstrated that inhibition of LIP by Treg was due to CTLA-4 dependent down-modulation of co-stimulatory B7-1 and B7-2 expression on DC, and that the ratio of Treg:DC in lymph nodes following reconstitution of lymphopenic mice with Treg critically determined the extent to which this occurred[186].

Bourgeois et al. reported that while only ~7% of CFSE-labeled DO11.10 TCR $Rag2^{-/-}$ transgenic CD4+ T cells remained undivided after 10-15 days post-transfer to BALB/c $Rag2^{-/-}$ hosts, co-transfer of either WT polyclonal CD4+CD25+ Treg or central memory CD4+ T cells at a 2:1 ratio with the responders increased the undivided proportion to ~50% in each case[306]. Importantly, under these conditions suppression of LIP appeared dependent on competition for IL-7, as $IL\text{-}7R^{-/-}$ polyclonal central memory cells or Treg

were unable to inhibit proliferation. Given that the DO11.10 cells and polyclonal Treg in this experiment likely had minimal overlap in their pMHC specificity, this suggests that the ability of Treg to suppress LIP in non-pMHC specific way is primarily through competition for IL-7 and their ability to do so is approximately equal to central memory T cells. Moving forward, it will be important for researchers in the field to fully clarify the role of Treg TCR specificity in controlling LIP of responding T cells.

1.3.3. LIP can drive autoimmune disease

LIP is a double-edged sword – while reconstitution of a diverse and functional T cell compartment following a lymphopenia-generating stimulus is important for the host, it can also promote autoimmune disease in a susceptible host[186,214,298,303,309-316]. Homeostatic cytokines like IL-7 or IL-15 can sensitize the TCR and potentiate TCR signals[317]. A lymphopenic host with an abundance of resources (high concentrations of IL-7[318], abundant relevant pMHC), along with low competition for those resources could be considered to have high "LIP potential" (discussed in Chapter 5). Such a situation can lead to hyper-stimulation of T cells and result in their outright activation. Accordingly, modulation of the T cell compartment in ways likely to reduce available T cell space/LIP potential can ameliorate or prevent such diseases.

1.3.3.1. Selected LIP-associated diseases and models

Multiple autoimmune diseases in humans and mice have been associated with lymphopenia. In the human population, these diseases include but are not limited to rheumatoid arthritis, type-1 diabetes, Crohn's disease, celiac disease, Sjögren's syndrome, and systemic lupus erythematosus (SLE) [316]. While lymphopenia is suspected to play a role in these diseases, situations in which disease clearly manifests during/immediately following reconstitution of an ablated T cell compartment offer a clearer picture of the role of LIP in driving inflammatory disease.

1.3.3.1.1. Immune reconstitution inflammatory syndrome in antiretroviral-treated human immunodeficiency virus infection

Infection with human immunodeficiency virus (HIV) results in a pathologic decrease in the population of peripheral CD4+ T cells[319], resulting in profound immunodeficiency and susceptibility of patients to multiple opportunistic infections. Treatment for HIV involves the use of highly active antiretroviral therapy (HAART), and following treatment of patients, viral load decreases and the CD4+ T cell population recovers and undergoes LIP to fill the niche.

Upon beginning HAART, approximately 16% of HIV patients (unstratified according to co-infections) develop inflammatory disease associated with reconstitution of the CD4+ T cell compartment, called immune reconstitution inflammatory syndrome (IRIS) [313,320]. IRIS occurs primarily in patients that have presented with an opportunistic infection, such as tuberculosis (TB, Mycobacterial infection), cryptococcal meningitis,

cytomegalovirus retinitis or Kaposi's sarcoma virus infection. The pathological findings are associated with the nature of the co-infection in the patient. For example, in patients with Mycobacterial infection, IRIS is associated with lymphadenitis[321], an increase in Mycobacterial antigen-specific IFN-γ^{+}TNF-α^{+}IL-2^{-} CD4+ T cells with a memory-phenotype, Th1-associated and non-specific cytokines, and KIR^{-} $\gamma\delta$ T cells[322,323]. It has been suggested that the syndrome may result from a general "cytokine storm" [324]. Another more recent study compared serum cytokines from TB-IRIS and non-IRIS patients and found that TNF, IFN-γ, and IL-6 were prominently increased in the IRIS group, with a strong trend toward increased TGF-β in the non-IRIS controls[325]. Risk factors associated with IRIS include a lower CD4+ T cell count upon initiation of HAART, particularly values below 50 cells/μL[320], and a shorter time between treatment for TB and initiation of HAART[313,326], presumably due to the presence of abundant Mycobacterial antigen in recently-treated patients.

Recently, a mouse model of *Cryptococcus neoformans*-mediated IRIS was described in which lymphopenic Rag$^{-/-}$ mice or controls were intranasally infected and subsequently received purified polyclonal CD4+ T cells[314]. In this model, the reconstituted, infected recipients exhibited significant weight loss and increased serum TNF-α, IFN-γ, and IL-6 at 8 days post-T cell transfer compared to non-lymphopenic controls or Rag$^{-/-}$ animals that did not receive CD4+ T cells. Importantly, even though pathogen burdens were similar between infected lymphopenic and non-lymphopenic controls, only the lymphopenic recipients developed disease, highlighting the ability of LIP of the CD4+ T cell compartment to promote overzealous inflammation against a concurrent infection[314].

1.3.3.1.2. Autoimmunity after PD-1$^{-/-}$ hematopoietic stem cell-mediated immune reconstitution in mice

As described in section 1.2.2.3.1, the C57BL/6 PD-1$^{-/-}$ mouse, spontaneously develops a late-onset mild lupus-like disease characterized by glomerulonephritis as well as arthritis[133]. Nishimura et al. reported a 30% incidence of glomerulonephritis by 6 months of age, all grade 1/3; and 40% incidence by 14 months with half scored as grade 1and half as grade 2. Notably aged B6 WT mice also showed 40% grade 1 glomerulonephritis by 14 months of age. Arthritis occurred in 40% of C57BL/6 PD-1$^{-/-}$ mice by 6 months of age, all grade 1/3; and in 100% by 14 mo with 50% grade 1, 25% grade 2, and 25% grade 3[133]. Maintenance of a colony of these PD-1$^{-/-}$ mice (predominantly < 6 months of age) in conventional animal facilities over several years has in general disclosed no macroscopically apparent phenotype, although they do have a mildly increased incidence of spontaneous death compared to WT controls (Anderson lab, unpublished observations). However, in stark contrast to this mild phenotype, our group showed that reconstitution of the lymphoid compartment of Rag$^{-/-}$ mice by transfer of PD-1$^{-/-}$ HSC gave rise to a rapid, severe, systemic autoimmune disease shortly after the first newly generated T cells emerged from the thymus[214] while WT HSC recipients were unaffected. This disease manifests macroscopically as weight loss (cachexia), kyphosis (hunched posture), dermatitis, ruffled fur/poor grooming, ocular lesions, and death. Microscopically it is characterized by infiltration of multiple tissues including liver, lung, esophagus, pancreas, heart, eye, and kidney with leukocytes including CD4+ and CD8+ lymphocytes. Significant elevation of a spectrum of serum cytokines was noted in PD-1$^{-/-}$ HSC compared to WT HSC recipient controls (IFN-γ, IL-13, TNF-α, IP-10, MIG,

MCP-1, VEGF) [214]. Flow cytometry revealed an almost complete skewing of the peripheral T cell population toward an effector-memory phenotype which was not seen in WT HSC recipients (~94% vs ~34% $CD44^{hi}CD62L^{lo}$) and total splenic T cells were significantly more abundant in PD-$1^{-/-}$ vs WT HSC recipients. Furthermore, disease could also be generated in $Rag^{-/-}$ recipients by transfer of PD-$1^{-/-}$ thymocytes but not splenocytes, suggesting that newly generated T cells (RTE) were critical for generation of disease in this model[214].

Consistent with a critical role for lymph node-dependent LIP in driving the disease, lymph node-deficient $Rag^{-/-}\gamma c^{-/-}$ and lethally irradiated $LT\alpha^{-/-}$ mice were not susceptible to disease following PD-$1^{-/-}$ HSC transfer, although splenocytes from diseased $Rag^{-/-}$ PD-$1^{-/-}$ HSC recipients could transfer disease secondarily to $Rag^{-/-}\gamma c^{-/-}$ hosts. Furthermore, while all adult $Rag^{-/-}$ recipients of PD-$1^{-/-}$ HSC developed disease, only 1/7 neonatal (d1) $Rag^{-/-}$ recipients of PD-$1^{-/-}$ HSC developed systemic autoimmunity. This protection of $Rag^{-/-}$ neonates correlated with a severe reduction in the extent to which T cells acquired a $CD44^{hi}CD62L^{lo}$ Tem phenotype (~19%) compared to in adult PD-$1^{-/-}$ stem cell recipients. One interpretation is that this finding highlights the contribution of available physical “space” to the LIP-potential (section 1.3.3) in a given host and the role of high LIP-potential in driving autoimmunity. Compared to the adult, the available lymphoid stroma and “space” in the neonate would be comparatively smaller, thus resources may be exhausted more rapidly by cells undergoing LIP as compared to in an adult. In other words the relatively small neonate may not provide adequate resources and space to facilitate sufficient unrestrained T cell activation during LIP to yield autoimmunity. The same explanation for lack of disease may apply to the PD-$1^{-/-}$ mouse in which the first

newly generated T cells seed the periphery normally during the neonatal period. Beyond anatomic size considerations, it was recently suggested that a subset of innate lymphoid cells (ILC3) expressing CD4 and the IL-7R are present in neonates and can act as inhibitors of CD8+ T cell LIP via an unknown mechanism dependent on IL-7R signaling[327]. It is possible that these ILC act as "sinks" for IL-7, reducing LIP-potential early in the neonatal period and decreasing the potential for high IL-7 concentrations to promote hyper-stimulation of T cells[318].

1.4. Potential mechanisms driving autoimmunity in lymphopenic PD-1$^{-/-}$ HSC recipients and unanswered questions

The severe autoimmune phenotype of adult Rag$^{-/-}$ PD-1$^{-/-}$ HSC recipients compared to the PD-1$^{-/-}$ mouse, neonatal Rag$^{-/-}$ PD-1$^{-/-}$ HSC recipients, or WT HSC recipients[214], suggests that one of the most critical roles for PD-1 described so far is in restraining activation of T cells during LIP. Importantly, only PD-1$^{-/-}$ HSC-mediated reconstitution of the lymphoid compartment or transfer of polyclonal PD-1$^{-/-}$ thymocytes, but not splenocytes, could cause disease, suggesting that RTE were the critical population. Our view is that peripheral T cells within the splenocytes sourced from an adult PD-1$^{-/-}$ mouse would have originated as RTE that first seeded the periphery in the neonatal period, a period of reduced LIP-potential that did not permit their unrestrained LIP. Thus they would have undergone relatively normal peripheral tolerance mechanisms to remove "dangerous" specificities from the population, convert them to pTreg, or anergize them, as evidenced by the lack of overt disease in C57BL/6 PD-1$^{-/-}$ mice. Indeed CD4+ FoxP3-

RTE are preferentially converted to pTreg compared to established peripheral Tcon[210,328]. In PD-1$^{-/-}$ HSC or thymocyte recipients, RTE would have not been subjected to peripheral tolerance mechanisms yet, and thus during LIP of these cells in an adult Rag$^{-/-}$ mouse, the normal mechanisms to induce peripheral tolerance may have failed due to the high LIP-potential and a strong push toward acquisition of effector function in the absence of PD-1.

While our previous data very strongly support the hypothesis that RTE drive the disease in this model, this has not been tested using a system in which the RTE can be purified and transferred separately of the MN T cells, such as the Rag2pGFP system. Furthermore, much of the literature suggests that the RTE population is poised for tolerance generation rather than autoimmunity[196]. Whether the RTE population emerging into the periphery of an adult, lymphoreplete mouse becomes rapidly tolerized or maintains a heightened autoimmune-generating potential is not known. If RTE purified from the periphery of lymphoreplete Rag2pGFP x PD-1$^{-/-}$ mice are able to drive disease in this model, it would suggest that despite their reported tolerogenic properties, in a host with high LIP-potential RTE are critically important targets for peripheral tolerance mechanisms that rely on PD-1 and these do not act immediately upon RTE upon contact with the periphery.

While PD-1 is thought to act as a rheostat on the TCR signal via recruitment of phosphatases, it has been suggested that PD-1/PD-L1 interactions can promote the generation of pTreg from Tcon and thus PD-1 signals may qualitatively regulate T cell fate as well[128,136,137]. This might be particularly important in the RTE population in order to facilitate seeding the periphery with Treg "protecting" self pMHC with which

members of the RTE population can make interactions with high affinity. If PD-1 truly controls pTreg generation during LIP this would provide a convenient potential explanation for disease in this model. However, whether PD-1 controls pTreg generation has never to our knowledge been carefully examined using a PD-1 deficient model – the work done has used PD-L1/PD-L2 deficient animals, and recombinant PD-L1-Ig. Given PD-L1's ability to interact with other molecules such as B7-1, the paradigm common in the literature that PD-1 controls pTreg generation is in our opinion not adequately supported by experimental evidence.

PD-1 signaling has also been suggested to inhibit the generation of Th17 responses[143,144], again potentially acting in a qualitative manner to determine T cell phenotype after activation. Indeed Th17 and induced Treg development occur along a cytokine axis: while TGF-β can promote Tcon conversion to Treg, the combination of TGF-β and IL-6 promotes the conversion of Tcon to Th17 cells[329]. Thus defective Treg conversion in PD-1$^{-/-}$ T cells could result in promotion of this inflammatory Th subset associated with various chronic autoimmune processes[330], and could potentially underlie the disease in the PD-1$^{-/-}$ HSC model.

The link between LIP and autoimmune disease is by now relatively well established. However in this PD-1$^{-/-}$ HSC transfer model, what are the antigenic determinants against which autoimmunity is generated? Although PD-1 mediates an inhibitory signal and thus PD-1$^{-/-}$ T cells might be expected to be more sensitive to negative selection, the possibility that defects in negative selection exist in PD-1$^{-/-}$ animals cannot be ignored. Thangavelu et al. demonstrated intact negative selection to a ubiquitous antigen (male antigen, HY) using the Marilyn TCR transgenic (anti-HY CD4)[331] or HY^{cd4} transgenic

(anti-HY CD8)[332] that had been crossed onto the PD-1$^{-/-}$ background[214]. The question of whether PD-1 deficiency inhibits proper thymic negative selection to tissue-restricted antigens (TRA) has not been addressed however, and escape of T cell clones with high affinity to TRA remains a possible contributor to autoimmunity in this model. Arguing against this possibility, analysis of TCR Vβ usage among T cells in the diseased HSC recipients revealed no obvious clonality to the T cell response[214]. Furthermore, the skewing of ~94% of the T cell population to a Tem phenotype also suggests a polyclonal response although this may simply reflect efficient acquisition of the memory phenotype due to strong LIP[270] in the absence of PD-1 and not be directly related to disease. However, in cases where transfer of mixed WT:PD-1$^{-/-}$ HSC to Rag$^{-/-}$ recipients was carried out,~40% WT T cells in the blood prevented disease and reduced Tem phenotype acquisition among the PD-1$^{-/-}$ cells[214]. Therefore a high proportion of T cells with the Tem phenotype appears to associate with disease development. Given that co-inhibitory molecules can inhibit LIP (section 1.3.2.2), it is possible that PD-1 can also control the strength of tonic TCR signals and thus the average TCR signal a PD-1$^{-/-}$ T cell receives in response to self pMHC may be shifted higher compared to a WT T cell. A range of affinities for self pMHC would be expected to be present within any newly generated polyclonal T cell TCR repertoire, and it might be that those on the higher end of the spectrum preferentially undergo LIP and, in the absence of PD-1, breach an activation threshold leading to acquisition of effector function and autoimmunity. The effect may be quantitative - acquisition of effector functions (cytokine production, etc.) by higher affinity T cells may occur on a WT background during LIP as well, but in the case of PD-1 deficiency a greater proportion of the T cell population crosses the threshold. Although

no obvious clonality was suggested in the Vβ panel analyses of diseased PD-1$^{-/-}$ HSC recipient T cells[214], this analysis strategy may be poor at detecting wide population-level shifts in TCR/self pMHC affinity: Vβ panel analyses of CD4+CD25+ vs CD25- T cell subsets have demonstrated no apparent differences in Vβ usage[178] even though the CD25+ Treg population is reported by numerous studies to be enriched for TCR with higher self pMHC affinity[66,68,69].

What cells drive autoimmunity in lymphopenic PD-1$^{-/-}$ HSC recipients? Both CD4 and CD8+ T cells infiltrated tissues in PD-1$^{-/-}$ HSC recipients. B6 Rag$^{-/-}$ K$^{b-/-}$ D$^{b-/-}$ recipients of PD-1$^{-/-}$ HSC developed disease symptoms with an incidence and onset identical to Rag$^{-/-}$ recipients, suggesting that CD8+ T cells were dispensable for autoimmunity in this model[214]. It should be noted that hematopoietic cells in the thymus and elsewhere derived from PD-1$^{-/-}$ HSC would still have expressed MHC Class I, and thus low numbers of CD8+ T cells managed to survive thymic selection. However if the disease was CD8+ T cell dependent one might reasonably expect a reduction in incidence or delayed onset of disease in this experiment due to inefficient CD8+ T cell emigration from the thymus and reduced MHC-I-expressing targets in the periphery, which was not the case. While CD4+ T cells are typically viewed as helpers, directing and promoting humoral and CD8+ T cell responses, CD4+ T cells can also acquire direct cytolytic properties mediated by the granzyme B/perforin granule exocytosis and FasL/Fas mechanisms[333-335]. Thus it is possible that CD4+ T cells are the key mediators of disease in this model via one or both of these killing pathways. Furthermore, the spectrum of up-regulated cytokines found in the serum of PD-1$^{-/-}$ HSC recipients[214] might suggest that a CD4+ T cell-mediated "cytokine storm" [336] drives the pathology in this model.

Importantly, these three potential mechanisms are not necessarily mutually exclusive. The term "cytokine storm" was initially coined to describe the cytokine dysregulation - classically increases in the serum cytokines IL-1, TNF-α, and IL-6 - associated with the pathology of graft-versus-host disease (GVHD) [337-339]. Indeed, the numerous parallels between allogeneic GVHD and autoimmunity after transfer of PD-1$^{-/-}$ HSC or thymocytes to lymphopenic recipient mice might suggest that the latter may essentially be best described as a model of syngeneic GVHD.

1.5. Overview and objectives of the thesis

The overarching goal of this work has been to characterize and explore why and how the triple combination of newly generated T cells, LIP, and PD-1 deficiency collaborate to drive severe systemic autoimmunity in lymphopenic recipients.

In **Chapter 2**, we asked whether PD-1 played a role in the generation of FoxP3+ regulatory T cells (either tTreg or pTreg) during LIP of T cells and if this led to autoimmunity in our PD-1$^{-/-}$ LIP-driven autoimmunity model. We initially hypothesized that PD-1 was important for the control of pTreg generation given previous, indirect reports in the literature supporting this notion. Furthermore, because some of the factors that have been reported to control pTreg and Th17 cell generation overlap, and PD-1 signals had also been linked to inhibition of Th17 generation, we also hypothesized that Th17 cells might become over-represented in lymphopenic PD-1$^{-/-}$ HSC recipients and contribute to autoimmunity. We found that PD-1 does not control the generation of tTreg or pTreg, that PD-1 deficiency does not affect Treg function, and that a hallmark cytokine

of the Th17 lineage, IL-17A, was dispensable for autoimmunity in our model. We also showed that purified Treg could inhibit autoimmunity in PD-1$^{-/-}$ HSC recipients, and that mixtures of PD-1$^{-/-}$ and WT polyclonal cells transferred to lymphopenic recipients resulted in a predominance of the PD-1$^{-/-}$ population upon their expansion.

In **Chapter 3**, we asked whether newly generated T cells/RTE purified from the periphery of Rag2pGFP x PD-1$^{-/-}$ mice were able to drive autoimmunity upon transfer to lymphopenic recipients. We also analyzed the role of host MHC Class II and potential T cell killing mechanisms involved in driving disease, namely the Fas:FasL and perforin-dependent granule exocytosis pathways. We hypothesized that: 1) The peripheral newly generated T cell/RTE population in a lymphoreplete mouse is not immediately tolerized and thus when purified from adult Rag2pGFP x PD-1$^{-/-}$ mice and transferred to lymphopenic recipients it would maintain enhanced disease-generating activity compared to established T cells. 2) Disease in PD-1$^{-/-}$ HSC recipients is primarily driven by CD4+ T cells and thus MHC class II expression is required for disease. 3) Disease in PD-1$^{-/-}$ HSC is primarily mediated by a "cytokine storm" and thus perforin expression in T cells and functional Fas in the host are dispensable for disease. We found that peripheral RTE/newly generated T cells maintain enhanced autoimmune potential relative to mature cells. MHC-II expression was required in the host for disease post-PD-1-/- HSC transfer, and disease was not dependent on either functional Fas in the host or perforin in the lymphocyte population.

In **Chapter 4**, we followed up on our hypothesis that disease in PD-1$^{-/-}$ HSC recipients may be driven by an exaggerated T cell response to self pMHC which would normally only provide a "tonic" signal for T cell survival as a mechanism of normal T cell homeostasis. Here we examined the hypothesis that PD-1 could control "tonic" TCR signals from non-agonist, self pMHC. For this analysis we used a competitive assay with mixed wild type and PD-1$^{-/-}$ TCR transgenic cells specific for the male antigen HY, which will undergo LIP when transferred to a female host. We found that in a mixture of WT and PD-1$^{-/-}$ HY-specific T cells undergoing LIP in a female (no HY) host, PD-1$^{-/-}$ T cells came to predominate. This was dependent on MHC class II expression in the host, but was independent of IL-7 as measured using anti-IL-7Rα blockade. We also determined that blockade of the IL-7Rα did not inhibit LIP, and was only effective in reducing the size of the T cell compartment or blocking autoimmunity-associated loss of weight when competition between T cells for resources was high.

Chapter 2

PD-1 is not required for natural or peripherally-induced regulatory T cells: Severe autoimmunity despite normal production of regulatory T cells

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2.1. Introduction

In order to be fully activated, a naïve $CD4^+$ T cell requires not only the antigen-specific signal through its TCR ("signal 1") but also accessory "co-stimulatory" signals ("signal 2") [76,340,341]. In the absence of sufficient co-stimulation, a T cell may become anergic, undergo apoptosis, or potentially convert to a regulatory or suppressive phenotype. The expression of co-stimulatory ligands on antigen presenting cells (APCs) can be modulated via triggering of APC-expressed innate immune receptors by damage or pathogen associated molecular patterns (DAMPs, PAMPs) [5,84]. In addition to co-stimulatory signals, co-inhibitory signals are delivered to the T cell upon synapse formation with an APC and the general view is that these oppose co-stimulatory signals[115,118]. For this reason the balance between co-stimulatory and co-inhibitory receptor-ligand interactions at the immune synapse dictates the magnitude of signal 2 and whether it reaches the threshold required to allow for full T cell activation. However, it is clear that T cell activation yields more than a binary outcome, as cells can acquire a range of effector (eg: Th1, Th2, Th17, Th9, Tfh) or suppressive (eg: Tr1, peripherally-induced Treg (pTreg)) phenotypes after encounter with APCs[102,342]. Although the cytokine milieu during the T cell-APC encounter is known to influence T cell phenotype selection, some evidence suggests that particular co-inhibitory signals are capable of promoting or preventing the development of particular T cell subsets.

PD-1 is a co-inhibitory receptor expressed on T cells, B cells, and antigen presenting cells[128,343]. PD-1 has two known ligands: PD-L1 is expressed widely on many hematopoietic and non-hematopoietic cells and tissues, while PD-L2 expression is restricted to hematopoietic cells. PD-1 deficient mice are prone to late onset autoimmune

disease with variable incidence which varies depending on the background strain carrying the mutation[132,133]. Likewise, blockade or deficiency of PD-L1 on APCs or tissue parenchyma can promote autoimmunity[143,344,345]. In terms of effects on specific T cell lineages, PD-L1:PD-1 signals have been shown to destabilize the phenotype of mature Th17 cells[144]. It was also recently shown that up-regulated PD-L1 expression on T cells following IL-27 treatment could, *in vitro* and *in vivo*, inhibit Th17 differentiation but not proliferation of adjacent naïve T cells[143]. Importantly, PD-L1 has been shown to promote the generation of pTreg cells from naïve T cells *in vitro*[136,137], and blockade of PD-L1 in an *in vivo* model of tumour-induced conversion of OT-II TCR transgenic T cells to pTreg cells abrogated the conversion[137]. Additionally, transfer of naïve FoxP3^{-} T cells to PD-L1$^{-/-}$PD-L2$^{-/-}$Rag$^{-/-}$ mice yielded severe lung autoimmunity with reduced frequency of FoxP3^{+} pTreg cells[136]. While its interaction with PD-1 has been generally considered to be the mechanism by which PD-L1 promotes pTreg cells, PD-L1 is also known to interact with the APC-expressed co-stimulatory ligand B7-1 with inhibitory effects[141,142]. The interaction of B7-1 with CD28 on T cells is the prototypical co-stimulatory interaction and while it seems to be required for pTreg cell generation through mediating the production of IL-2[346], several studies have provided evidence that strong CD28 co-stimulation can inhibit pTreg cell generation[137,347,348]. Thus, the possibility exists that PD-L1 modulates pTreg cell conversion not by interaction with PD-1 but instead via its interaction with B7-1.

In contrast to the relatively mild, late-onset autoimmunity seen in PD-1$^{-/-}$ mice[132,133], we have previously described a severe, multi-organ autoimmune disease phenotype following reconstitution of the lymphoid compartment of adult lymphopenic

Rag$^{-/-}$ mice by adoptive transfer of PD-1 deficient hematopoietic stem cells (HSC) [214], uncovering a critical role for PD-1 in the control of self-reactivity in recent thymic emigrant populations during lymphopenia induced proliferation (LIP). We considered that disease might result from a deficiency in Treg cell generation, and particularly pTreg cell generation given the published data on the role of PD-L1 in that process. However, the question remains whether PD-1 is primarily important for restraining effector T cells or directing pTreg cell conversion in a cell intrinsic manner. Herein we have found that PD-1 deficiency does not lead to a T cell intrinsic defect in the *in vivo* conversion of conventional T cells (Tcon) to pTreg cells during LIP, nor does it lead to Treg-cell functional deficits. Our data are consistent with the hypothesis that PD-1 primarily functions to restrain the expansion of both Tcon and pTreg cells, and disease in PD-1$^{-/-}$ HSC recipients results from effector T cell expansion beyond the ability of Treg cells to control them.

2.2. Materials and Methods

2.2.1. Mice and adoptive transfer experiments

B6.129S7-Rag1tm1Mom/J (Rag$^{-/-}$), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1), B6.129P2-Il10tm1Cgn/J (IL-10$^{-/-}$), B6.Cg-Foxp3tm2(EGFP)Tch/J (FoxP3^{EGFP}), C57BL/6-TgN(OT-I)-RAG1^{tm1Mom}, C57BL/6-TgN(OT-II.2a)-RAG1^{tm1Mom}, B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J (TCR$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-*Pdcd1*$^{-/-}$ (PD-1$^{-/-}$; backcrossed 11 generations to C57BL/6) were originally generated by Prof. T. Honjo and colleagues[133]. C57BL/6J-*il17*$^{-/-}$ (IL-17$^{-/-}$)

mice[349] were kindly provided by Defu Zeng (Beckman Research Institute, City of Hope, Duarte, CA). PD-1$^{-/-}$ x IL-17$^{-/-}$ mice were generated by crossing the above PD-1$^{-/-}$ and IL-17$^{-/-}$ mice. FoxP3^{EGFP} x PD-1$^{-/-}$ mice were generated by crossing the above PD-1$^{-/-}$ and FoxP3^{EGFP} mice. FoxP3^{EGFP} (CD45.1^{+}/CD45.2^{+}) mice used for tracking experiments were the male F1 progeny of the cross between a female FoxP3^{EGFP} and a male CD45.1. TCR transgenic B6 Rag2$^{-/-}$ Marilyn mice[331] were bred at the University of Alberta. C57BL/6-BTLA$^{-/-}$ (BTLA-/-) mice have been previously described[145]. Animals were maintained and experiments were performed in accordance with the guidelines of the animal care and use committee at the University of Alberta.

Thymocytes or splenocytes for injection were prepared by disruption in HBSS (Gibco) + 2% Fetal bovine serum (FBS, Sigma-Aldrich) through a 70 µm nylon cell strainer into a 50 mL conical tube. Cells were centrifuged at ~300 x g for 5-10 minutes at room temperature and red blood cell lysis was performed by resuspending cells in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) with incubation for 5 minutes, followed by addition of ~10 volumes of HBSS + 2% FBS, and 2 cycles of centrifugation at ~300 x g for 5-10 minutes, and resuspension in PBS with no additives. If cells were prepared for further manipulation (e.g. staining and sorting) they were instead washed and resuspended in HBSS + 2% FBS. For experiments involving adoptive transfer of HSC, fetal liver cells (FLC) were used as a source of HSC. Timed pregnancies were established and fetal livers were harvested at 14-15 days post coitus. On ice, fetal livers were disrupted by repeated trituration through a 5 mL serological pipet, followed by filtration through a 70 µm nylon mesh filter basket. Cells were then centrifuged at ~300 x g for 10 minutes at 4 degrees C, and resuspended according to their

intended further use. FLC were used either fresh or frozen. For immediate use for *in vivo* transfers, FLC were resuspended at 50 x 10^6 cells/mL in PBS, placed on ice, and injected as soon as possible into recipient animals. 1.5 x 10^7 fetal liver cells were transferred intravenously to $Rag^{-/-}$ recipients. For freezing, cells were resuspended at 2 x 10^8 cells/mL in 90% fetal bovine serum (FBS) + 10% DMSO, frozen in a -1°C per minute rate controlled cell freezing apparatus in a -80°C freezer, and transferred to the vapor phase of a liquid nitrogen tank for long-term storage.

Macroscopic signs of disease in HSC recipients included cachexia/weight loss (>15%), hunched appearance, ruffled fur, dermatitis, and ocular lesions. Recipient mice were no longer considered disease free when two or more of the above symptoms were evident. For pTreg-cell conversion experiments, 2 x 10^6 of the mixed WT and PD-$1^{-/-}$ $CD4^+$ T cells (1:1) as indicated in figure legends were transferred to $Rag^{-/-}$ recipients intravenously.

For antibiotic treatment experiments, mouse water was supplemented with 0.5 g/L Vancomycin hydrochloride (Pharmaceutical partners of Canada), 1 g/L metronidazole (Alberta health services), 1 g/L ampicillin disodium (Novopharm), 1 g/L Neomycin sulfate (Sigma Aldrich) beginning four weeks prior to HSC injection. 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 MRS agar plates and cultured under anaerobic or aerobic conditions at 37°C for 24 to 48 h. Mice continued to receive medicated water for the duration of the experiment post-HSC transplantation.

2.2.2. Real time PCR

For gene expression analyses, animals were perfused through the heart with PBS and tissues were collected into tubes containing ceramic beads (Lysing matrix D, MP Biomedicals) and flash frozen in liquid nitrogen. For RNA extraction, tissues were disrupted in TriZol (Invitrogen) using the FastPrep system (MP Biomedicals) followed by extraction of RNA from the aqueous phase using RNeasy mini columns (Qiagen). 1 µg of total RNA was treated with amplification grade DNase I (Invitrogen), and cDNA was prepared using anchored oligo-(dT) (dT_{18}(VN))-primed reverse transcription using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's supplied protocols. Semi-quantitative real-time RT-PCR was performed using Bio-Rad iQ SYBR green supermix (Bio-Rad, Hercules, CA) according to the manufacturer's supplied protocols and recommended cycling conditions (annealing temperature used for all primer sets: 60°C), on a Bio-Rad MyiQ real time PCR instrument. For template, 5 µL of 1/3 diluted heat-inactivated reverse transcription reaction per 25 µL PCR reaction was used without further purification. All data were normalized to the expression of *gapdh* and are expressed as relative fold change (RFC) compared with the average of the control group, calculated using the ΔΔCt method. Clustered image maps were generated from fold-change data using CIMMiner (http://discover.nci.nih.gov/cimminer/). Primer sets used were: *gapdh* F: AGCCTTCTCCATGGTGGTGAAGAC; *gapdh* R: CGGAGTCAACGGATTTGGTCG; *cd3ε* F: TCTCGGAAGTCGAGGACAGT; *cd3ε* R: TTGAGGCTGGTGTGTAGCAG; *foxp3* F: GGCCCTTCTCCAGGACAGA; *foxp3* R: GCTGATCATGGCTGGGTTGT; *il17a* F: GCTCCAGAAGGCCCTCAGA; *il17a* R: AGCTTTCCCTCCGCATTGA; *cd11c* F: ATGTTGGAGGAAGCAAATGG; *cd11c* R:

CCTGGGAATCCTATTGCAGA; *il4* F: ACAGGAGAAGGGACGCCAT; *il4* R: GAAGCCCTACAGACGAGCTCA; *ifng* F: GCATTCATGAGTATTGCCAAG; *ifng* R: GGTGGACCACTCGGATGA; *tnfa* F: CATCTTCTCAAAATTCGAGTGACAA; *tnfa* R: TGGGAGTAGACAAGGTACAACCC; *il12p35* F: CATCGATGAGCTGATGCAGT; *il12p35* R: CAGATAGCCCATCACCCTGT; *il10* F: GGTTGCCAAGCCTTATCGGA; *il10* R: ACCTGCTCCACTGCCTTGCT; *il6* F: ATGGATGCTACCAAACTGGAT; *il6* R: TGAAGGACTCTGGCTTTGTCT.

2.2.3. Antibodies, flow cytometry and FACS

For flow cytometric staining and sorting, fluorophore-labeled antibodies against the following markers were obtained from eBioscience (San Diego, CA) unless otherwise indicated: CD4 (RM4-5), TCRb (H57-597), CD45.1 (A20), CD45.2 (104), CD8 (53-6.7), FoxP3 (FJK-16s). Antibodies were used at manufacturer's recommended concentrations. Flow cytometric staining always used an Fc block cocktail to block nonspecific staining. Fc block cocktail consisted of 3 mL each of normal mouse, rat, and hamster serum, with addition of 0.3 mg of anti-CD16/32 antibody (clone 2.4g2, BioXCell). For staining blood or cells, 20 μL of Fc block cocktail was combined with 30-50 μL of blood or prepared cells in 5 mL FACS tubes (BD), incubated for 5 minutes at RT, and then appropriate fluorochrome-labeled antibodies were added (final volume – 100 μL). Following incubation at RT protected from light to allow for staining (generally ~30 min, depending on antibodies), cells were resuspended in 2 mL ACK buffer (blood) or HBSS + 2% FBS (other cells) and centrifuged at RT for 5 minutes at 300 x g. Supernatant was decanted and cells were resuspended in 0.5-1mL HBSS + 2% FBS for analysis. For cell sorting, a

BD Influx cell sorter was employed using the Spigot software (Beckton Dickinson, Franklin Lakes, NJ). Cells were resuspended in HBSS + 20% FBS + 10 mM HEPES and sorted directly into FBS supplemented with 10 mM HEPES. Sorted $CD4^+GFP^-$ cells from $FoxP3^{EGFP}$ mice and crosses were routinely 99.9% GFP negative. Standard flow cytometry was performed using a BD LSR II instrument. Flow cytometric data analysis was performed using FlowJo (Treestar software, Portland, OR). Statistical analysis was performed using Graphpad Prism v5.0 software.

2.2.4. In vitro proliferation/suppression assays

For *in vitro* evaluation of Treg cell function, $FoxP3^{EGFP}$ or $FoxP3^{EGFP}$ x PD-$1^{-/-}$ $CD4^+GFP^+$ ("Treg") and $CD4^+GFP^-$ ("Responder") populations were purified by FACS. Responder cells were labeled with 5 μM Celltrace™ Violet proliferation dye (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. 1 x 10^5 labeled responders were cocultured with the indicated ratios of Treg cells and 2 x 10^5 antigen presenting cells (splenocytes from $Rag^{-/-}$ mice) in the presence of 1 μg/mL soluble anti-CD3ε antibody (145-2C11, functional grade purified, eBioscience) for 4-5 days. For some experiments, unpurified total splenocytes from CD45.1 mice, labeled as above, were used as responders. Dead cells were excluded from the analysis using propidium iodide staining or the Live/Dead Fixable Yellow dead cell stain kit (Invitrogen).

2.3. Results

2.3.1. Disease in PD-1 deficient HSC recipients is not dependent on IL-17 expression

We recently developed a model of lymphopenia-driven autoimmunity whereby transfer of PD-1$^{-/-}$ HSC to Rag deficient recipients resulted in a rapid and severe multi-organ autoimmune disease shortly after the first newly generated T cells began to emerge from recipient thymi and underwent lymphopenia induced proliferation (LIP) [214]. Given previous data linking PD-L1 to the inhibition of Th17 conversion from naïve T cells and the destabilization of the Th17 phenotype[143,144], we hypothesized that disease in PD-1$^{-/-}$ deficient HSC recipients might result from increased Th17 cell development. To address this possibility, we first performed gene expression analysis of highly lymphocyte-infiltrated tissues (liver and kidney) from HSC recipients to analyze the levels of cytokine and other inflammation-associated messages (Fig. 2-1 A). As anticipated, the levels of *cd3ε* mRNA were significantly higher in the infiltrated liver and kidney of the PD-1$^{-/-}$ HSC recipients compared with WT (p=0.024). The *ifng* and *il4* transcripts were likewise increased in the diseased PD-1$^{-/-}$ HSC recipients. However, *il17a* message was not significantly elevated in PD-1$^{-/-}$ HSC recipients compared with WT HSC recipients (Fig. 2-1 A and B; liver and kidney; p=0.85 and p=0.54, respectively) suggesting that T cells producing IL-17 were not a major component of the inflammatory infiltrates. To more definitively examine a potential role for IL-17 in the disease seen in PD-1$^{-/-}$ HSC recipients, we generated PD-1$^{-/-}$ x IL-17$^{-/-}$ animals and transferred their fetal liver cells into Rag$^{-/-}$ recipients. PD-1$^{-/-}$ x IL-17$^{-/-}$ HSC recipients developed macroscopic disease with manifestations that were indistinguishable from PD-1$^{-/-}$ HSC recipient controls (Fig.

2-1 C), including dermatitis, ocular disease, and weight loss, confirming the results of the gene expression experiments.

2.3.2. Autoimmunity is not driven by exaggerated responses to gut microbiota or a deficiency in IL-10

It has been suggested that PD-L1 expression can promote the development of a regulatory subset of IL-10 producing Tr1 cells[350]. We reasoned that PD-1 deficiency might cause disease during LIP of T cells due to insufficient IL-10 production by conventional and/or regulatory subsets of T cells, thereby subverting a normal feedback inhibition mechanism of the T cell response or a functional mechanism of suppressors. We therefore examined *il10* mRNA levels in infiltrated tissues of HSC recipients, and found that *il10* transcripts were actually much higher in PD-1$^{-/-}$ HSC recipients compared with recipients of WT HSC (Fig. 2-1 A). These data did not rule out the possibility that IL-10 deficiency in lymphocytes outside of the examined infiltrated tissues may underlie disease, including in the gut where IL-10 plays a key role in maintenance of homeostasis[351]. Therefore we transferred fetal HSC from IL-10$^{-/-}$ animals into Rag$^{-/-}$ recipients and monitored their health status. Though recipients showed signs of colitis and aged recipients were prone to dermatitis, they did not develop systemic autoimmunity comparable to that seen in PD-1$^{-/-}$ HSC recipients (Fig. 2-1 C). Thus disease in PD-1$^{-/-}$ HSC recipients was not simply a result of IL-10 deficiency. Given that intestinal flora stimulate acute proliferation of T cells in a lymphopenic host[289,352], we further explored whether exaggerated inflammatory responses to intestinal microbiota could underlie the disease seen in PD-1$^{-/-}$ HSC recipients, via, for example, the systemic

release of inflammatory mediators. We thus employed an antibiotic treatment protocol of the Rag$^{-/-}$ HSC recipient animals which was able to completely eliminate culturable aerobic and anaerobic bacteria from fecal samples[353], as expected[352,354,355]. Such a regimen has been previously demonstrated to block the flora-dependent form of rapid homeostatic proliferation[352,356], and influence the severity of other autoimmune disease models including experimental autoimmune encephalomyelitis (EAE) [355] and autoimmune arthritis[357]. Antibiotic treated PD-1$^{-/-}$ HSC recipients developed disease indistinguishable from non-treated recipients (Fig. 2-1 D) suggesting that autoantigens may drive disease in our model of LIP-driven autoimmunity rather than responses to flora.

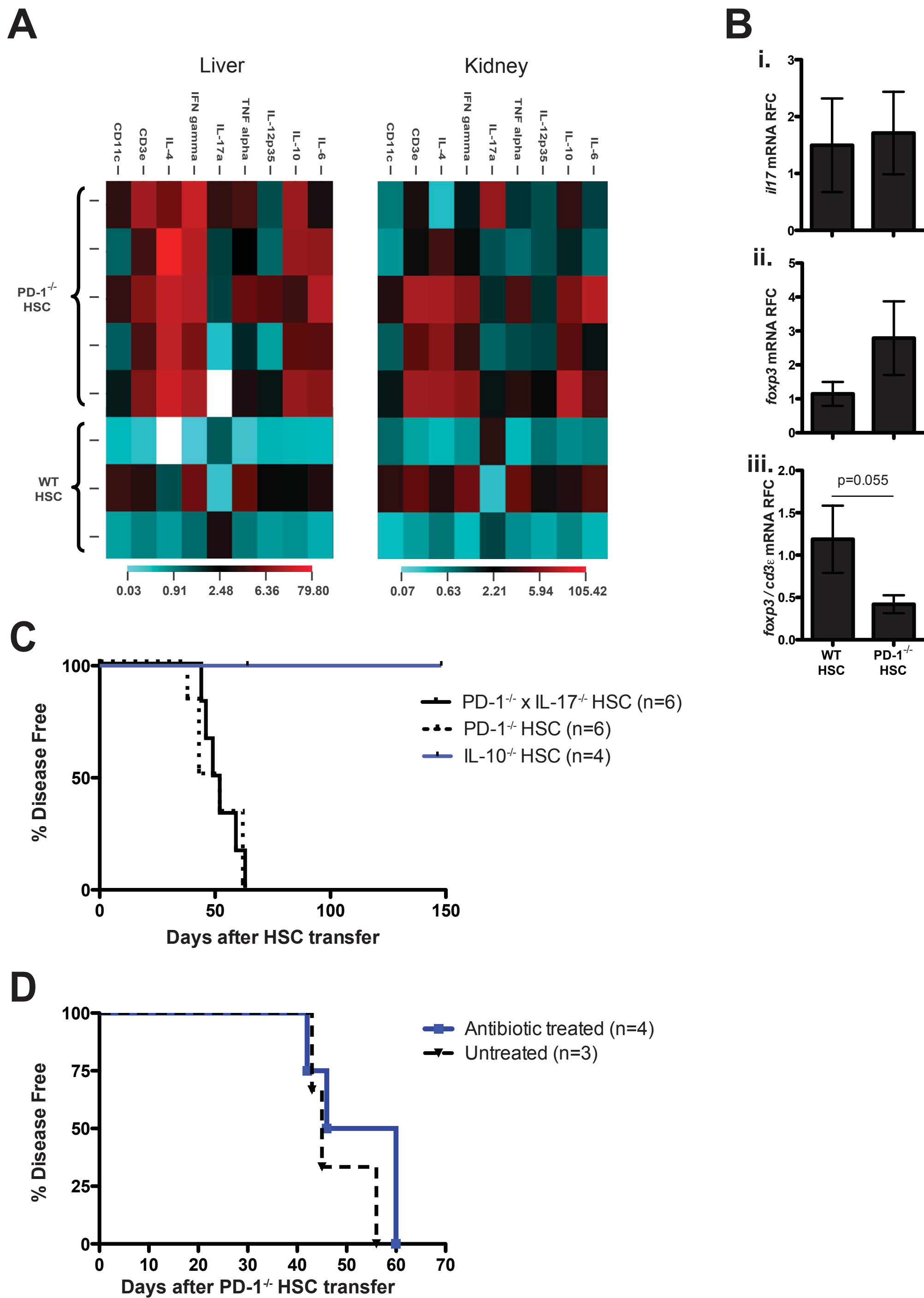


Figure 2-1. Inflammatory and regulatory cytokines in disease post HSC transfer. HSC from WT vs. various knockout donors were transferred to Rag$^{-/-}$ recipient animals and recipients were monitored for disease. Medicated water contained 0.5 g/L

Vancomycin hydrochloride, 1 g/L metronidazole, 1 g/L ampicillin, and 1 g/L neomycin sulfate. No culturable aerobic or anaerobic flora were detected in fecal cultures from medicated animals. (A) Clustered image map representation of transcript level data from real-time PCR analysis of selected inflammation associated genes within liver and kidney of the indicated HSC recipient animals. PD-1$^{-/-}$ HSC recipients were diseased at the time of sacrifice. Scale represents fold change relative to the average of the control group. White squares = not detected. Each row denotes an individual animal. (B) Transcript levels of i) *il17a*, ii) *foxp3* and iii) ratio of *foxp3/cd3ε* mRNA in liver of WT and diseased PD-1$^{-/-}$ HSC recipients from (A). Data are shown as mean +/- SEM (n=3-5 mice per group) and are representative of 2 independent experiments. Statistical analysis was performed using Student's *t*-test. (C) Kaplan Meier survival curve analysis of Rag$^{-/-}$ recipients of IL-10$^{-/-}$ (data are from one experiment with n=4 mice), PD-1$^{-/-}$ and PD-1$^{-/-}$ x IL-17$^{-/-}$ HSC (data pooled from two experiments, n=6 mice per group). Macroscopic signs of disease in HSC recipients included cachexia/weight loss (>15%), hunched appearance, ruffled fur, dermatitis, and ocular lesions. Recipient mice were no longer considered disease free when two or more of the above symptoms were evident. (D) Kaplan Meier survival curve analysis of antibiotic-treated or untreated Rag$^{-/-}$ recipients of PD-1$^{-/-}$ HSC, analyzed as in (C). Data in (D) are from one experiment with n=3-4 mice per group.

2.3.3. PD-1$^{-/-}$ HSC recipients have a reduced frequency but not absolute number of FoxP3^{+} Treg cells

To begin to address whether disease in PD-1$^{-/-}$ HSC recipients resulted from a deficiency in Treg cells, we analyzed WT and PD-1$^{-/-}$ animals, as well as HSC recipients (sacrificed at the time of disease onset, ~40-45 days post HSC transplant) for Treg cell populations. In PD-1$^{-/-}$ animals (non-HSC recipients) we found a small but significant ($p<0.0001$) increase in the proportion of splenic CD4^{+} T cells that expressed FoxP3 (Treg) compared with WT animals (Fig. 2-2 A). It should be noted that these WT and PD-1$^{-/-}$ mice were not littermates, and therefore the influence of factors other than PD-1 on Treg-cell frequency (e.g. differences in microbiome) cannot be excluded. In contrast, in diseased PD-1$^{-/-}$ HSC recipients, we found a lower frequency of CD4^{+} Treg cells compared with WT HSC recipients (Fig. 2-2 B and C). Interestingly, although the frequency of CD4^{+}FoxP3^{+} cells was lower in PD-1$^{-/-}$ HSC recipients, the calculated absolute numbers of Treg cells were not significantly different between the groups (Fig. 2-2 E-G), suggesting that rather than an outright deficiency in Treg cells, non-Treg cells had preferentially expanded over Treg cells. Furthermore, the difference in frequency between PD-1$^{-/-}$ and WT groups was not seen if the analysis was restricted to the CD4^{+}CD25^{+}FoxP3^{+} population (Fig. 2-2 D and F). We did detect a significantly increased proportion of CD25-expressing FoxP3^{-} cells in the PD-1$^{-/-}$ HSC recipients, consistent with an autoimmune diseased state (Fig. 2-2 H). In concordance with the FACS data, our transcriptional analyses revealed that there was a trend towards elevated *foxp3* mRNA in the liver and kidney of diseased PD-1$^{-/-}$ HSC recipients compared with WT HSC recipients (Fig. 2-1 B, (ii)), while the ratio of *foxp3/cd3ε* transcripts was elevated in the WT HSC recipient liver with a p-value approaching statistical significance

(p=0.055, Fig. 2-1 B, (iii)), again suggesting a preferential expansion of Tcon over Treg cells in PD-1$^{-/-}$ HSC recipients.

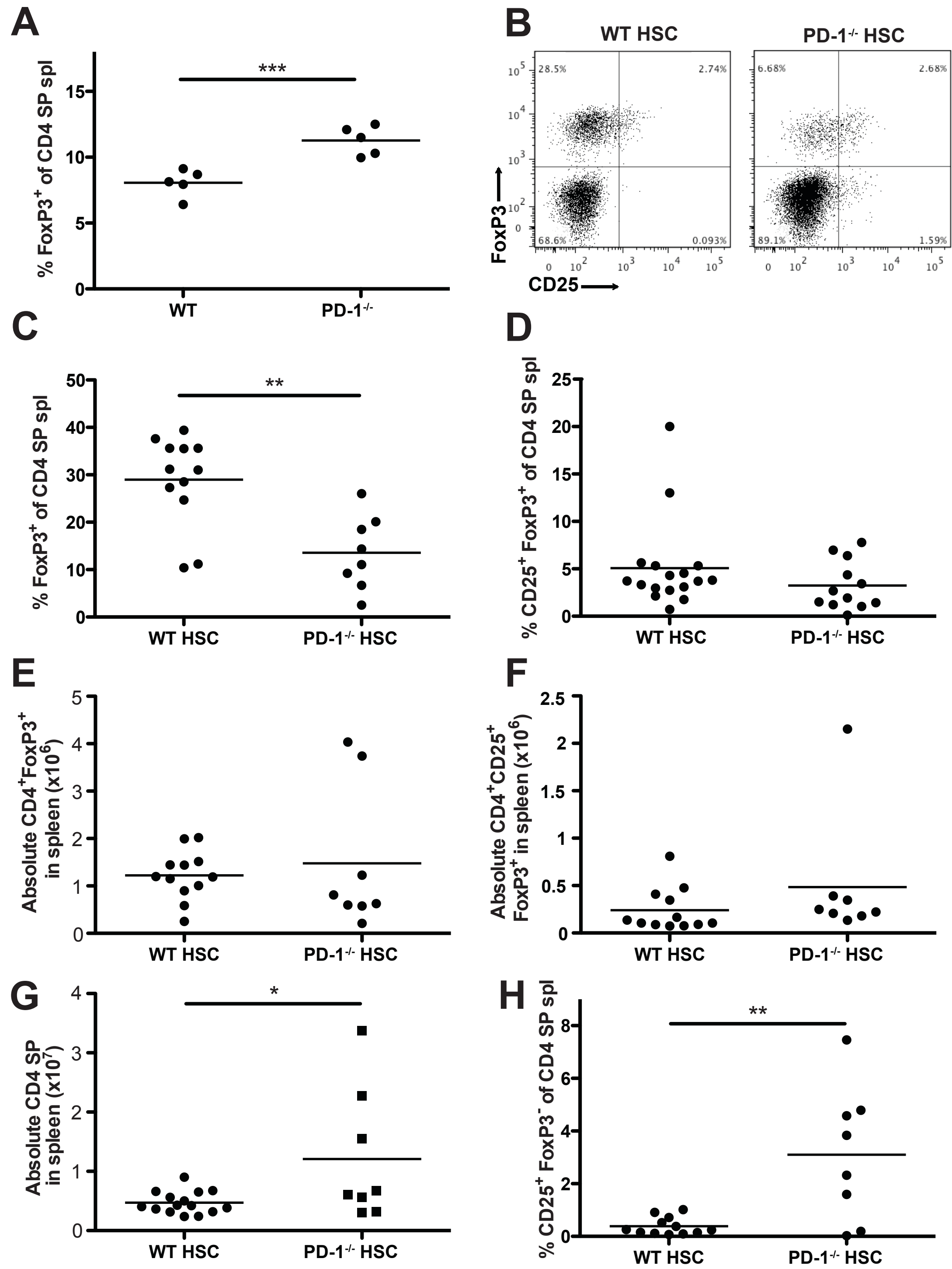


Figure 2-2. PD-1 deficiency leads to increased conventional T cells without a reduction in T cells expressing FoxP3.

Peripheral lymphocyte populations were analyzed from diseased PD-1$^{-/-}$ HSC recipients or WT HSC recipient controls by flow cytometry. (A) % FoxP3^{+} cells in CD4 SP splenocytes (spl) from WT or PD-1$^{-/-}$ animals (non-HSC recipients). (B) Representative flow cytometry plots (n=8-12 mice per group) of FoxP3 vs. CD25 expression in WT and PD-1$^{-/-}$ diseased HSC recipient CD4 SP splenocyte populations. (C) FoxP3^{+} cells in CD4 SP splenocytes from WT or PD-1$^{-/-}$ HSC recipients. (D) CD4^{+}CD25^{+}FoxP3^{+} cells in CD4 SP splenocytes from HSC recipients. (E) Total CD4^{+}FoxP3^{+} cells and (F) CD4^{+}CD25^{+}FoxP3^{+} cells in CD4 SP splenocytes from the indicated HSC recipients. (G) Absolute numbers of CD4 SP cells in the spleens of HSC recipients. (H) % FoxP3^{-} CD25^{+} cells in CD4 SP splenocytes from the indicated HSC recipients. (A-G) Data are from (A) one experiment, n=5 per group, or (C-H) pooled from at least two independent experiments, n=8-15 mice per group. Each point in a scatter plot depicts data from an individual mouse, horizontal bar depicts mean. ** $p<0.01$, *** $p<0.0001$, one-way ANOVA.

2.3.4. PD-1$^{-/-}$ Treg cells suppress in vitro and in vivo, and PD-1$^{-/-}$ Tcon are not resistant to suppression

In order to address whether CD4^{+}FoxP3^{+} cells deficient in PD-1 lacked suppressor activity, we crossed the PD-1$^{-/-}$ transgene onto the FoxP3^{EGFP} transgenic mouse background to allow FACS-based purification of live CD4^{+}FoxP3^{+} cells. *In vitro* suppression assays were performed using sorted CD4^{+}FoxP3^{+} cells ("suppressors") from the spleen of FoxP3^{EGFP} (hereafter referred to as WT) and FoxP3^{EGFP} x PD-1$^{-/-}$ (PD-1$^{-/-}$) animals, using WT CD4^{+}FoxP3^{-} (GFP^{-}) cells as responders with polyclonal activation. We found no difference in the *in vitro* suppressive activity of PD-1$^{-/-}$ vs WT Treg cells under these conditions (Fig. 2-3 A, (i)). We reasoned that additional cells normally

present in the mouse may influence the suppressive activity of Treg cells, and thus we performed similar assays using bulk splenocytes as responders rather than purified $CD4^{+}FoxP3^{-}$ T cells. Under these conditions we similarly observed no difference in the suppressive activity of PD-$1^{-/-}$ vs WT Treg cells (Fig. 2-3 A, (ii)). In order to examine whether PD-$1^{-/-}$ T cells might instead simply be resistant to Treg-cell suppression*,* we set up in vitro suppression assays using all possible combinations of WT or PD-$1^{-/-}$ Treg cells and responder T cells. In terms of percentage of proliferated cells in the assay, no significant reduction in the ability of PD-$1^{-/-}$ T cells to be suppressed compared with WT T cells was observed in this assay (Fig. 2-3 B). In order to address whether PD-$1^{-/-}$ Treg cells were functional *in vivo,* we asked whether supplying supra-physiological levels of PD-$1^{-/-}$ Treg cells at the same time as PD-$1^{-/-}$ HSC to $Rag^{-/-}$ recipients could prevent the development of autoimmunity. While PD-$1^{-/-}$ splenocytes and $CD4^{+}CD25^{+}$ WT cells (each containing 1 x 10^{6} $TCR\beta^{+}$ cells) were both able to completely suppress disease, $CD4^{+}CD25^{-}$ WT cells were not, suggesting that PD-$1^{-/-}$ Treg cells within the splenocyte population delivered were functional (Fig. 2-3 C). Importantly, transfer of PD-$1^{-/-}$ HSC to TCR transgenic mice or $TCR^{-/-}$ mice demonstrated that B cells or monoclonal T cells were unable to block disease progression (Appendix 1). An additional approach was followed using purified $FoxP3^{EGFP}$ WT or PD-$1^{-/-}$ $CD4^{+}FoxP3^{+}$ cells (1 x 10^{6}) transferred to recipient animals 2 weeks following adoptive transfer of HSC. This strategy was used to avoid over-expansion of the Treg cell populations prior to the first waves of HSC-derived newly generated T cells emerging from the thymus, to avoid obscuring any subtle functional differences between the WT and PD-$1^{-/-}$ $FoxP3^{+}$ Treg cells. WT and PD-$1^{-/-}$ $FoxP3^{+}$ cells suppressed disease equivalently even in this setting, again indicating that the

WT and PD-$1^{-/-}$ Treg cells were equally functional *in vivo* (Table 2-1). Finally, to test the possibility that Treg cells that have developed within the context of lymphopenia-driven autoimmunity were dysfunctional, we purified $CD4^{+}FoxP3^{+}$ Treg cells from the spleen of diseased PD-$1^{-/-}$ HSC recipients or matched WT HSC recipients and compared their suppressive function *ex vivo* (Appendix 2). Treg cells purified from diseased PD-$1^{-/-}$ HSC recipients were found to be equally suppressive, in terms of the proportion of responder $CD4^{+}$ and $CD8^{+}$ T cells proliferated, compared with those purified from WT HSC recipients in these assays (Appendix 2). We did note a small reduction in the calculated mean fluorescence intensity (MFI) of the proliferated responder cells after culture with PD-$1^{-/-}$ Treg cells vs. WT Treg cells, however this small effect is unlikely to underlie the dramatic disease in PD-$1^{-/-}$ HSC recipients. As it has been demonstrated that Treg-cell function can be influenced by inflammatory states[358,359], this may reflect the effects of the inflammatory milieu from which the PD-$1^{-/-}$ Treg cells were purified. Taken together, these data indicate that PD-1 is not required for Treg-cell function, and that Treg cells (WT or PD-$1^{-/-}$), and not other lymphocytes, have the capacity to block disease.

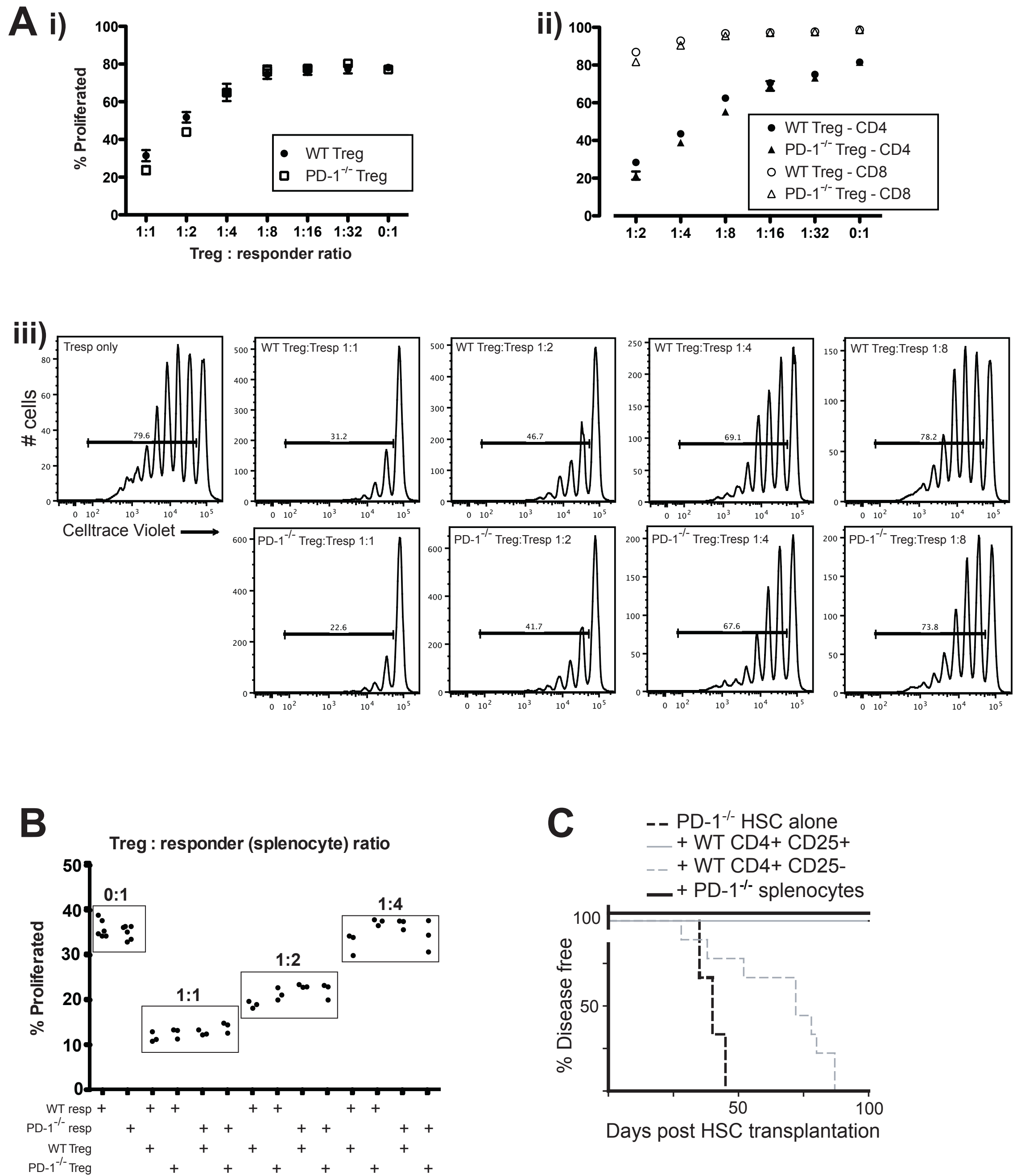


Figure 2-3. PD-1 deficiency does not reduce suppressive function or sensitivity to suppression. (A) i) Suppression assays were performed using proliferation dye-labeled purified WT CD4^{+}GFP^{-} T cells purified from FoxP3^{EGFP} mice as responder cells and CD4^{+}GFP^{+} T cells from FoxP3^{EGFP} or FoxP3^{EGFP} x PD-1$^{-/-}$ mice as suppressors, in the presence of splenocytes from Rag$^{-/-}$ animals (as APCs) and soluble anti-CD3e antibody (1 μg/mL). Proliferation values are expressed as the percentage of generation 0-7 T cells

that had proliferated at least once. ii) Suppression assays were performed as in i) except using unpurified total labeled splenocytes from $CD45.1^+$ animals as responders and without additional APCs. Proliferation values are expressed as percentage of $CD45.1^+$ $CD4^+$ or $CD8^+$ responder T cells that had proliferated at least once. (iii) Representative Celltrace violet dye-dilution plots from the experiment depicted in i). Data in i)-iii) are representative of two independent experiments, with each condition assayed in triplicate, using cells pooled from 4-5 mice per group. (B) Suppression assays were performed as in (A) with all combinations of WT and $PD\text{-}1^{-/-}$ Treg cells and responder T cells. Labeled boxes indicate the ratio of Treg cells:responder T cells. Data are from one experiment. (C) $PD\text{-}1^{-/-}$ whole splenocytes containing 1 x 10^6 $TCR\beta^+$ cells (approximately 3 x 10^6 splenocytes) (n=5 mice per group), or $1x10^6$ FACS sorted WT $TCR\beta^+CD4^+CD25^+$ (n=3 mice per group) or $TCR\beta^+CD4^+CD25^-$ cells (n=9 mice per group) were adoptively transferred along with $PD\text{-}1^{-/-}$ HSC to $Rag^{-/-}$ recipient animals, and animals were monitored for disease. Controls received $PD\text{-}1^{-/-}$ HSC alone (n=3 mice per group). Data are from one experiment. * $p<0.05$, two-tailed Student's *t*-test. ** $p<0.01$, one-way ANOVA with Tukey's multiple comparison test.

Table 2-1. Analysis of disease incidence among recipients of HSC with or without WT or $PD\text{-}1^{-/-}$ $FoxP3^+$ Treg.

Group	**Days disease-free**
WT HSC (n=2)	>129, >129
$PD\text{-}1^{-/-}$ HSC (n=3)	58, 73, 80
$PD\text{-}1^{-/-}$ HSC + WT Treg (n=3)	97, 129, >127
$PD\text{-}1^{-/-}$ HSC + $PD\text{-}1^{-/-}$ Treg (n=3)	>106, 110, >129

FACS purified $CD4^+FoxP3^+$ WT or $PD\text{-}1^{-/-}$ Treg ($1x10^6$) were transferred to $PD\text{-}1^{-/-}$ HSC recipients 2 weeks after HSC adoptive transfer and animals were monitored for disease.

2.3.5. PD-1 is not required for pTreg conversion and instead limits accumulation of Tcon and pTreg cells

It has been previously reported that PD-L1, a ligand for PD-1, is important in controlling the development of pTreg cells[136,137]. However, it has not been conclusively established that the role of PD-L1 in pTreg-cell development is dependent on PD-1, an important caveat given that PD-L1 can interact with other molecules such as B7-1[138-142]. Therefore in order to address the question of whether a cell-intrinsic defect in pTreg-cell conversion exists in PD-1$^{-/-}$ T cells, and whether such a defect might underlie the disease in PD-1$^{-/-}$ HSC recipients, we purified CD4 SP, GFP negative thymocytes or splenocytes from FoxP3^{EGFP} (WT, CD45.1^{+}/CD45.2^{+}) and FoxP3^{EGFP} x PD-1$^{-/-}$ (CD45.2^{+}) animals, mixed them at equal ratios, injected them i.v. into Rag$^{-/-}$ recipient animals and monitored the proportion of Treg cells and Tcon in peripheral blood. Both WT and PD-1$^{-/-}$ T cells underwent homeostatic expansion in the recipient animals as evidenced by an increase in their numbers in the blood over time. Recipients of CD4^{+}GFP^{-} cells experienced weight loss, hunched posture, and diarrhea. Notably, the PD-1$^{-/-}$ CD4^{+} T cells came to predominate over the WT cells, regardless of whether the input T cells were splenocyte or thymocyte derived (Fig. 2-4 A). Although the proportion of CD4^{+} cells that expressed FoxP3 steadily increased over the course of each experiment, there was no significant difference between the proportion of WT and PD-1$^{-/-}$ CD4 SP T cells that had converted to pTreg cells, as measured by the acquisition of FoxP3 expression (Fig. 2-4 B). Therefore, the total number of PD-1$^{-/-}$ pTreg cells present in the thymocyte recipient mice came to be significantly ($p=0.0349$) higher than WT pTreg cells by 55-63 days post transfer (Fig. 2-4 C). However, a striking difference was observed between the ability of CD4 SP FoxP3^{-} thymocytes to convert into pTreg cells compared with splenocytes, with

approximately 6% of thymocyte-derived CD4 SP cells from each group expressing FoxP3 by ~60d post-transfer compared with ~0.3% of splenocyte-derived CD4 SP (Fig. 2-4 D). Together, these data indicate the PD-1 does not control pTreg-cell conversion and instead restrains the expansion of conventional T cells and pTreg cells, and that newly generated T cells have increased potential to convert to pTreg cells.

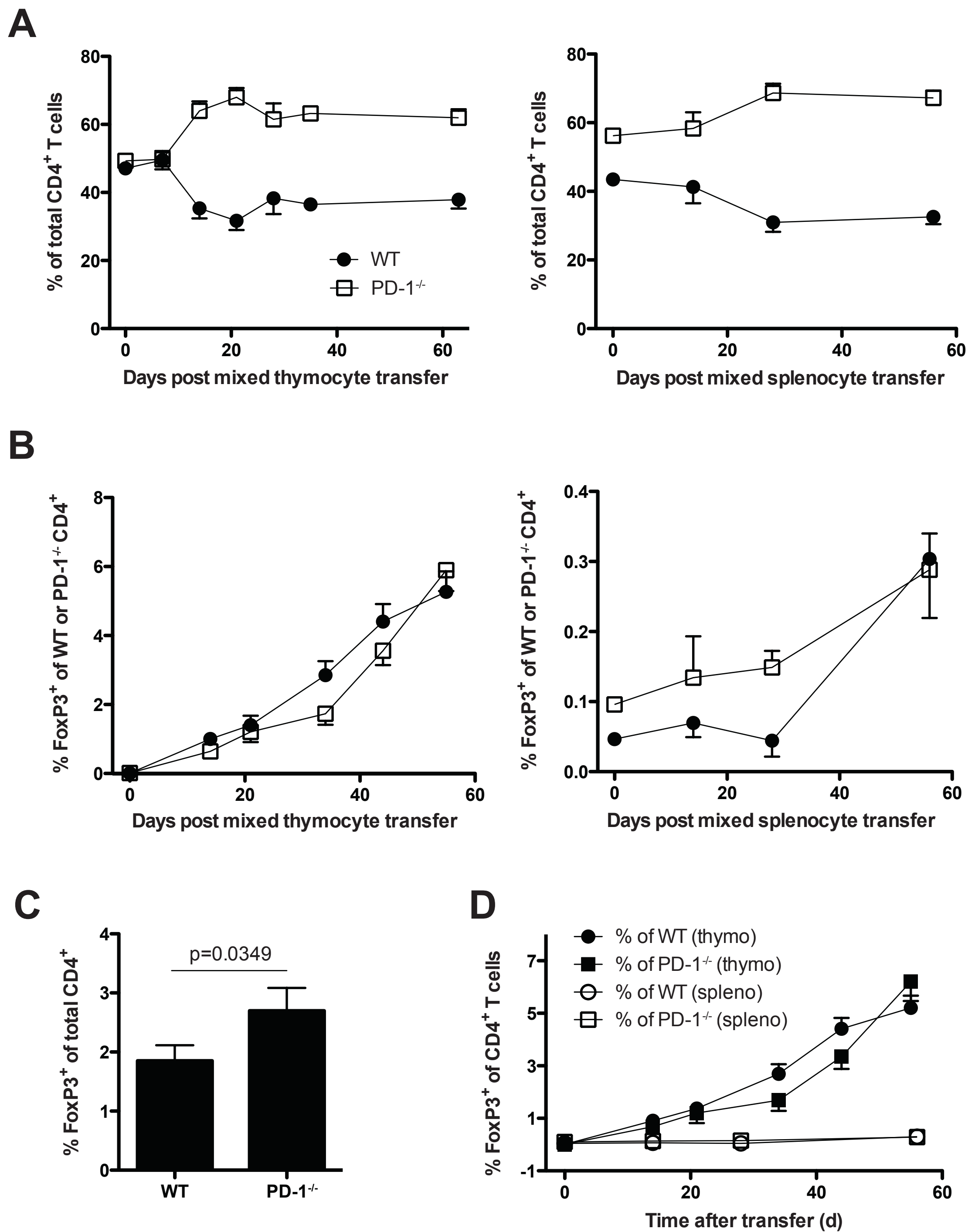


Figure 2-4. PD-1 limits accumulation of pTreg and Tcon but is not required for conversion to pTreg.

2 x 10^6 mixed $FoxP3^{EGFP}$ (WT, $CD45.1^+CD45.2^+$) and $FoxP3^{EGFP}$ x $PD\text{-}1^{-/-}$ ($PD\text{-}1^{-/-}$, $CD45.2^+$) CD4 SP GFP^- thymocytes (n=5 per group) or splenocytes (n=6 per group) were injected into $Rag^{-/-}$ recipient animals and peripheral blood was monitored for abundance of WT and $PD\text{-}1^{-/-}$ $CD4^+$ T cells and the frequency of $FoxP3^+$ (GFP^+) pTreg cells among each of the WT or $PD\text{-}1^{-/-}$ $CD4^+$ populations by flow cytometry. (A) Frequency of WT or $PD\text{-}1^{-/-}$ $CD4^+$ T cells relative to the total $CD4^+$ population in recipients of mixed CD4 SP thymocytes (left) and splenocytes (right). (B) Frequency of $FoxP3^+$ cells among WT and $PD\text{-}1^{-/-}$ CD4 SP thymocyte (left) and splenocyte-derived (right) T cell populations in recipient animals. (C) Proportion of all $CD4^+$ T cells in mixed thymocyte recipients at day 55-63 post-transfer that are WT $FoxP3^+$ or $PD\text{-}1^{-/-}$ $FoxP3^+$ ($p=0.0349$, paired Student's *t*-test, n=9 per group). (D) Comparison of the proportion of $CD4^+$ T cells derived from splenocytes or thymocytes that acquired FoxP3 expression over time *in vivo*. (A, B and D) Thymocyte data are representative of two independent experiments, n=5 mice/per group; splenocyte data are from a single experiment, n=6 mice per group. (C) Depicts pooled data from two experiments.

2.3.6. PD-1 dependent reduction in pTreg cells and Tcon is abrogated by PD-L1 blockade

Thus far our data indicated that T cell expression of PD-1 reduces rather than enhances the number of pTreg cells. We next examined whether PD-1's interaction with PD-L1 is required to reduce pTreg cells by assessing the proportion of pTreg cells that are WT vs. $PD\text{-}1^{-/-}$ when PD-L1 is blocked. We purified $CD4^+CD62L^{hi}GFP^-$ T cells from $FoxP3^{EGFP}$ ($CD45.1^+CD45.2^+$) and $FoxP3^{EGFP}$ x $PD\text{-}1^{-/-}$ ($CD45.2^+$) mouse splenocytes, mixed them, and injected them into $Rag^{-/-}$ recipient animals together with monoclonal anti-PD-L1 (10F.9G2) or isotype control treatment every 2 days from day -2 until

sacrifice (d35) at which time the spleen was harvested and splenocytes analyzed by flow cytometry. Mice receiving anti-PD-L1 treatment had moderately increased weight loss compared with those receiving isotype control antibody (Fig. 2-5 A). There was no significant difference in the proportion of $CD4^+$ T cells that converted to pTreg cells within each of the WT or PD-$1^{-/-}$ cell populations, either with anti-PD-L1 or isotype control treatment (Fig. 2-5 B, C). When we analyzed the proportion of the total $CD4^+$ T cell population that were WT $FoxP3^+$ or PD-$1^{-/-}$ $FoxP3^+$ however, we noted that within the isotype control treated animals the PD-$1^{-/-}$ $FoxP3^+$ population was more than threefold larger than the WT $FoxP3^+$ population, while treatment with anti-PD-L1 was able to restore the WT and PD-$1^{-/-}$ $FoxP3^+$ populations to equal proportions (Fig. 2-5 B). Similarly, when the total proportions of all PD-$1^{-/-}$ vs WT CD4 SP T cells among the total $CD4^+$ population were examined, the PD-$1^{-/-}$ T cells were enriched by a similar margin and this enrichment was prevented by PD-L1 blockade (Fig. 2-5 C). This indicates that the antibody treatment was indeed effective in blocking PD-L1 and reversing the PD-1 dependent inhibition of T cell accumulation in the WT cell population. Together, these data indicate that PD-1 is not required for conversion to pTreg cells, but instead limits the accumulation of pTreg cells as it does conventional T cells.

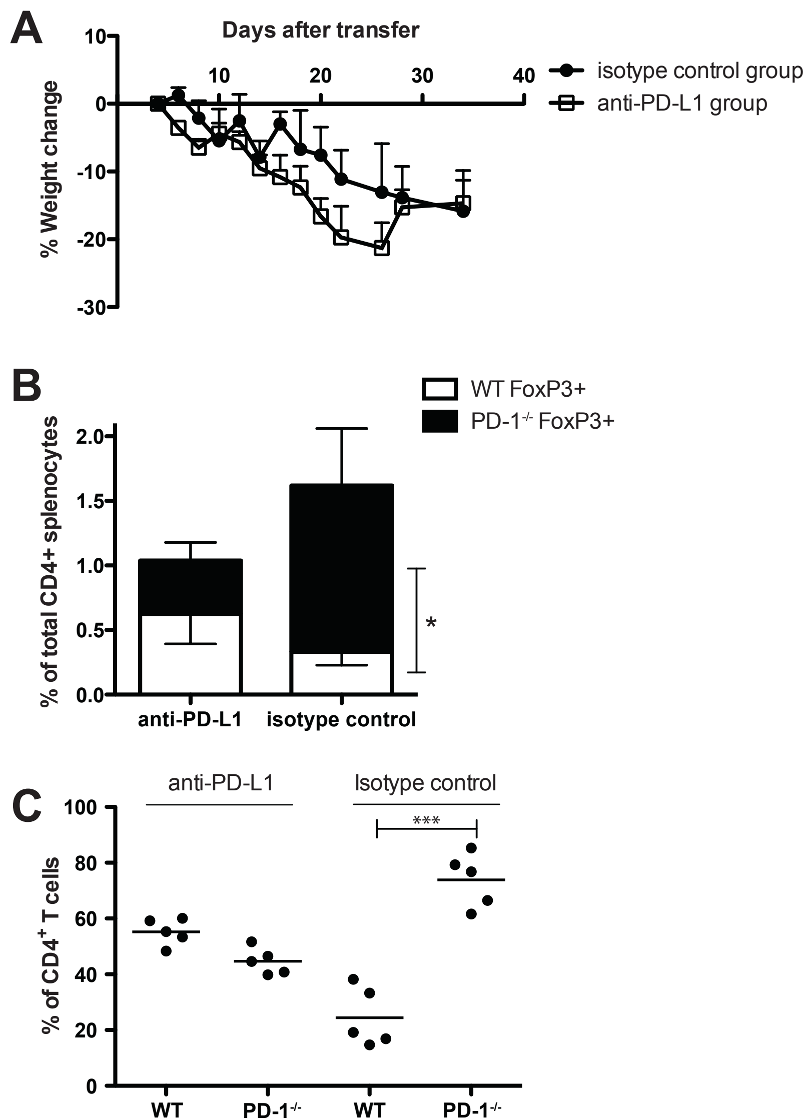


Figure 2-5. PD-L1 blockade releases both conventional T cells and pTreg from PD-1 dependent inhibition but does not decrease generation of pTreg.

2 x 10^6 mixed FoxP3^{EGFP} (WT, CD45.1$^+$CD45.2$^+$) and FoxP3^{EGFP} x PD-1$^{-/-}$ (PD-1$^{-/-}$, CD45.2$^+$) CD4$^+$CD62L^{hi} GFP$^-$ splenocytes were injected into Rag$^{-/-}$ recipient animals. Recipient animals (n=5/group) were treated every other day starting on day -2 with 200 µg of anti-PD-L1 mAb (clone 10F.9G2) or isotype control (Rat IgG2a, clone 2A3) i.p. (A) Comparison of weight change in recipient animals over the course of the experiment. (B) Proportion of WT or PD-1$^{-/-}$ FoxP3$^+$ (GFP$^+$) cells within the total CD4$^+$ T cell populations in splenocytes of recipient animals treated with anti-PD-L1 or isotype control

and sacrificed at d35, as determined by flow cytometry. * $p < 0.05$, Student's *t*-test. (C) Proportion of WT or PD-$1^{-/-}$ $CD4^+$ T cells among the total $CD4^+$ population in splenocytes of recipient animals treated with anti-PD-L1 or isotype control and sacrificed at d35, as determined by flow cytometry. (A-C) Data are shown as mean +/-SEM (n=5 mice/group) from one experiment *** $p < 0.0001$, Student's *t*-test.

2.4. Discussion

Autoimmunity can be considered to be the result of a complex interplay of factors. Clearly, the self-reactive potential of the T cell repertoire can potentiate autoimmunity as occurs in AIRE knockout mice[45]. Other factors such as deficiency of molecules involved in restraining the immune response, for example PD-1, also contribute. In contrast to PD-1 deficient animals which develop late onset autoimmunity with variable incidence[132,133], we recently demonstrated that in a situation where the lymphoid compartment of adult $Rag^{-/-}$ mice was reconstituted by transfer of PD-$1^{-/-}$ HSC, rapid lymphopenia-driven expansion of PD-1 deficient T cells led to severe, multi-organ autoimmune disease[214]. Notably, this disease also manifested (albeit at a lower frequency) upon transfer of PD-$1^{-/-}$ thymocytes to lymphopenic animals, but did not occur upon transfer of PD-$1^{-/-}$ splenocytes, which we consider would have largely previously undergone other peripheral tolerance mechanisms (i.e. anergy, deletion, or conversion to pTreg cells). These findings indicated that PD-1 was of particular importance for control of self-tolerance in newly generated T cells and provided the precedent that regulators of

TCR signals are critical to prevent lymphopenia-potentiated autoimmunity. Deficiencies in other TCR regulators, TGFβR and PTPN2, are now also known to synergize with lymphopenia to drive autoimmunity[298,352,360].

Our transcriptional analyses showed that *il17a* was not highly expressed in the infiltrated organs of PD-1$^{-/-}$ HSC recipients suggesting that IL-17 producing T cells were not a significant component of the inflammatory infiltrate in these organs. Because these findings were limited to liver and kidney and left some doubt about the systemic role of IL-17 in disease pathogenesis in our model, we also generated and transferred IL-17$^{-/-}$ x PD-1$^{-/-}$ HSC and the disease incidence and onset were identical to those seen with PD-1$^{-/-}$ HSC. This suggests that IL-17 is dispensable for the disease in this model. Transfer of IL-10$^{-/-}$ HSC also did not cause systemic autoimmune disease, suggesting that IL-10 production is dispensable for systemic peripheral tolerance during homeostatic proliferation. We did note some dermatitis in the animals after significant aging and sporadic colitis as has been described in conventional IL-10$^{-/-}$ mice[361] and would be expected due to the important role of IL-10 in intestinal homeostasis. Given the role of the intestinal microbiota in driving acute proliferation during lymphopenia and in contributing to the autoimmunity seen in several models[289,352,355,357], we also asked whether depletion of the intestinal microflora of Rag$^{-/-}$ recipient animals prior to PD-1$^{-/-}$ HSC transplantation via antibiotic administration would influence the disease severity in our LIP model. Our results demonstrated that such a treatment regimen did not influence the disease and indicate that a response to microbiota-associated antigens or ligands for innate pattern recognition receptors (PRR) is unlikely to be the major mechanism of disease in this model.

Our initial examinations did not find any difference in the thymic output of FoxP3^{+} cells or the absolute number of FoxP3^{+} cells in the periphery of PD-1$^{-/-}$ animals or the diseased PD-1$^{-/-}$ HSC recipients, although we did find them at a lower frequency in the periphery of PD-1$^{-/-}$ HSC recipients than in WT HSC recipients (Fig. 2-2). Similar findings were obtained by analysis of *foxp3* mRNA levels in infiltrated organs of HSC recipients (Fig. 2-1 B), together suggesting that PD-1$^{-/-}$ HSC recipients are not deficient in the absolute numbers of Treg cells, but instead that the proportion of Treg cells among all T cells becomes lower in the setting of PD-1 deficiency due to expansion of non-Treg cells. This indicated that in PD-1$^{-/-}$ HSC recipients, thymic Treg-cell generation was not inhibited and that a likely effect of PD-1 deficiency was the disproportionate expansion of effector T cells. However, Haribhai et al. [170] demonstrated that both tTreg and pTreg cells were required to maintain immune homeostasis and we could not exclude that pTreg cells were deficient in the diseased animals without using a transgenic system to specifically monitor the conversion of conventional T cells to pTreg cells *in vivo*.

Several studies have indicated that PD-L1, a known ligand of PD-1, can contribute to generation of pTreg cells[136,137]. However, PD-L1 is also known to interact with B7-1 to inhibit immune responses[141,142] and pTreg-cell generation can be impacted depending on signals mediated through CD28, which may be affected by the B7-1:PD-L1 interaction. Furthermore, the common use of CD25 negative selection as a strategy to purify non-Treg cells as a substrate for monitoring pTreg-cell conversion (via acquisition of FoxP3 expression) is problematic in that one cannot distinguish cells which acquired FoxP3 expression during the assay from cells which were CD25^{-}FoxP3^{+} initially and either survived or expanded. In addition, there is the potential for an inflammatory milieu

generated by complete PD-L1/L2 deficiency to inhibit pTreg-cell generation, independent of a cell-intrinsic role for PD-1 in the pTreg-cell conversion process. To avoid these issues, we endeavored to examine the *in vivo* conversion potential of WT and PD-1$^{-/-}$ conventional CD4 SP T cells, highly purified by FACS on the basis of FoxP3 negativity, to pTreg cells simultaneously in the same mouse during LIP, a setting where we have demonstrated that peripheral tolerance mechanisms mediated by PD-1 are key to the maintenance of self tolerance[214]. This approach also has the benefit of specifically assaying the T cell-intrinsic effects of PD-1 deficiency. Following the mixture of separately trackable WT and PD-1$^{-/-}$ CD4^{+}FoxP3^{-} cell populations and injection into recipient mice, we found that the rate of conversion to pTreg cells was equivalent between the PD-1$^{-/-}$ and WT populations in the recipient mice, regardless of whether the Tcon were purified from splenocytes or thymocyte populations (Fig. 2-4 A). Indeed, in the mixed thymocyte recipient animals the proportion of all CD4^{+} cells that were PD-1$^{-/-}$ pTreg cells was significantly higher at day 55-63 post-transfer compared with WT pTreg cells (Fig. 2-4 D), and a similar result was seen in the isotype control-treated splenocyte recipients (Fig. 2-5 B). Interestingly, consistent with 2 recently published reports of increased propensity for recent thymic emigrants to become pTreg cells[210,328], we also found that the rate of conversion of thymocyte-derived Tcon was much higher than the corresponding splenocytes (Fig. 2-4 C). We consistently found that regardless of their source (thymocytes or splenocytes), PD-1$^{-/-}$ cells, when mixed with WT cells and injected into the same animals, came to outnumber the WT cells (Fig. 2-4 B). The reduced expansion of PD-1-expressing WT T cells compared with PD-1$^{-/-}$ cells in mixed cell recipients could also be abrogated by blockade of PD-L1, suggesting that PD-L1/PD-1

signals indeed regulate this effect on T cell numbers (Fig. 2-5 B and C). These data further suggested that reduced restraint on effector T cell proliferation lead to disease in PD-1$^{-/-}$ HSC recipients as opposed to a tTreg or pTreg-cell deficiency. Another study examining the role of PD-1 in the generation of pTreg cells was recently published which, contrary to our results, reported a role for PD-1 in the generation of pTreg cells[362]. The discrepancy between our results may stem from the use by Chen et. al. of a transgenic reporter system in which GFP is fused to the FoxP3 protein, which has been shown to lead to deficiencies in Treg cells and altered Treg-cell transcriptional profiles[363,364], whereas we have employed a bicistronic reporter system to avoid these challenges.

We did not detect substantial functional differences between PD-1$^{-/-}$ and WT Treg cells using a combination of *in vitro* suppression assays as well as *in vivo* adoptive transfer experiments. PD-1$^{-/-}$ and WT Treg cells were suppressive *in vitro* (Fig. 2-3 A and B), including when purified from diseased HSC recipients (Appendix 2). Furthermore, we could not detect any *in vitro* resistance of PD-1$^{-/-}$ conventional T cells to suppression by Treg cells (Fig. 2-3 B). By supplying supra-physiological levels of either PD-1$^{-/-}$ or WT Treg cells to PD-1$^{-/-}$ HSC recipients, we could completely suppress the disease (Fig. 2-3 C). Notably, we also adjusted the number and timing of Treg cell transfer to the PD-1$^{-/-}$ HSC recipients (1 x 10^6 cells, 2 weeks after HSC transfer) such that we were not supplying such a large number so as to obscure potential subtle functional differences between WT and PD-1$^{-/-}$ Treg cells – under these conditions WT and PD-1$^{-/-}$ Treg cells were also equally functional (Table 2-1).

The importance of understanding the scope of PD-1's role in mediating peripheral

tolerance is underscored by the emergence of PD-1 blockade as a therapeutic strategy for cancer[365]. It has been unclear whether the bulk of the success seen in blockade of PD-1 is due to an effect on releasing inhibition of effector T cells and thus potentiating anti-cancer T cell immunity, or by interfering with Treg cell generation or function and thus subverting this potential mechanism of tumour immune evasion. Certainly, the present data would suggest the former. Taken together, our data suggest that PD-1 does not intrinsically control the conversion of Tcon to pTreg cells nor does it influence the suppressive function of Treg cells, and that disease in our model of lymphopenia-potentiated autoimmunity after PD-1$^{-/-}$ HSC transplantation is the result of a failure to intrinsically control the activation and/or expansion of T cells in the absence of PD-1.

Chapter 3

PD-1 deficient CD4+ newly generated T cells drive autoimmunity during lymphopenia-induced proliferation independent of Fas and perforin-dependent killing

3.1. Introduction

Thymic selection processes can be viewed as the first “filtration” step on the developing T cell repertoire. Although these processes serve to remove the majority of strongly self-reactive T cells from the developing repertoire or convert them to tTreg, some self-reactive conventional T cell (Tcon) clones escape[366]. This is, for example, evidenced by the ability to induce autoimmune diseases such as myelin-oligodendrocyte glycoprotein $(MOG)_{35-55}$-induced experimental autoimmune encephalomyelitis in mice[70]. Peripheral tolerance mechanisms such as anergy, deletion, ignorance or conversion to peripherally generated Treg (pTreg) are thus important for the establishment and maintenance of immune tolerance and can be viewed as a second filter on the peripheral T cell repertoire.

In a lymphopenic host, T cells undergo a process known as lymphopenia-induced proliferation (LIP), which is facilitated and regulated in its extent by the availability of “resources” for T cells that together define T cell “space” [216]. These include peptide-MHC complexes (derived from self or otherwise) that can mediate at least a weak, “tonic” signal through the TCR, as well as homeostatic cytokines such as IL-7 and IL-15. Regulatory T cells (Treg) [186,292,296,303-308], co-stimulatory molecules such as CD28[296,297], as well as molecules with known co-inhibitory activity (eg: BTLA[299], Lag3[300], TGFBRII[298,360]) can modulate the kinetics of LIP and the maximum size of the T cell compartment. In addition, LIP can promote autoimmune disease and LIP of $TGFBRII^{-/-}$ T cells results in autoimmunity[298].

Programmed death-1 (PD-1), a co-inhibitory receptor expressed on activated T cells, enters the immune synapse upon T cell:APC interaction and is known to recruit the

phosphatase SHP2 upon receptor ligation which can dampen proximal TCR signaling cascades[129-131]. In contrast to the C57BL/6 PD-$1^{-/-}$ mouse, which displays a relatively mild phenotype characterized by development of a lupus-like disease with spontaneous arthritis and glomerulonephritis upon aging[133], we have shown that reconstitution of the lymphoid compartment of lymphopenic adult $Rag1^{-/-}$ animals via transfer of PD-$1^{-/-}$ hematopoietic stem cells (HSC) leads to a rapid, severe, and lethal systemic autoimmune disease soon after the first newly generated T cells, or recent thymic emigrants (RTE), emerge into the periphery[214]. The disease is characterized by infiltration of CD4+ and CD8+ T cells into multiple organs, although $Rag1^{-/-}$ $K^{b-/-}D^{b-/-}$ animals remain fully susceptible suggesting that MHC-I restricted CD8+ T cells are dispensable for disease. Macroscopically the disease is characterized by kyphosis, cachexia, diarrhea, and skin and ocular lesions. Interestingly, PD-$1^{-/-}$ HSC reconstitution of day 1 $Rag^{-/-}$ neonates results in a drastically reduced incidence of disease[214], suggesting that limited T cell "space" due to small anatomic size (e.g.: of lymph nodes) or other factors can limit the aberrant activation of T cells promoted by LIP. Indeed lymph node-deficient $Rag^{-/-}\gamma c^{-/-}$ or irradiated $LT\alpha^{-/-}$ hosts were also resistant to disease after PD-$1^{-/-}$ HSC transfer[214]. Transfer of PD-1 deficient thymocytes to adult $Rag1^{-/-}$ mice likewise results in autoimmunity, however transfer of splenocytes from mature PD-$1^{-/-}$ mice does not result in disease. These data suggest that the RTE/newly generated T cell population, which has not yet been subject to peripheral tolerance mechanisms, has greater autoimmune potential than established peripheral T cells and that PD-1 is critically important for controlling their activity during LIP. However, several lines of evidence suggest that newly generated T cells have properties that promote tolerance[196]. It is not clear whether

newly generated T cells in an adult retain a heightened potential for the generation of autoimmunity after their emergence into the periphery, or whether exposure of newly generated T cells to a lymphoreplete environment leads to their rapid tolerization. Herein, we have taken advantage of the Rag2pGFP transgenic mouse strain in which GFP is expressed during early T cell development and remains detectable as a marker of newly generated lymphocytes after their emergence into the periphery[203,204]. PD-1$^{-/-}$ peripheral newly generated T cells or established T cells were purified and tested for their ability to drive autoimmunity upon transfer into lymphopenic hosts. We found that purified peripheral PD-1$^{-/-}$ newly generated T cells are similar to thymocytes in their ability to drive systemic autoimmunity upon transfer to lymphopenic hosts. Using transgenic lymphopenic hosts lacking Fas or MHC-II expression, or PD-1$^{-/-}$ donors lacking Perforin expression, we also show that host MHC-II expression is required for disease after PD-1$^{-/-}$ HSC transfer, and that Fas- and perforin-dependent killing mechanisms are dispensable for disease in this model. Taken together, our data suggest that even in a lymphoreplete adult host, peripheral newly generated T cells retain a heightened potential for LIP-driven autoimmunity in the absence of PD-1, which is mediated by CD4+ T cells, likely via a cytokine-dependent mechanism.

3.2. Methods:

3.2.1. Mice

B6.129S7-*Rag1*^{tm1Mom}/J (Rag1$^{-/-}$, Rag$^{-/-}$), B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J (FoxP3^{EGFP}, used in the present manuscript as WT) , B6.MRL-*Fas*^{lpr}/J (Fas^{lpr}), C57BL/6-*Prf1*^{tm1Sdz}/J (Prf1$^{-/-}$),

and B6.129S2-*Ciita*^{tm1Ccum}/J (CiiTA$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-*Pdcd1*$^{-/-}$ (backcrossed 11 generations to C57BL/6) were originally generated by Prof. T. Honjo and colleagues[133]. FoxP3^{EGFP} x *Pdcd1*$^{-/-}$ mice were generated by crossing the above FoxP3^{EGFP} and B6-*Pdcd1*$^{-/-}$ mice and are referred to in the present manuscript simply as PD-1$^{-/-}$. PD-1$^{-/-}$ x Prf1$^{-/-}$ mice were generated by crossing the above PD-1$^{-/-}$ and Prf1$^{-/-}$ mice without any selection for the FoxP3^{EGFP} transgene. Rag$^{-/-}$ x CiiTA$^{-/-}$ mice were generated by crossing the above Rag$^{-/-}$ and CiiTA$^{-/-}$ mice. Fas^{lpr} x Rag$^{-/-}$ mice were generated by crossing the above Fas^{lpr} and Rag$^{-/-}$ mice. B6.Rag2pGFP (Rag2pGFP) mice[203,204] were kindly provided by Pamela Fink (University of Washington, Seattle, WA). Rag2pGFP x PD-1$^{-/-}$ mice were generated by crossing Rag2pGFP mice with the above B6-*Pdcd1*$^{-/-}$, with screening and selection of breeders for high GFP expression. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.2. Cell preparations and adoptive transfer experiments

For experiments involving transfer of thymocytes or peripheral T cells, recipient NK cells were depleted (to avoid potential NK-mediated killing of the input cells[367-369]) by treatment on days -4, -1, and +2 with 0.3 mg per mouse of anti-NK1.1 (PK136, Bioceros) injected intraperitoneally. Thymocytes or splenocytes for injection were prepared by disruption in HBSS (Gibco) + 2% Fetal bovine serum (FBS, Sigma-Aldrich) through a 70 μm nylon cell strainer into a 50 mL conical tube. Cells were centrifuged at ~300 x g for 5-10 minutes at room temperature and red blood cell lysis was performed by resuspending cells in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM

Na_2EDTA) with incubation for 5 minutes, followed by addition of ~10 volumes of HBSS + 2% FBS, and 2 cycles of centrifugation at ~300 x g for 5-10 minutes, and resuspension in PBS with no additives. If cells were prepared for further manipulation (e.g. staining and sorting) they were instead washed and resuspended in HBSS + 2% FBS. For experiments involving adoptive transfer of HSC, fetal liver cells (FLC) were used as a source of HSC. Timed pregnancies were established and fetal livers were harvested at 14-15 days post coitus. On ice, fetal livers were disrupted by repeated trituration through a 5 mL serological pipet, followed by filtration through a 70 μm nylon mesh filter basket. Cells were then centrifuged at ~300 x g for 10 minutes at 4 degrees C, and resuspended according to their intended further use. FLC were used either fresh or frozen. For immediate use for *in vivo* transfers, FLC were resuspended at 50 x 10^6 cells/mL in PBS, placed on ice, and injected as soon as possible into recipient animals. For freezing, cells were resuspended at 2 x 10^8 cells/mL in 90% fetal bovine serum (FBS) + 10% DMSO, frozen in a -1°C per minute rate controlled cell freezing apparatus in a -80°C freezer, and transferred to the vapor phase of a liquid nitrogen tank for long-term storage. 1.5 x 10^7 fresh or frozen fetal liver cells were transferred intravenously to the indicated recipients.

3.2.3. Definition of disease and data analysis

Macroscopic signs of disease in HSC/thymocyte/peripheral cell recipients included cachexia/weight loss (>15%), kyphosis (hunched appearance), ruffled fur, dermatitis, ocular lesions, and diarrhea. Recipient mice were no longer considered disease free when two or more of the above symptoms were evident, or if mice lost ≥20% body weight. Kaplan-Meier survival curve analysis of disease onset/incidence was performed using

Graphpad Prism v5.0 software. Probability values reported for survival curve comparisons were calculated using the Mantel-Cox method. For thymocyte experiments, calculation of weight loss for disease determination was performed relative to weights at d0 or d1 relative to cell transfer. For HSC experiments, calculation of weight loss was determined relative to initial weight measurements taken prior to d30. Unless otherwise indicated, animals were used at 8-16 weeks of age. All animals were maintained and experiments were performed in accordance with the guidelines of the animal care and use committee at the University of Alberta.

3.2.4. Antibodies, flow cytometry, and FACS

For flow cytometric staining and sorting, fluorophore-labeled antibodies against the following markers were obtained from eBioscience (San Diego, CA) unless otherwise indicated: CD4 (RM4-5), TCRb (H57-597), CD8 (53-6.7), PD-1 (J43). Antibodies were used at manufacturer's recommended concentrations. Flow cytometric staining always used an Fc block cocktail to block nonspecific staining. Fc block cocktail consisted of 3 mL each of normal mouse, rat, and hamster serum, with addition of 0.3 mg of anti-CD16/32 antibody (clone 2.4g2, BioXCell). For staining blood or cells, 20 μL of Fc block cocktail was combined with 30-50 μL of blood or prepared cells in 5 mL FACS tubes (BD), incubated for 5 minutes at RT, and then appropriate fluorochrome-labeled antibodies were added (final volume – 100 μL). Following incubation at RT protected from light to allow for staining (generally ~30 min, depending on antibodies), cells were resuspended in 2 mL ACK buffer (blood) or HBSS + 2% FBS (other cells) and centrifuged at RT for 5 minutes at 300 x g. Supernatant was decanted and cells were

resuspended in 0.5-1mL HBSS + 2% FBS for analysis. For cell sorting, a BD Influx cell sorter was used controlled with Spigot software (Beckton Dickinson, Franklin Lakes, NJ). Briefly, for sorting source cells were stained and resuspended in HBSS + 20% FBS + 10 mM HEPES and sorted directly into FBS supplemented with 10 mM HEPES. Standard flow cytometric analysis was performed using a BD LSR II instrument. Flow cytometric data analysis was performed using FlowJo (Treestar software, Portland, OR).

3.2.5. Statistical analysis

Statistical analysis was performed using Graphpad Prism v5.0 software. Details of statistical tests used are provided in figure legends. However, in general, for comparisons of two groups, Student's T test was used. In cases of comparison of groups with unequal variances, Welch's correction was applied. For multiple group comparisons, One way ANOVA with Tukey's multiple comparison test was used.

3.3. Results

3.3.1. Newly generated CD4 T cells express lower levels of PD-1 compared to mature cells.

PD-1 expression appears to be critical for the generation of immune tolerance during LIP of newly generated T cells. We previously showed that newly generated T cells in a lymphopenic setting (i.e. in a lymphopenic recipient of WT HSC) had a high

proportion of PD-1 expressing T cells that diminished over time[214]. Whether the higher PD-1 expression on newly generated T cells was dependent on exposure to the lymphopenic environment or is an intrinsic property of newly generated T cells even in lymphoreplete mice has not been fully assessed. We therefore examined PD-1 expression in the steady state in both TCRβ+ thymocytes and peripheral splenocytes from 10-12 wk old adult Rag2pGFP animals (Figure 3-1). Unexpectedly, peripheral newly-generated CD4 SP splenocytes expressed very little PD-1 while approximately 15% of established or "mature" GFP- CD4 SP T cells were found to be PD-1 positive (Figure 3-1 A). Likewise, mean fluorescence intensity (MFI) of PD-1 staining was significantly higher in the CD4 SP GFP- compared to the GFP+ population (Figure 3-1 B). Neither established nor newly-generated T cells within the splenic CD8 SP population expressed appreciable levels of this co-inhibitor. Within the thymocyte population, approximately 90% of the CD4 SP and 85% of the CD8 SP were GFP+, with the remainder presumably representing mature cells that had recirculated from the periphery back to the thymus although it is conceivable that at least a subset could represent cells that failed to exit the thymus for longer than 3 weeks post-VDJ recombination[204]. GFP+ thymic CD4 and CD8 SP cells expressed slightly higher levels of PD-1 (3.1% and 2.6% positive, respectively, Figure 3-1 A) compared to their splenic counterparts (both 1.3%), although this difference was not statistically significant. Similar to what was seen in splenocytes, the thymic CD4 SP GFP- population contained a sizeable population of PD-1+ cells (26%) and thus these more established cells had higher overall PD-1 expression compared to the GFP+ newly generated T cell population (Figure 3-1 A, B). Unlike their splenic counterparts, there was a trend toward increased PD-1 positivity and overall PD-1

expression in the thymic CD8 SP GFP- cells compared to the GFP+ cells although this difference was not statistically significant (Figure 3-1). Together, these data indicate that while PD-1 is up-regulated on a subset of established T cells – particularly the CD4 SP population - following a period of residency in the periphery (possibly due to encounter with antigen) only very low/barely detectable levels of PD-1 are expressed on newly generated T cells. Thus the heightened PD-1 expression on newly generated T cells seen in lymphopenic HSC recipients is not an intrinsic characteristic of these cells (i.e. it is lymphopenia-induced). Furthermore, the data draw attention to the fact that a significant portion (10-15%) of thymic T cells are established cells, consistent with the known ability of peripheral T cells to recirculate back to the thymus[370].

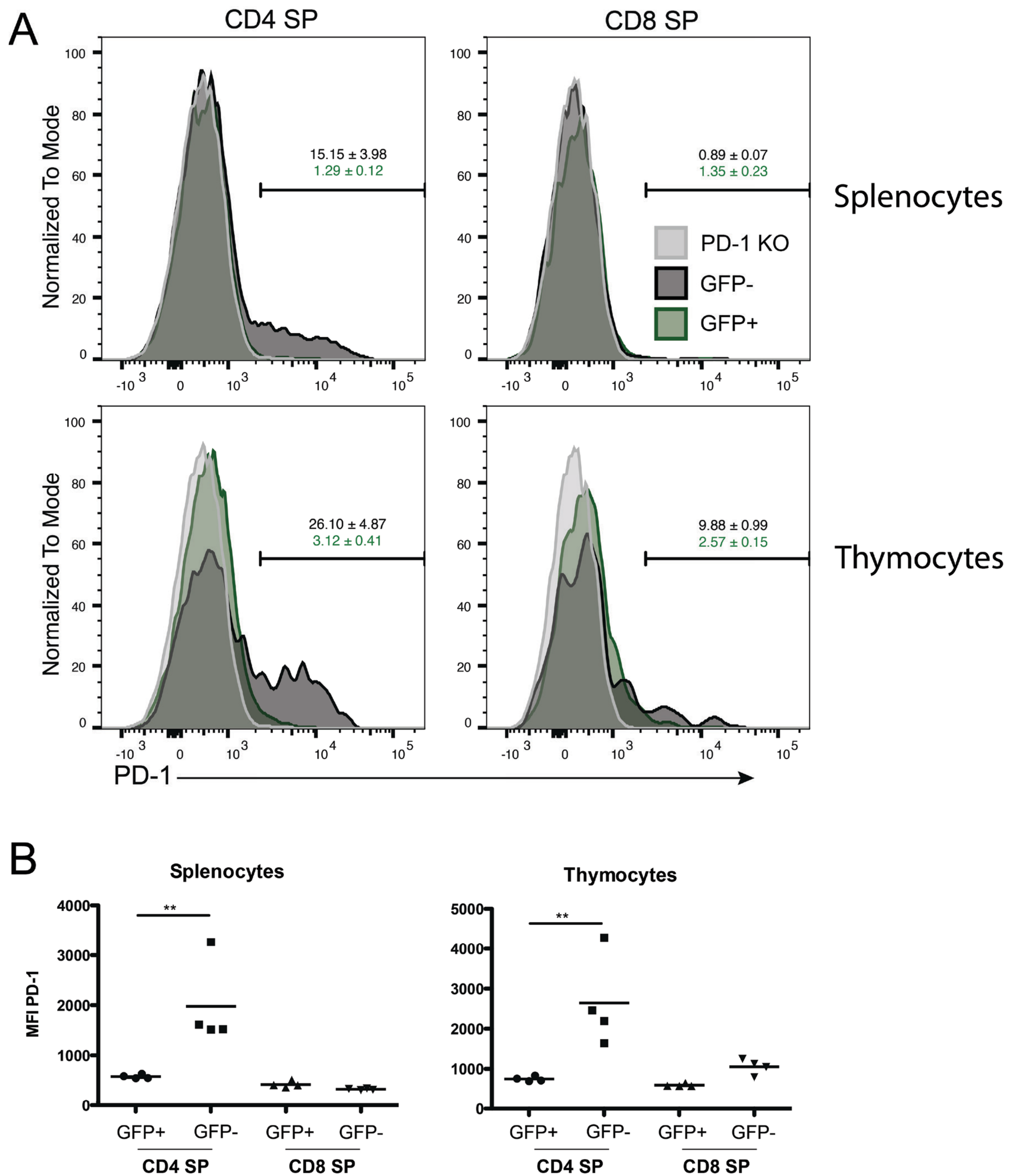


Figure 3-1. Newly generated T cells in both thymus and spleen express lower steady-state levels of PD-1 compared to mature T cells

A) Representative flow cytometric analysis of splenocytes and thymocytes from 10-12 wk old adult Rag2pGFP mice for PD-1 expression among TCRβ+CD4 or CD8 SP cells that are GFP+ (newly generated T cells) or GFP- (mature cells). T cells from PD-1$^{-/-}$ mice were also stained as a control for background. Values above gates are the group

average %PD-1 positive cells within the mature GFP- population (black text) or GFP+ population (green text), ± SEM. B) Mean fluorescence intensity of anti-PD-1 staining in CD4 or CD8 SP, GFP+ or GFP- T cells from Rag2pGFP splenocytes or thymocytes. **$p<0.01$, One-way ANOVA with Tukey's multiple comparison test.

3.3.2. Purified peripheral newly generated T cells preferentially drive autoimmunity compared to established T cells upon transfer to lymphopenic hosts.

Our previous studies showed that newly generated T cells exported from the thymus into a lymphopenic environment are critically dependent on PD-1 to prevent autoimmunity; in contrast, PD-1 was not needed in established T cells. However, it is unknown whether steady state newly generated T cells in immunocompetent mice retain this heightened potential for autoimmunity or if instead the peripheral tolerance process is rapid and newly generated T cells are immediately tolerized upon export to a lymphoreplete environment. In order to test the hypothesis that steady state peripheral RTE / newly generated T cells had increased ability to drive autoimmunity upon transfer to a lymphopenic host, we generated the B6 Rag2pGFP x PD-1$^{-/-}$ cross and purified the CD4 and CD8 SP, GFP+ or GFP- populations from splenocytes of ~6 week old animals by fluorescence activated cell sorting (FACS). 1 x 10^6 purified cells or thymocytes containing an equivalent number of SP T cells (~8-9 x 10^6 total cells) were transferred to NK depleted recipient Rag$^{-/-}$ animals and the recipients were monitored for disease. NK depletion with monoclonal anti-NK1.1 was carried out in an attempt to maximize the

number of injected T cells that survived, as other studies in our lab and others[367-369] have suggested that even syngeneic cells can be subject to NK killing after *in vitro* manipulation and injection. Beginning at around day 20 after transfer, mice that received either PD-1$^{-/-}$ thymocytes or purified GFP+ newly generated T cells began to develop autoimmune disease while the recipients of established cells were relatively spared (Fig. 3-2 A). Similarly, while all recipient mice lost weight beginning almost immediately after cell transfer until around 2 weeks post-transfer, after this point PD-1$^{-/-}$ established T cell recipients began to regain their lost weight while the thymocyte and newly-generated T cell recipients clearly and significantly diverged from the established cell recipients and continued to lose weight (Figure 3-2 B). Kaplan-Meier survival curve analysis of disease onset and incidence between groups revealed a significant difference between the three survival curves with p=0.003 (Fig 3-2 A). Further statistical analyses revealed that the PD-1 KO GFP+ newly generated cell recipient vs established cell recipient curves were significantly different (p=0.02) while PD-1 KO thymocyte recipient vs PD-1 KO GFP+ newly generated cell recipient curves were not significantly different (p=0.087) despite a trend towards a slightly earlier disease onset time in the former. Despite the clearly higher propensity of newly generated vs. established cells to drive autoimmunity, it should be mentioned that a low proportion of the GFP- established T cell recipients also met the criteria for disease albeit at a much later time point relative to the other groups (Figure 3-2 A). Approximately 40% of the purified GFP+ newly generated cells were CD4 SP and 56% were CD8 SP (a CD4:CD8 ratio of ~0.7:1), whereas the purified GFP- population contained approximately 80% CD4 SP and 17% CD8 SP (CD4:CD8 ratio ~4.7:1, Figure 3-2 C). These values from Rag2pGFP x PD-1$^{-/-}$ peripheral cells differ

substantially from the ratios previously reported in the literature for WT Rag2pGFP peripheral GFP+ and GFP- T cells in ~6 week old animals[204]. Specifically, the ratio of CD4:CD8 SP cells in WT GFP+ T cells was about 3:1 and in GFP- cells it was about 2.5:1. This may suggest that in the absence of PD-1, although MHC Class I appears to be largely dispensable for disease following transfer of PD-1$^{-/-}$ HSC[214], the differences in the proportions of CD4 vs CD8 SP cells in the purified cell populations used in this experiment could potentially contribute to the differences in the ability of newly generated vs. established T cells to drive disease. Thus it is important to determine whether this autoimmune disease model depends on CD4 or CD8 SP cells (or both).

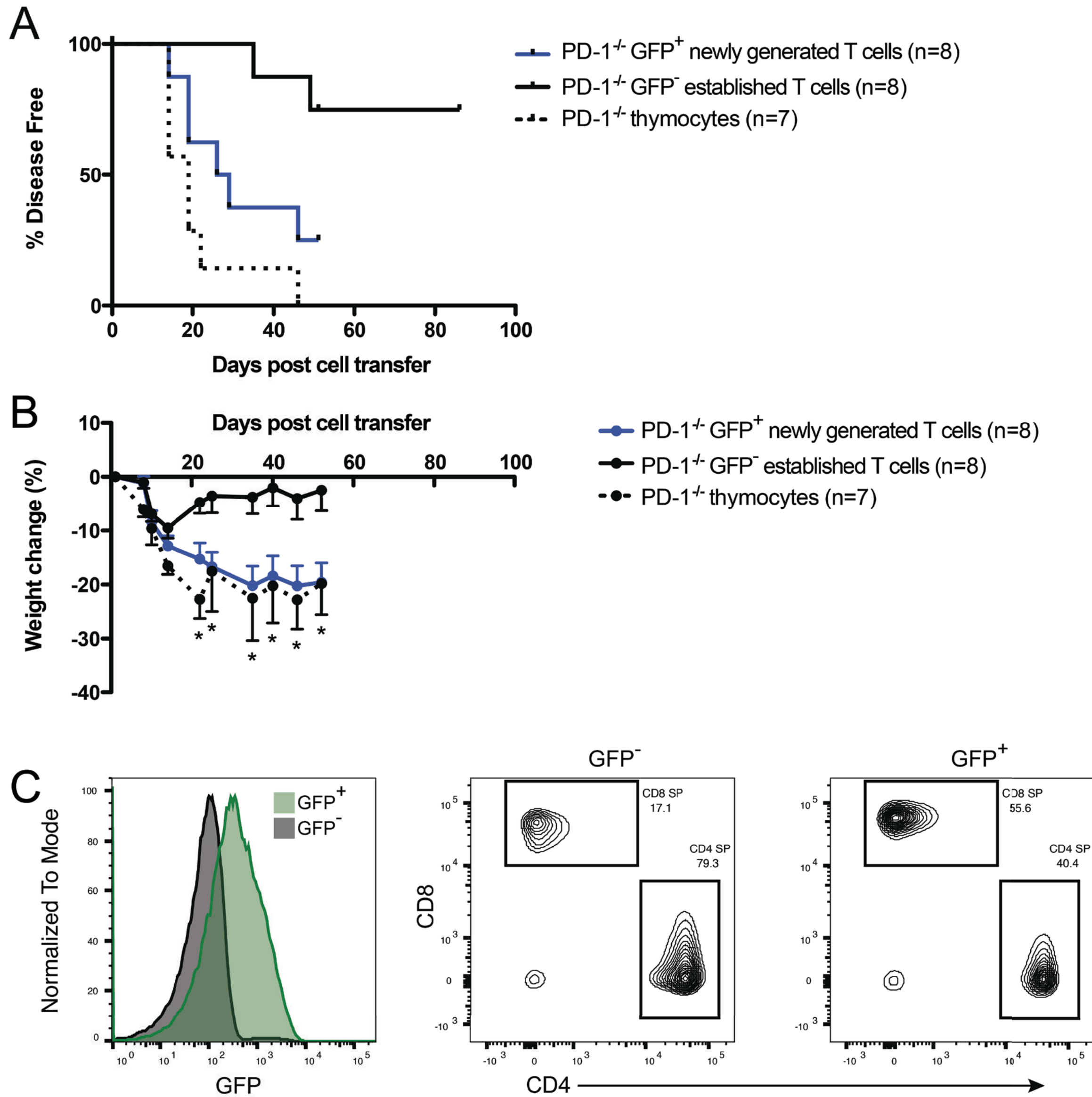


Figure 3-2. Peripheral PD-1$^{-/-}$ newly generated T cells preferentially drive autoimmunity in lymphopenic recipient animals.

CD4 and CD8 SP, GFP+ and GFP- cells were sorted from ~6 wk old Rag2pGFP x PD-1$^{-/-}$ splenocytes. 1 x 10^6 sorted CD4 and CD8 SP GFP+ or GFP- cells, or unfractionated thymocytes containing an equivalent number of CD4 and CD8 SP cells (~8-9 x 10^6) were injected into NK depleted adult Rag$^{-/-}$ recipients and mice were monitored for disease symptoms. A) Disease incidence in recipients of thymocytes, GFP+ newly generated T cells or GFP- established T cells. Survival curve comparison demonstrated a significant difference between groups with p=0.003. Data are combined from two independent

experiments, starting n values are indicated in the legend. B) Weight changes in recipients of indicated cells from two independent experiments from a), ± SEM. Weight observations for which similar time points were available between experiments (± 2d) were combined and used for this analysis. *p<0.05, GFP+ newly generated T cells vs GFP- established T cells, One-way ANOVA with Tukey's multiple comparison test. C) Representative GFP expression (left panel) and CD4 and CD8 SP proportions (right 2 panels) in purified cell populations used in A and B.

3.3.3. Host MHC Class II is required for disease upon transfer of PD-1$^{-/-}$ HSC

Although adult Rag1$^{-/-}$ K$^{b-/-}$D$^{b-/-}$ mice were fully permissive for the development of autoimmune disease following transfer of PD-1$^{-/-}$ HSC[214], in that experiment the developing thymocytes were MHC Class I sufficient and thus low numbers of CD8+ T cells were able to undergo selection processes and emerge into the periphery (G. Thangavelu, personal communication). Importantly, no perturbations in the time to disease onset or severity were reported compared to Rag$^{-/-}$ hosts, as would be reasonably expected if the disease were CD8+ T cell dependent. Nevertheless, in order to investigate the involvement of CD4+ T cells in this disease model we generated the cross between Rag$^{-/-}$ and MHC Class II transactivator deficient (CiiTA$^{-/-}$) mice, which are deficient in most MHC-II expression[13]. To test the requirement for MHC Class II in the host for disease post PD-1$^{-/-}$ HSC transfer, we transferred PD-1$^{-/-}$ HSC to Rag$^{-/-}$ or Rag$^{-/-}$ x CiiTA$^{-/-}$ hosts and monitored the mice for signs of disease. While Rag$^{-/-}$ mice developed

systemic autoimmune disease around d44 after transfer, $Rag^{-/-}$ x $CiiTA^{-/-}$ were completely spared (Figure 3-3 A). Similarly, the pronounced weight loss encountered in the $Rag^{-/-}$ recipient group was not seen in the $Rag^{-/-}$ x $CiiTA^{-/-}$ recipients and indeed the latter group actually gained weight for much of the experiment (Figure 3-3 B) despite CD8 SP T cell and B cell development by d46 post-transfer (Figure 3-3 C). These data indicate that MHC-II expression in the host is required for disease after $PD\text{-}1^{-/-}$ HSC transfer, suggesting disease is dependent on CD4+ T cells.

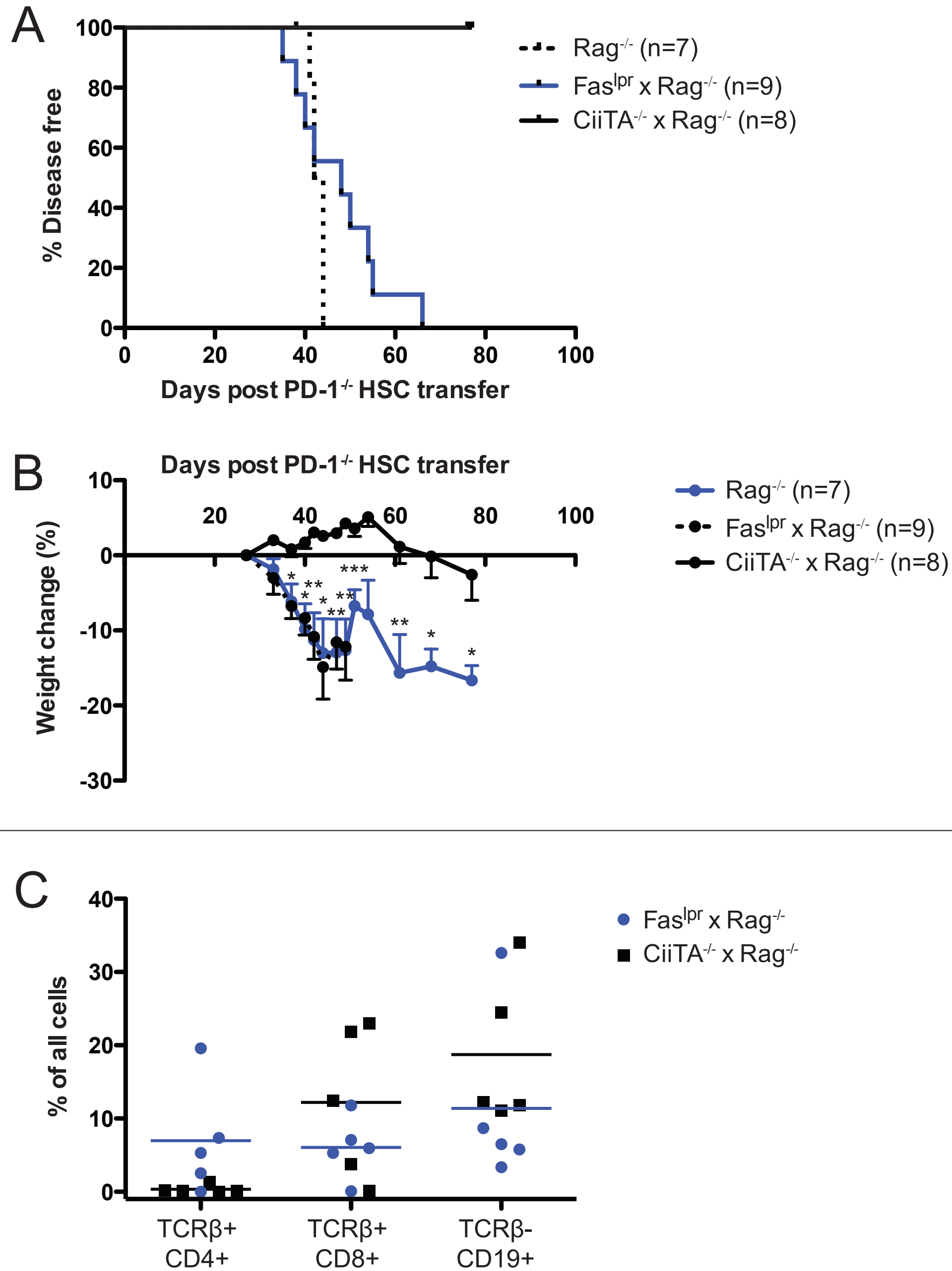


Figure 3-3. Systemic autoimmunity in lymphopenic PD-1$^{-/-}$ HSC recipients requires host MHC Class II but not Fas.

PD-1$^{-/-}$ HSC were transferred to Rag$^{-/-}$, Fas^{lpr} x Rag$^{-/-}$, or CiiTA$^{-/-}$ x Rag$^{-/-}$ recipients and mice were monitored for disease symptoms. A) Kaplan-Meier survival curve analysis of disease incidence among HSC recipients groups are significantly different with p=0.0002. Data are combined from two independent experiments, with starting numbers

per group indicated in the legend. B) Weight changes in recipients relative to d27 post-transfer, combined from 2 independent experiments, ± SEM. Weight observations for which similar time points were available between experiments (± 2d) were combined. For each timepoint, only data from groups with n>2 are presented. *p<0.05, **p<0.01, ***p<0.001, Fas^{lpr} x $Rag^{-/-}$ vs. $CiiTA^{-/-}$ x $Rag^{-/-}$, One-way ANOVA with Tukey or Dunn's multiple comparison test (d27-49) or Student's T test (d>51). C) % of all peripheral blood cells that were CD4+ or CD8+ T cells or CD19+ B cells in Fas^{lpr} x $Rag^{-/-}$ and $CiiTA^{-/-}$ x $Rag^{-/-}$ recipients from one experiment in A,B above, measured at d46 post-HSC transfer.

3.3.4. Host Fas expression is not required for autoimmunity post-PD-1^-/- HSC transfer

Two major pathways of T cell killing, namely the FasL-Fas and perforin-dependent pathways, have been described[371]. In order to begin to elucidate which, if any, of these pathways are involved in mediating autoimmunity in the PD-1-/- HSC model, we first crossed the B6-Fas^{lpr} mouse, which lacks functional Fas expression[372], with $Rag1^{-/-}$ mice to generate the double mutant strain Fas^{lpr} x $Rag^{-/-}$. Transfer of PD-$1^{-/-}$ HSC to these double mutants revealed that host Fas expression was completely dispensable for the generation of autoimmunity in this model (Fig. 3-3 A, C). It should be noted that one Fas^{lpr} x $Rag^{-/-}$ recipient depicted in Fig. 3-3 C had a robust B cell population but very few T cells at d46, and had delayed development of disease (kyphosis, ocular lesions) by d66 post transfer. Reconstitution of the T cell compartment may thus have been delayed in this recipient. No statistically significant difference in the survival curves of the $Rag^{-/-}$

and Fas^{lpr} x Rag$^{-/-}$ recipient groups was detected. Similarly, weight loss in Fas^{lpr} x Rag$^{-/-}$ hosts was indistinguishable from that seen in the Rag$^{-/-}$ hosts (Fig. 3-3 B). Thus, host Fas expression is not required for disease post PD-1$^{-/-}$ HSC transfer.

3.3.5. Perforin expression in the lymphoid compartment is not required for LIP-driven autoimmunity after transfer of PD-1$^{-/-}$ thymocytes.

Next we examined whether perforin expression in lymphocytes was required for disease following transfer of PD-1$^{-/-}$ thymocytes to a lymphopenic host. We generated the cross between perforin knockout (Prf1$^{-/-}$) and PD-1$^{-/-}$ mice. The double knockout animals we were able to generate resulted from heterozygous crosses and never successfully bred in our facility as homozygous double knockouts (unpublished observations). Nevertheless, we transferred thymocytes from the Prf1$^{-/-}$ x PD-1$^{-/-}$ or PD-1$^{-/-}$ mice to Rag$^{-/-}$ recipients and monitored the animals for disease and weight loss. Prf1$^{-/-}$ x PD-1$^{-/-}$ thymocyte recipients all developed autoimmune disease between d13 and 21 post-transfer, while the PD-1$^{-/-}$ thymocyte recipients had a more delayed course of disease with lower incidence (Fig. 3-4 A, top, p=0.01). Furthermore, weight loss (Figure 3-4 A, bottom) was significantly greater in the Prf1$^{-/-}$ x PD-1$^{-/-}$ thymocyte recipients, and in general the severity of symptoms, particularly diarrhea, was greater in this group compared to PD-1$^{-/-}$ thymocyte recipients. It should also be mentioned that in addition to this experiment, we also performed 2 small pilot experiments to question whether Prf1$^{-/-}$ was required for disease in the HSC transfer model. In one experiment, Prf1$^{-/-}$ x PD-1$^{-/-}$ T cell depleted bone marrow cells were transferred to Rag$^{-/-}$ hosts. In the other, Prf1$^{-/-}$ HSC were transferred to Rag$^{-/-}$ hosts treated intraperitoneally with 200 µg/mouse monoclonal blocking anti-PD-1 antibody every 2d from d25 until termination. In both of these

experiments recipients developed autoimmunity (data not shown) supporting the notion that perforin expression in T cells is not required for disease in this model.

Because of the potential for functional redundancy between the Fas-FasL and perforin-dependent killing pathways, we also tested whether adoptive transfer of cells from diseased $Rag^{-/-}$ $Prf1^{-/-}PD\text{-}1^{-/-}$ thymocyte recipients to our Fas^{lpr} x $Rag^{-/-}$ hosts would result in the development of autoimmunity. Indeed, transfer of splenocytes+lymph node cells from the diseased $Prf1^{-/-}PD\text{-}1^{-/-}$ thymocyte recipients to $Rag^{-/-}$ or Fas^{lpr} x $Rag^{-/-}$ mice resulted in the robust development of autoimmunity in both groups with no statistically significant difference upon comparison of their survival curves (Figure 3-4 B, left panel, p=0.14). Although both recipient groups lost weight, by d22 post-adoptive transfer the Fas^{lpr} x $Rag^{-/-}$ hosts had lost significantly more weight than the $Rag^{-/-}$ recipients (25% vs. 15%, Figure 3-4 B, right panel), suggesting that Fas in the host may actually play an immunoregulatory role. Taken together, these data show that neither perforin expression in T cells nor Fas expression in the host are required for LIP- and newly-generated T cell-driven autoimmunity in the context of PD-1 deficiency.

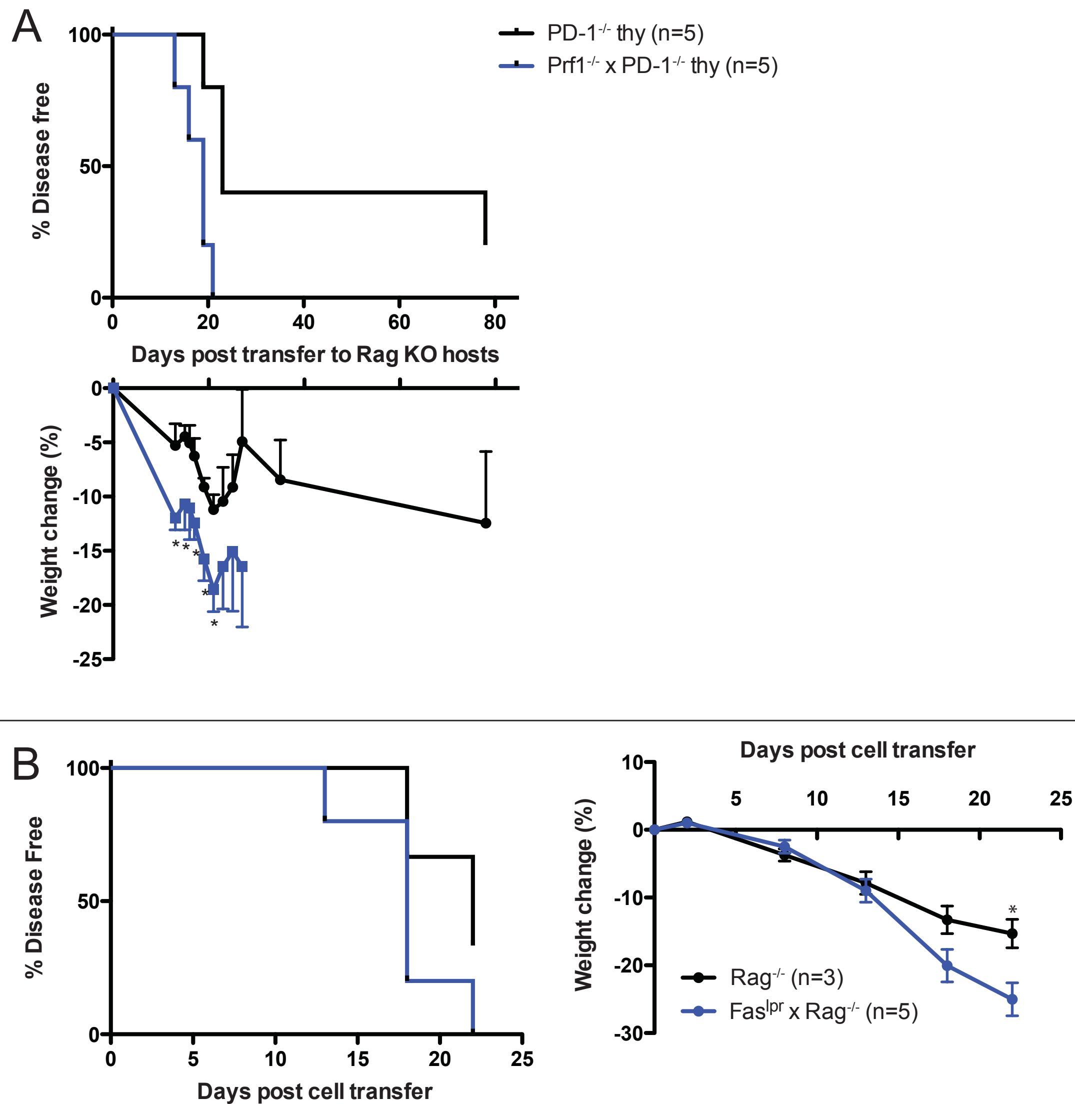


Figure 3-4. Systemic autoimmunity after PD-1$^{-/-}$ thymocyte transfer to lymphopenic recipients is independent of Perforin-mediated killing.

10 x 10^{6} Thymocytes from PD-1$^{-/-}$ or Perforin$^{-/-}$ x PD-1$^{-/-}$ (Prf1$^{-/-}$ x PD-1$^{-/-}$) animals were transferred i.v. to NK depleted adult Rag$^{-/-}$ recipients and mice were monitored for disease symptoms. A) Top: Kaplan-Meier survival curve analysis of disease incidence among recipients of the indicated thymocytes. Survival curve comparison demonstrated a significant difference between the two groups with p=0.01. Bottom: weight change among the recipients in (a), ± SEM. Data are from a single experiment, starting n values are indicated in the legend. B) Splenocytes and inguinal lymph node cells were harvested from 3 diseased Prf1$^{-/-}$ x PD-1$^{-/-}$ thymocyte recipients from the experiment depicted in (a)

at d28 post-transfer, and 20 x 10^6 cells were transferred i.v. into NK depleted, adult $Rag^{-/-}$ or Fas^{lpr} x $Rag^{-/-}$ recipients. One Fas^{lpr} x $Rag^{-/-}$ recipient received 10 x 10^6 cells. Mice were monitored for disease symptoms. Left panel: Survival curve comparison. No significant difference between the two groups was detected (p=0.14). Right panel: Weight change among cell recipients, ± SEM. *$p<0.05$, Student's T test. Data are from a single experiment with starting n values in the legend.

3.4. Discussion

Mechanisms of T cell homeostasis function to maintain a diverse repertoire of sufficient size for effective immune surveillance of the host. Reconstitution of the T lymphocyte compartment of a lymphopenic host by hematopoietic stem cell transplant or transfer of T cells results in LIP as the cells expand to fill the available niche defined by available pMHC and cytokine "resources". The relative abundance of resources in a host with respect to the numbers of competitors for those resources can be considered as a way to define the "severity" of lymphopenia in that host or its "LIP-potential". Importantly, LIP is strongly associated with the promotion of autoimmune or inflammatory disease[186,214,298,303,309-316,373]. Such promotion of autoimmunity may result from potentiation of TCR signaling by high concentrations of homeostatic cytokines like IL-7 and IL-15[317,318] along with unimpeded access to self pMHC due to decreased competition from other Tcon or Treg. Thus, perturbations which would be expected to increase the LIP potential of a host, such as decreased Treg, increased homeostatic

cytokines, or decreases in co-inhibitory signals would be expected to increase the potential for such autoimmunity, and *vice versa*. We have previously demonstrated that PD-1$^{-/-}$ hematopoietic stem cells or thymocytes, but not established peripheral cells (ie: splenocytes), could promote severe systemic autoimmunity upon transfer to adult Rag$^{-/-}$ hosts[214], suggesting that newly generated T cells were the critical mediators of disease in this model. Our lab recently found that the frequency of CD4 SP T cells to a peripheral neoself antigen decline with time post-HSC transfer[353]. We consider that newly generated T cells have not been subjected to peripheral tolerance mechanisms and thus may contain an increased proportion of strongly self-reactive cells compared to established T cells that arose in a host with low LIP-potential upon initial seeding of its peripheral T cell compartment (ie: during the neonatal period) and were tolerized. Indeed neonatal Rag$^{-/-}$ mice, which by virtue of anatomic size (small lymph nodes, reduced overall resources) and competition by specialized populations of innate lymphoid cells for IL-7[327] could be considered to have relatively low LIP-potential, rarely developed severe autoimmunity after reconstitution with PD-1$^{-/-}$ HSC[214]. Together, the data suggest that newly-generated T cells are a particularly important population to control during establishment or recovery of the T cell compartment and that PD-1 is critically involved in that control. On the other hand, much of the published literature describing newly generated T cells / RTE suggests that they have functional properties geared toward promotion of tolerance[196]. Herein we directly tested whether newly generated T cells purified from the periphery had an increased ability to drive autoimmunity compared to established cells. This was done by purifying GFP+ and GFP- T cells from Rag2pGFP x PD-1$^{-/-}$ transgenic mice, in which GFP expression marks newly generated T cells for

approximately 3 weeks following TCR rearrangement[203,204], and transferring them to $Rag^{-/-}$ recipients. In support of our hypothesis, we found that newly generated PD-$1^{-/-}$ T cells isolated from the periphery of adult animals were indeed capable of generating autoimmune disease (particularly cachexia, kyphosis, and diarrhea) similar to PD-$1^{-/-}$ thymocytes, and much more efficiently than established T cells which contained approximately 1.3% contaminating GFP+ cells (Figure 3-2). These data indicate that steady state newly generated T cells have a dangerous autoimmune potential even when their initial export from the thymus is into a lymphoreplete environment. Although PD-1 is critical for tolerance in newly generated T cells, we did not, in the steady state, detect PD-1 expression above background on the peripheral newly generated T cell population of Rag2pGFP mice by flow cytometry and only low expression in thymic GFP+ T cells (Fig. 3-1). In contrast, both the peripheral CD4 SP and thymic CD4 and CD8 SP established (GFP-) T cell population contained sizeable populations that robustly expressed PD-1. Previous data from our lab assessing PD-1 expression in steady state RTE (i.e. in WT lymphoreplete mice) used CD24 as the marker to define RTE and the data suggested RTE have higher PD-1. However, based on our current data with the Rag2pGFP mouse and the fact that only a small fraction of newly generated T cells expressed CD24 and established T cells can also express CD24, we conclude that steady state newly generated T cells do not have increased PD-1. Together with other data from our lab suggesting that PD-1 can regulate LIP and the response to tonic pMHC signals[374] (Chapter 4), these observations suggest that PD-1 is up-regulated during LIP as a negative feedback mechanism. Finally, it is worth noting that because a significant portion (10-15%) of thymic T cells are established cells based on lack of GFP expression,

consistent with the previously described ability of peripheral T cells to recirculate back to the thymus[370], the use of the term "recent thymic emigrants" to describe GFP+ cells in the Rag2pGFP transgenic model as is common in the literature might be somewhat imprecise as presumably some cells emerging from the thymus in an adult are also GFP-. Therefore although all peripheral GFP+ cells are RTE, all RTE are not necessarily GFP+.

It was previously shown that MHC-I deficient Rag$^{-/-}$ hosts were fully susceptible to disease post-PD-1$^{-/-}$ HSC transfer, suggesting that CD8+ T cells are dispensable for the development of autoimmunity in this system. We also found that in comparison to ~6 week old WT Rag2pGFP mice in which the GFP+ and GFP– T cell compartments contained about a 3:1 and 2.5:1 ratio, respectively, of CD4:CD8 SP T cells[204], GFP+ T cells in ~6 week old Rag2pGFP x PD-1 mice contained a far lower ratio of CD4:CD8 T cells (~0.7:1) with a increased ratio in the GFP- T cell population (~4.7:1, Figure 3-2 C). This change in ratios in the absence of PD-1 might suggest that PD-1 is particularly important for controlling the homeostasis of CD4 SP cells and thus these cells have undergone more divisions and have diluted out their GFP in the PD-1$^{-/-}$ animals. In order to further explore the question of which cells are driving autoimmunity during LIP in this model system, we transferred PD-1$^{-/-}$ HSC into Rag$^{-/-}$ x CiiTA$^{-/-}$ mice which lack the majority of MHC-II expression[13]. Here we found that the MHC-II deficient hosts were completely protected from disease (Fig 3-3 A, B). Taken together, these data suggest that the CD4 SP population is the key population responsible for promoting disease in this model.

While CD4+ T cells are most commonly considered as "helpers" of the immune response, numerous studies have suggested that they can in some circumstances acquire

cytolytic effector function via up-regulation of killing mechanisms typically associated with CD8+ cytotoxic T lymphocytes (CTL) such as the perforin and Fas ligand (FasL) pathways[375]. In order to explore which, if any, of these killing mechanisms were important for the autoimmune pathology seen in our model, we generated Fas-deficient $Rag^{-/-}$ hosts (Fas^{lpr} x $Rag^{-/-}$) as well as perforin-deficient $PD\text{-}1^{-/-}$ donors ($Prf1^{-/-}$ x $PD\text{-}1^{-/-}$). Neither Fas^{lpr} x $Rag^{-/-}$ recipients of $PD\text{-}1^{-/-}$ HSC, nor recipients of $Prf1^{-/-}$ x $PD\text{-}1^{-/-}$ thymocytes were spared from disease (Fig. 3-3 and Fig. 3-4 A). Furthermore, disease in $Prf1^{-/-}$ x $PD\text{-}1^{-/-}$ thymocyte recipients was exacerbated compared to $PD\text{-}1^{-/-}$ thymocyte recipients (Fig. 3-4 A), suggesting that perforin-dependent effector pathways may actually play an immunoregulatory role during LIP. Importantly, to rule out functional redundancy between Fas-FasL and perforin-dependent killing pathways we also tested whether we could generate autoimmunity in Fas^{lpr} x $Rag^{-/-}$ hosts via adoptive transfer of peripheral cells from diseased recipients of $Prf1^{-/-}$ $PD\text{-}1^{-/-}$ thymocytes. Indeed, all of the recipients of these cells developed autoimmunity which was more severe in the Fas^{lpr} x $Rag^{-/-}$ compared to $Rag^{-/-}$ recipients (Fig. 3-4 B) suggesting that Fas may also play an immunoregulatory role during LIP. Indeed preliminary data from our lab suggests that WT HSC may be able to induce autoimmune disease in Fas^{lpr} x $Rag^{-/-}$ recipients. Taken together, these data strongly suggest that neither the FasL-Fas or perforin-dependent granule exocytosis pathways are involved in mediating the autoimmune pathology in this model. We did consider the possibility that Granzyme B produced by T cells activated during LIP might contribute to disease, for example through the mannose-6-phosphate receptor[376]. However, examination of Granzyme B expression in WT vs $PD\text{-}1^{-/-}$ HSC recipients at the peak of disease (d45) demonstrated no significant difference in splenic

CD4 or CD8 SP T cell populations although a small trend toward increased expression in PD-1$^{-/-}$ HSC recipient CD8 SP T cells was noted (Appendix 3).

Although our data do not rule out a contributing role for CD8 SP T cells, the lack of susceptibility of MHC-II deficient hosts to disease despite generation of CD8+ T cells and B cells (Fig. 3-3 C) and the broad up-regulation of a number of pro-inflammatory cytokines in PD-1$^{-/-}$ HSC recipients (IFN-γ, IL-13, TNF-α, IP-10, MIG, MCP-1, VEGF) [214] along with the lack of requirement for either perforin- or Fas- dependent killing pathways for autoimmunity in this model suggest that the immune pathology seen is primarily a CD4 T cell and "cytokine-storm" dependent phenomenon. Several similarities exist between the LIP-driven autoimmunity described herein and certain clinical syndromes, such as immune reconstitution inflammatory syndrome (IRIS) in HIV patients experiencing a rebound of the CD4+ T cell compartment after treatment with antiviral drugs[313,320] or graft-versus-host disease (GVHD) post-allogeneic bone marrow transplantation. Notably a mouse model of IRIS was recently described in which transfer of purified CD4 SP T cells to *Cryoptococcus*-infected lymphopenic mice yields weight loss and systemic inflammatory disease associated with cytokine dysregulation[314]. In addition, the striking increases in the chemokines IP-10 and MIG described previously in our model[214] are also observed in clinical chronic GVHD[377]. Furthermore, the term "cytokine-storm" was originally used to describe the syndrome of cytokine dysregulation (particularly IL-6, IL-1 and TNF-α) which is associated with and greatly contributes to GVHD pathology[336,339]. Indeed, early preliminary data from our lab suggests that lack of responsiveness to IL-1β in the T cell compartment, through deficiency of MyD88, may prevent or retard autoimmunity post-PD-1$^{-/-}$ HSC transplant. One could make the

argument that our model of LIP-driven autoimmunity after transfer of PD-1$^{-/-}$ newly generated T cells to a lymphopenic host might actually be viewed as a model of syngeneic GVHD. Thus, beyond the insights it offers into the role of co-inhibitory molecules in establishing tolerance, further characterization of this model may lead to translatable insights to treat cytokine-driven systemic autoimmunity and inflammatory disease.

Chapter 4

PD-1 controls tonic TCR signaling and lymphopenia-induced proliferation of T lymphocytes

4.1. Introduction

The number of cells in and diversity of the peripheral T lymphocyte pool is controlled by intra- and interclonal competition for resources, which together define T cell "space" [216]. Such resources include homeostatic cytokines like IL-7 and IL-15, but also peptide:MHC (pMHC) complexes; often self pMHC, with which the TCR can interact and receive at least a weak "tonic" signal to promote T cell survival. In lymphopenic hosts, recovery of the T cell compartment occurs via a process known as lymphopenia-induced proliferation (LIP), where resources are in excess and T cells expand to fill the available niche. While the term lymphopenia lacks a precise quantitative character, one can consider the extent to which LIP can occur, or "LIP potential" of a host, as a ratio of the available resources to the number of competitors for those resources. Thus, provision of competition for a particular pMHC can reduce LIP potential for other T cells that recognize the same pMHC[233,283,292]. Similarly, Treg, which may be viewed as "super"-competitors for pMHC, can inhibit LIP[186,292,296,303-308]. Positive or negative regulation of the strength of the TCR signal a lymphocyte receives in response to a given pMHC, for example by blocking/reducing co-inhibition[214,298-300,360] or co-stimulation[296], respectively, can modulate LIP potential. Finally, a neonatal host might be viewed to have a low LIP potential due to small anatomic size (low absolute quantities of resources) as well as the recently described presence of an innate lymphoid cell population that may act as a sink for IL-7[327].

Importantly, LIP is strongly associated with acquisition of an effector-memory phenotype in T cells, including the ability to rapidly mediate effector function[270], and in the context of concurrent infections or deficiencies of co-inhibitory pathways, LIP can

result in overzealous T cell responses and autoimmune disease[186,214,298,303,309-316,373]. This association between LIP and inappropriate inflammation may be rationalized by considering that the conditions present in lymphopenia - including reduced competition for access to a given T cell's cognate or tonic pMHC, along with high concentrations of IL-7 and IL-15 (which have been suggested to potentiate TCR signals[317,318]) - may conspire to increase the frequency with which T cells can productively interact with pMHC and generate an abnormally strong TCR signal when they do so. Thus autoimmunity or inflammatory disease secondary to LIP may ultimately derive from a stochastically greater chance of a given T cell crossing the threshold for the induction of its effector functions, and on a system-wide basis, this may result in a cytokine storm, for example. In a clean conventional housing environment, reconstitution of the lymphoid compartment of an otherwise normal $Rag^{-/-}$ mouse with B6 hematopoietic stem cells (HSC) does not typically result in the development of apparent autoimmune disease[214,374], which is perhaps not surprising given that vertebrates have been evolving alongside T cell-depleting stimuli and infections (e.g. retroviruses) for millions of years and thus clearly there has been selective pressure against mounting productive immune responses against self when such situations occur. However, in stark contrast, we recently demonstrated that reconstitution of adult $Rag^{-/-}$ mice with HSC deficient in the co-inhibitory molecule PD-1[378] results in a rapid, severe, and frequently lethal systemic autoimmune disease soon after the first newly generated T cells emerge from the thymus[214]. Autoimmunity also results from transfer of $PD\text{-}1^{-/-}$ newly generated T cells to $Rag^{-/-}$ recipients (Chapter 3), but not established peripheral cells from adult $PD\text{-}1^{-/-}$ mice, which, on the B6 background, develop only a mild lupus-like autoimmune disease upon

aging[133]. Because thymic central tolerance is not perfect and some cells with higher affinity for self pMHC escape[366], we consider that the newly generated T cell population has a higher overall potential for driving autoimmune disease during LIP compared to established cells that developed in a lymphoreplete adult environment or an environment with low LIP potential (i.e. the neonate) and thus underwent more normal peripheral tolerance mechanisms (deletion, anergy, ignorance, conversion to pTreg). In other words, the newly generated T cell population as a whole would be expected to have a higher average affinity for self pMHC compared to established cells. One hypothesis to explain disease in our model is that in the "three-strikes" scenario of high LIP potential, PD-1 deficiency, and a periphery seeded with newly-generated T cells, a stochastically greater number of the T cells on the higher end of the self pMHC affinity spectrum receive a strong enough signal to acquire effector function such as cytokine production and the magnitude of the response gives rise to a systemic cytokinemia. On the other hand, when PD-1 is sufficient, its restraint on the magnitude of the TCR signal[129-131] limits the frequency of such spurious activation preventing a pathological systemic effect. Although multiple co-inhibitory molecules other than PD-1 have been shown to control LIP[298-300,360] and our data suggest that PD-1 does as well[214,374] (Chapter 3), whether they do so *in vivo* by controlling TCR signals mediated by tonic self pMHC is unknown. Indeed, PD-1 is known to be up-regulated on *bona fide* activated T cells and it is unclear whether the generally weak, tonic signals provided by interaction with self pMHC can drive its expression. Furthermore, because of the polyclonal repertoire generated post-HSC transplant in our disease model, we cannot rule out that the response might be directed to foreign pMHC (although depletion of gut microbiota in recipients does not

protect from disease[374] (Chapter 2)) or pMHC derived from tissue-restricted antigens against which developing PD-1$^{-/-}$ T cells failed to be appropriately negatively selected in the thymus. Herein we examined whether PD-1 is able to control the response to tonic pMHC using transfer of male antigen (HY)-specific Marilyn CD4+ TCR transgenic T cells[331] to MHC-II sufficient and deficient (MHC Class II transactivator (CiiTA) knockout[13]) lymphopenic hosts lacking the male antigen HY. We also examined whether PD-1 deficiency had any effects on signaling through IL-7Rα during LIP. We found that Marilyn PD-1$^{-/-}$ CD4+ T cells have a proliferative advantage relative to Marilyn WT cells in the presence of only tonic or both tonic and cognate pMHC, and that this was independent of IL-7 signaling. Furthermore we show that IL-7Rα blockade does not substantially inhibit proliferation in early phases of LIP when competition is low, and that reduction of LIP potential by blocking IL-7 signals can ameliorate weight loss after transfer of high- (30 x 10^6) but not low-dose (10 x 10^6) PD-1$^{-/-}$ thymocytes to a lymphopenic host. These findings suggest that IL-7 is particularly important for controlling T cell homeostasis during situations of high competition for limited pMHC but is not a required factor for LIP to occur. Thus PD-1 can control tonic pMHC signals during LIP, and modulation of LIP-potential via mechanisms independent of PD-1 can offset the severity of LIP-driven autoimmunity precipitated by PD-1 deficiency.

4.2. Materials and Methods

4.2.1. Mice

B6.129S7-*Rag1*^{tm1Mom}/J (Rag1$^{-/-}$, Rag$^{-/-}$), B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J (FoxP3^{EGFP}), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) and B6.129S2-*Ciita*^{tm1Ccum}/J (CiiTA$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-*Pdcd1*$^{-/-}$ (backcrossed 11 generations to C57BL/6) were originally generated by Prof. T. Honjo and colleagues[133]. FoxP3^{EGFP} x *Pdcd1*$^{-/-}$ mice were generated by crossing the above FoxP3^{EGFP} and B6-*Pdcd1*$^{-/-}$ mice and are referred to in the present manuscript simply as PD-1$^{-/-}$. Rag$^{-/-}$ x CiiTA$^{-/-}$ mice were generated by crossing the above Rag$^{-/-}$ and CiiTA$^{-/-}$ mice. Marilyn Rag2$^{-/-}$ CD4+ anti-IA^{b}-HY TCR transgenic mice (called Marilyn herein) were originally generated by Lantz and colleagues[331] and were originally obtained from the NIAID exchange program. Marilyn-CD45.1, referred to herein as Marilyn WT, were generated by crossing the above Marilyn mice with the above CD45.1 mice. Marilyn-PD-1$^{-/-}$ mice were generated by crossing the above Marilyn mice with PD-1$^{-/-}$ mice. All Marilyn mice used as donors were female. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care.

4.2.2. Cell preparations and adoptive transfer experiments

For experiments involving transfer of thymocytes, recipient NK cells were depleted (to avoid potential NK-mediated killing of the input cells[367-369]) by treatment on days -4, -1, and +2 with 0.3 mg per mouse of anti-NK1.1 (PK136) injected

intraperitoneally. Thymocytes or splenocytes for adoptive transfer or staining were prepared by disruption in HBSS (Gibco) + 2% Fetal bovine serum (FBS, Sigma-Aldrich) through a 70 μm nylon cell strainer into a 50 mL conical tube. Cells were centrifuged at ~300 x g for 5-10 minutes at room temperature and red blood cell lysis was performed by resuspending cells in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) with incubation for 5 minutes, followed by addition of ~10 volumes of HBSS + 2% FBS, centrifugation at ~300 x g for 5-10 minutes at RT, and resuspension in PBS (*in vivo* use or CTV labeling) or HBSS+2% FBS (staining, *in vitro* use). For labeling with Celltrace violet (CTV, ThermoFisher), WT and PD-$1^{-/-}$ polyclonal or Marilyn cells were counted and mixed as required, centrifuged at ~300 x g for 5-10 minutes, and resuspended rapidly in prewarmed (37°C) PBS containing 5 μM CTV such that the final cell concentration was below ~40 x 10^6 cells per mL. Following a 20 minute incubation in a 37°C water bath protected from light, sufficient FBS to achieve a final concentration of 2% FBS was added to the staining reaction and the cells incubated on ice for 5 minutes. Cells were washed twice and resuspended in enriched DMEM (high glucose DMEM + 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 50 μM 2-mercaptoethanol, 10% FBS) and incubated in a 50 mL conical tube with loosened cap in a 37°C, 5% CO_2, humidified incubator for at least 15 minutes. For subsequent *in vivo* use cells were washed once with PBS and resuspended in PBS on ice for immediate intravenous tail vein injection into recipient mice. For *in vitro* restimulation assays, cells were resuspended in E-DMEM at 2 x 10^6 cells/mL and seeded 200 μL per well into a 96 well, round bottom plate. A final concentration of 16 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and

1.4 μM ionomycin (Sigma-Aldrich) were added for 2h, at which time brefeldin A (eBioscience, 3 μg/mL) and monensin (eBioscience, 2μM final) were added for a further 2h prior to surface staining, fixation, intracellular staining, and analysis.

4.2.3. Definition of disease and data analysis

Macroscopic signs of disease in thymocyte recipients included cachexia/weight loss (>15%), kyphosis (hunched appearance), ruffled fur, dermatitis, ocular lesions, and diarrhea. Recipient mice were no longer considered disease free when two or more of the above symptoms were evident, or if mice lost ≥20% body weight. Kaplan-Meier survival curve analysis of disease onset/incidence was performed using Graphpad Prism v5.0 software. Probability values reported for survival curve comparisons were calculated using the Mantel-Cox method. For thymocyte experiments, calculation of weight loss for disease determination was performed relative to weights at d0 or d1 relative to cell transfer. Unless otherwise indicated, animals were used between 6-20 weeks of age. All animals were maintained and experiments were performed in accordance with the guidelines of the animal care and use committee at the University of Alberta.

4.2.4. Antibodies and flow cytometry

For flow cytometric staining, fluorophore-labeled antibodies against the following markers were obtained from eBioscience (San Diego, CA) unless otherwise indicated: CD4 (RM4-5), TCRβ (H57-597), CD8 (53-6.7), PD-1 (J43), CD5 (53-7.3), IL-7Rα/CD127 (A7R34), IFN-γ (XMG1.2), Bcl2 (10C4). Antibodies were used at

manufacturer's recommended concentrations. Flow cytometric staining always used an Fc block cocktail to block nonspecific staining. Fc block cocktail consisted of 3 mL each of normal mouse, rat, and hamster serum, with addition of 0.3 mg of anti-CD16/32 antibody (clone 2.4g2, BioXCell). For surface staining of blood or cells, 20 µL of Fc block cocktail was combined with generally ~30-50 µL of blood or prepared cells in 5 mL FACS tubes (BD), incubated for 5 minutes at RT, and then appropriate fluorochrome-labeled antibodies were added (final volume – 100 µL). Following incubation at RT protected from light to allow for staining (generally ~30 min, depending on antibodies), cells were resuspended in 2 mL ACK buffer (if blood) or HBSS + 2% FBS (for other cells) and centrifuged at RT for 5 minutes at 300 x g. Supernatant was decanted and if no intracellular staining was required, cells were resuspended in 0.5-1 mL HBSS + 2% FBS for analysis. For intracellular staining, cells were fixed and permeabilized using the eBioscience FoxP3 Fixation/permeabilization buffer kit according to supplied protocols, blocked with Fc block cocktail, and stained for intracellular markers as was done for surface staining but performing 2 washes with eBioscience 1X permeabilization buffer instead of HBSS + 2% FBS. After the final wash, cells were resuspended in HBSS + 2% FBS and analyzed on the flow cytometer. Standard flow cytometric analysis was performed using a BD LSR II instrument. Flow cytometric data analysis was performed using FlowJo (Treestar software, Portland, OR). Anti-IL-7Rα treatment *in vivo* was carried out by biweekly intraperitoneal injection of 0.5mg of anti-IL-7Rα clone A7R34 (BioXcell for polyclonal PD-1$^{-/-}$ thymocyte disease model experiments; or generated by Louis Boon for mixed Marilyn experiments) or Rat IgG2a isotype control (2A3, BioXcell or generated by Louis Boon).

4.2.5. Statistical analyses

For statistical analyses we used Graphpad Prism 5.0 software. Unless otherwise noted Student's T test was used for comparisons. In the case of unequal variances, T test was performed using Welch's correction. For comparisons of multiple groups, One way ANOVA with Tukey's multiple comparison test was used.

4.3. Results

4.3.1. Polyclonal PD-1$^{-/-}$ T cells have a competitive advantage over WT T cells in lymphopenic hosts despite similar proliferation kinetics

We have previously demonstrated that transfer of a mixture of Treg-depleted polyclonal WT and PD-1$^{-/-}$ CD4+ T cells purified from the thymocyte or splenocyte population (including CD62L^{hi} selected cells) into lymphopenic Rag$^{-/-}$ hosts resulted in predominance of the PD-1$^{-/-}$ population in the periphery during LIP[374] (Chapter 2). In the same study we also demonstrated that the proportion of WT and PD-1$^{-/-}$ cells could be equalized by treatment of hosts with anti-PD-L1, releasing the WT population from cell-intrinsic PD-1-dependent inhibition. These data indicated that the effect on population size was mediated through the PD-L1:PD-1 interaction. To begin to explore whether PD-1 controls T cell abundance during LIP by controlling tonic TCR signals in response to pMHC and verify our model, we generated the cross between Rag$^{-/-}$ and MHC Class II transactivator deficient (CiiTA$^{-/-}$) mice, which are MHC-II deficient[13]. We transferred

mixed, Celltrace Violet (CTV) proliferation dye-labeled WT and PD-1$^{-/-}$ thymocytes (40 x 10^6) to Rag$^{-/-}$ and CiiTA$^{-/-}$ x Rag$^{-/-}$ recipient animals and monitored their proliferation and abundance in the periphery at days 3, 5 (blood), and 7 (spleen) post-transfer. Within each of the Rag$^{-/-}$ and CiiTA$^{-/-}$ x Rag$^{-/-}$ recipient groups and CD4+ and CD8+ populations, the kinetics of proliferation between WT and PD-1$^{-/-}$ T cells unexpectedly appeared similar as judged by dilution of CTV dye (Figure 4-1 A-C). Deficiency of MHC-II led to a significantly delayed or reduced course of proliferation of CD4+ T cells at all time points analyzed (Fig. 4-1 C top, WT d3, 5, 7; p=0.02, 0.001, 0.03, respectively). There was a parallel trend toward reduced proliferation of CD8+ T cells in MHC-II deficient hosts as well (compare Fig. 4-1 B, d5 top vs bottom and see Fig. 4-1 C, lower panel) suggesting that CD4+ T cells may "help" LIP of CD8+ cells. The ratio of PD-1$^{-/-}$ to WT T cells significantly increased over time post-transfer in the CD8+ T cell populations in both MHC-II sufficient and deficient hosts (Figure 4-1 D). In the CD4+ population, during the period in which the cells appeared to be proliferating in a host MHC-II dependent manner (i.e. ~d5) there was a trend to an increase in the ratio of PD-1$^{-/-}$:WT cells in MHC-II sufficient hosts, while at the same time period in the MHC-II deficient hosts no similar ratio increase was seen. After this period, proliferation that was independent of host MHC-II was detected (Fig. 4-1 A) with a corresponding trend towards an increase in the PD-1$^{-/-}$:WT cell ratio. Comparison of the percentage of all cells within splenocytes at day 7 post-transfer that were CD4+ or CD8+ T cells disclosed that the CD4+ population was significantly less numerous in recipients lacking MHC-II, as expected[219,275,276], while CD8+ T cells were not significantly affected (Fig. 4-1 E). Finally, it is known that LIP of T cells can result in acquisition of a memory phenotype

including the ability to rapidly produce IFN-γ upon *in vitro* restimulation[270,272,296,297]. As we have provided evidence suggesting autoimmune disease following transfer of PD-1$^{-/-}$ HSC or newly generated T cells to lymphopenic hosts is driven via a cytokine-dependent mechanism (Chapter 3), we asked whether PD-1$^{-/-}$ T cells in the mixed thymocyte recipients had increased propensity to produce IFN-γ. Day 7 post-transfer splenocytes from the mixed thymocyte recipients were restimulated *ex vivo* with PMA/ionomycin and IFN-γ expression was measured by flow cytometry (Fig. 4-1 F). No difference in IFN-γ expression was detected between WT and PD-1$^{-/-}$ T cells. Taken together, these data suggest that PD-1$^{-/-}$ cells have a cell-intrinsic competitive advantage over WT T cells during LIP, and that some cells within a polyclonal CD4+ thymocyte population can undergo substantial LIP in the periphery in MHC-II deficient-hosts, with the caveat that some MHC-II sufficient thymic APC may have been co-transferred with the thymocytes and/or there is the possibility of low-level occult MHC-II expression in the CiiTA$^{-/-}$ x Rag$^{-/-}$ hosts, notably in the eye and brain[379] and in some lymph node DC populations[380].

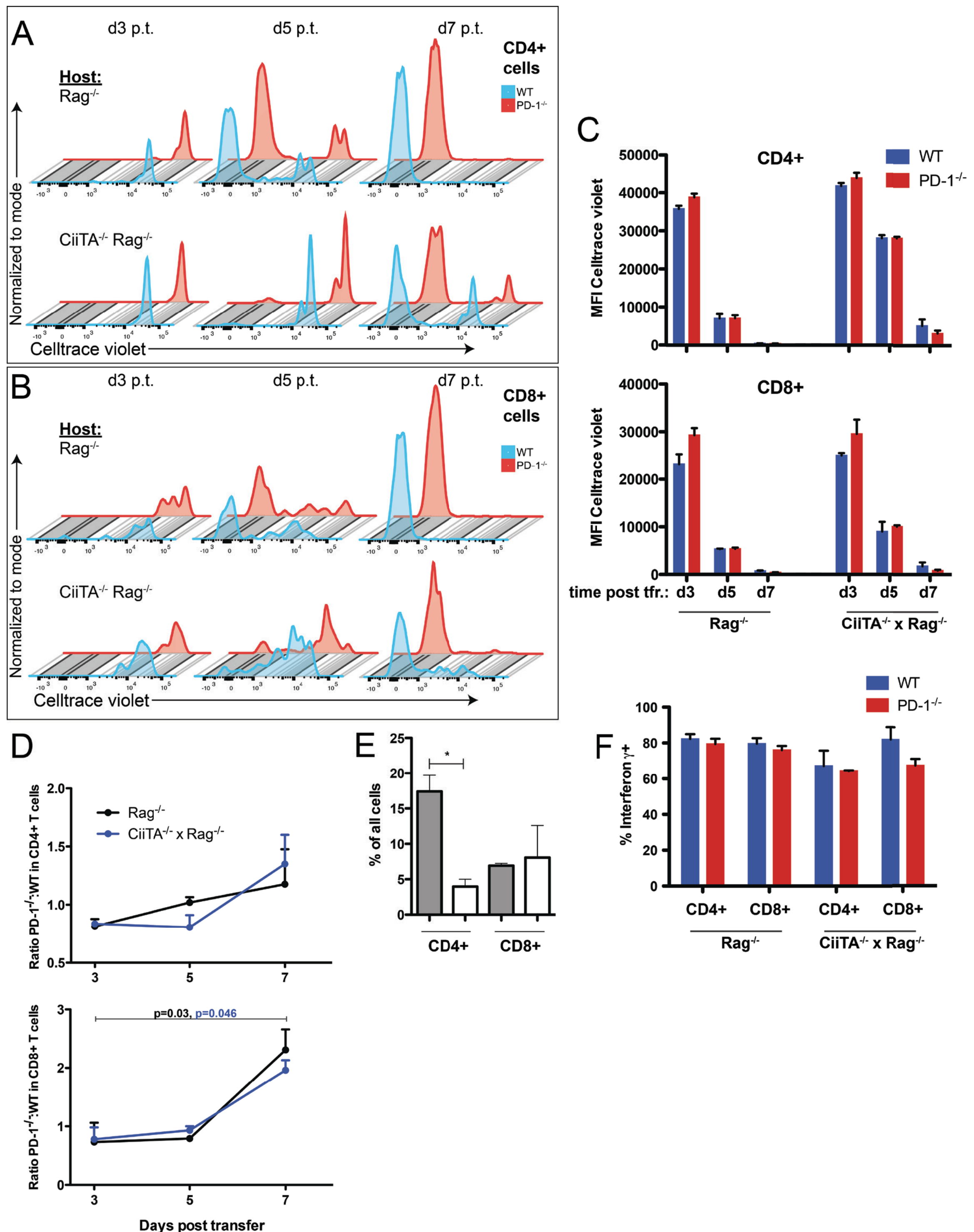


Figure 4-1. Mixed polyclonal thymic WT and PD-1$^{-/-}$ T cells proliferate with similar kinetics but PD-1$^{-/-}$ T cells come to predominate.

40 x 10^6 labeled mixed polyclonal thymocytes from WT or PD-1$^{-/-}$ mice were transferred to Rag$^{-/-}$ (n=3) or CiiTA$^{-/-}$ x Rag$^{-/-}$ (n=2) recipients. A) Representative histograms

comparing *in vivo* proliferation of WT and PD-1$^{-/-}$ CD4+ and B) CD8+ T cells in blood (d3, 5) and spleen (d7). C) Quantitation of proliferation dye mean fluorescence intensity in populations from a) and b). D) PD-1$^{-/-}$:WT cell ratios within CD4+ and CD8+ T cells from the indicated recipients. p values calculated using Student's T test. E) Percent of all cells that are CD4+ or CD8+ in Rag$^{-/-}$ (shaded) or CiiTA$^{-/-}$ x Rag$^{-/-}$ (unshaded) recipients in splenocytes at d7 post-cell transfer. *$p<0.05$ Student's T test. F) Percent of CD4+ and CD8+ T cells expressing IFNγ upon PMA/ionomycin restimulation in splenocytes of recipients at d7 post-cell transfer. Data are from one experiment.

4.3.2. PD-1 controls LIP in response to tonic self pMHC-II independent of IL-7 signals

In order to examine whether PD-1 controls LIP in response to tonic self pMHC signals in the absence of potential responses to cognate antigens by PD-1$^{-/-}$ cells from a polyclonal repertoire, we previously generated the Marilyn male antigen-specific CD4+ TCR transgenic cross with B6-CD45.1 (CD45.1+, "Marilyn-WT") mice as well as B6-PD-1$^{-/-}$ mice (CD45.2+, Marilyn-PD-1$^{-/-}$)[119]. We also generated MHC-II deficient CiiTA$^{-/-}$ x Rag$^{-/-}$ mice. We transferred a mixture (40 x 10^6 cells) of Marilyn-WT and Marilyn-PD-1$^{-/-}$ thymocytes labeled with Celltrace violet proliferation dye into NK-depleted Rag$^{-/-}$ females (only tonic signals possible) or males (systemic cognate HY antigen present) and CiiTA$^{-/-}$ x Rag$^{-/-}$ females or males, with or without biweekly anti-IL-7Rα treatment and monitored their relative proliferation and abundance over time (Fig. 4-2).

By day 3 post-transfer, only barely detectable levels of proliferation had occurred within female Rag$^{-/-}$ recipients with none in the female Rag$^{-/-}$ anti-IL-7Rα treated group (Fig. 4-2 A, B top panel), and no difference between the WT and PD-1$^{-/-}$ cells was apparent nor was there any change in the ratio of PD-1$^{-/-}$ to WT T cells in these recipients relative to the initial seeding ratio, regardless of anti-IL-7Rα treatment (Fig. 4-2 C). In contrast, in the presence of systemic cognate antigen all of the cells within the Rag$^{-/-}$ males; regardless of anti-IL-7Rα treatment, had proliferated extensively and had already almost completely diluted out their CTV dye (Fig. 4-2 A, B). This corresponded with a decreased ratio of PD-1:WT cells at this early time point which was not influenced by IL-7Rα blockade (Fig. 4-2 C). Proliferation had also clearly occurred albeit to a reduced extent within CiiTA$^{-/-}$ x Rag$^{-/-}$ male recipients, which may be due to small numbers of MHC-II sufficient thymic DC that were transferred along with the thymocytes or host non-CiiTA dependent MHC-II expression.

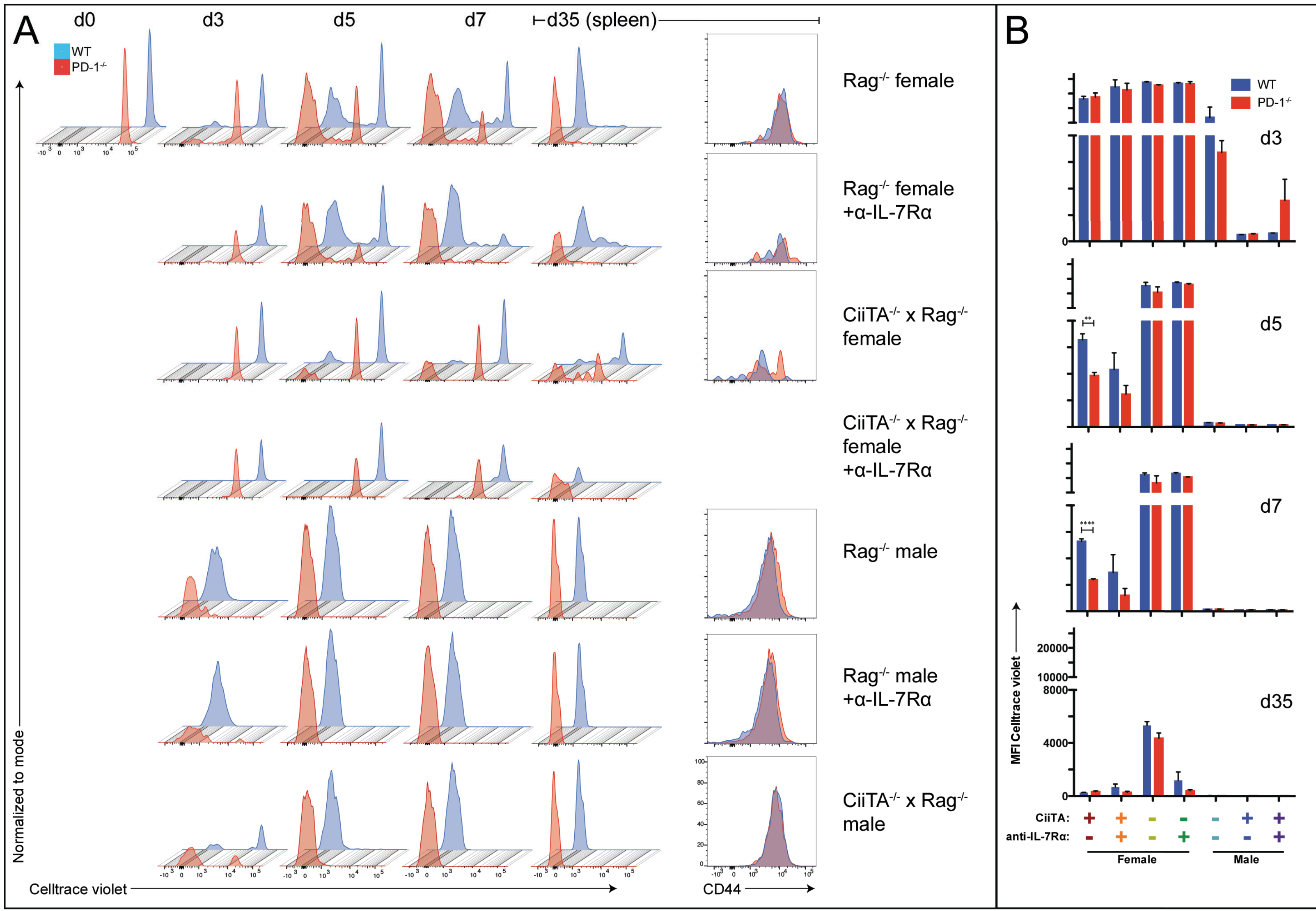


Figure 4-2, page 1

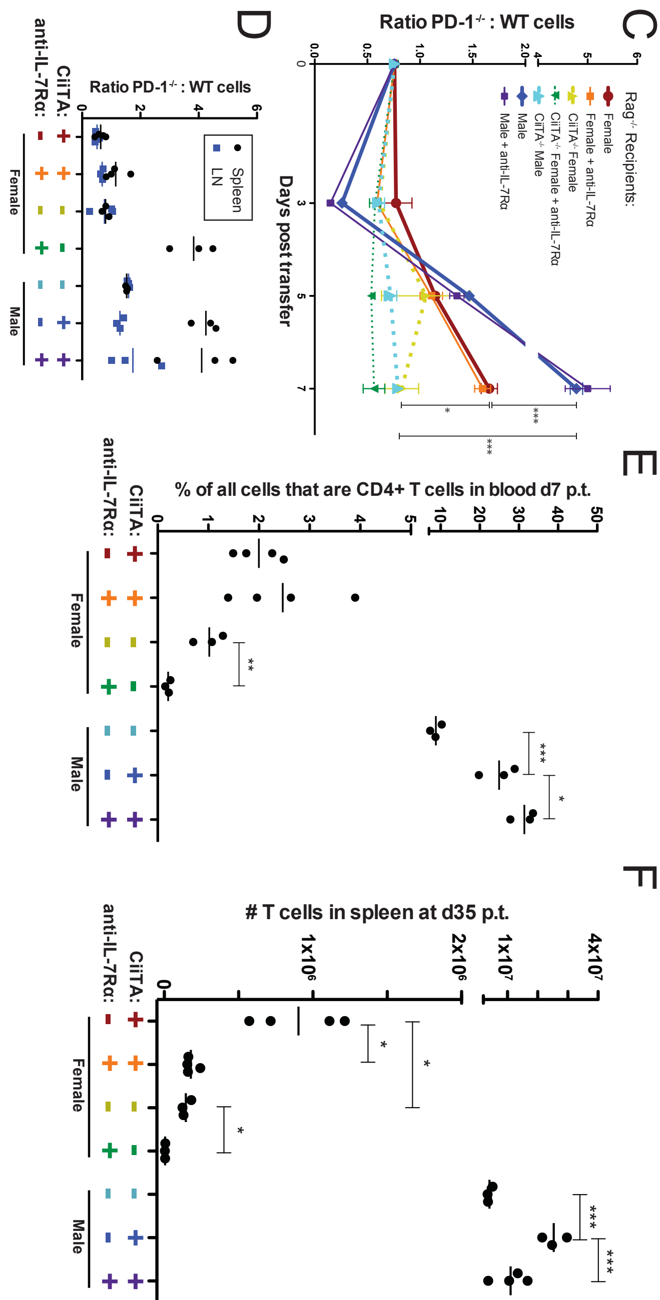


Figure 4-2. Monoclonal CD4+ HY-specific PD-1$^{-/-}$ T cells out-proliferate and out-number WT cells during LIP in response to tonic or cognate pMHC-II but independent of IL-7 signals.

40 x 10^6 Celltrace violet (CTV)-labeled mixed Marilyn WT (CD45.1+) and PD-1$^{-/-}$ (CD45.2+) thymocytes were transferred i.v. to Rag$^{-/-}$ or CiiTA$^{-/-}$ x Rag$^{-/-}$ male or female

hosts with or without anti-IL-7Rα treatment. A) Representative flow cytometry histograms of CTV in TCRβ+CD4+ WT or PD-1$^{-/-}$ cells from blood collected at 3, 5, and 7 days and spleen at 35 days post-transfer (p.t.) and overlaid histograms of CD44 expression in WT or PD-1$^{-/-}$ T cells in the spleen at d35 p.t. B) Mean fluorescence intensity of CTV in WT and PD-1$^{-/-}$ CD4+ T cell populations of the indicated recipient groups from a), ± SEM. **p<0.01, ****p<0.0001, Student's T test. C) Ratio of PD-1$^{-/-}$ to WT CD4+ T cells in blood among indicated recipients at d3-7 p.t. ± SEM. Broken lines and thin lines depict CiiTA$^{-/-}$ and anti-IL-7Rα treated hosts, respectively. Colors correspond to recipient legends in b) and d)-f). D) Ratios of PD-1$^{-/-}$ to WT CD4+ T cells in spleen and inguinal LN at d35 post-transfer. LN in female CiiTA$^{-/-}$ + anti-IL-7Rα group had too few cells to calculate. E) Comparison of the % of all cells that were CD4+ T cells in blood of indicated recipient groups at d7 p.t. *p<0.05, ***p<0.001, One way ANOVA with Tukey's multiple comparison test. **p<0.01, Student's T test. F) Absolute numbers of CD4+ T cells in spleen of recipient mice at d35 p.t. *p<0.05, Student's T test. ***p<0.001 One way ANOVA with Tukey's multiple comparison test. Data presented from non-anti-IL-7Rα treated female Rag$^{-/-}$ and CiiTA$^{-/-}$ x Rag$^{-/-}$ recipients are from one experiment but representative of two independent experiments. Male recipient and anti-IL-7Rα treatment data are from one experiment.

By day 5, PD-1$^{-/-}$ T cells had undergone significantly more proliferation than WT cells in Rag$^{-/-}$ female recipients (Fig. 4-2 A, B) and the ratio of PD-1$^{-/-}$:WT cells started to increase (Fig. 4-2 C). Furthermore, in these recipients we detected up-regulation of PD-1 expression within the WT cell population at this time point among the extensively proliferated cells (Fig. 4-3 A). Although PD-1$^{-/-}$ cells were proliferating more and coming to predominate by d5, Bcl2 expression was slightly reduced in PD-1$^{-/-}$ cells compared to WT cells and in general Bcl2 appeared to be depressed in response to anti-IL-7Rα treatment. However, IL-7Rα blockade together with a lack of MHC-II lead to the strongest decrease in Bcl2 expression in both WT and PD-1$^{-/-}$ cells (Fig. 4-3 E). We also noted that CD5, commonly used as a marker of TCR affinity the expression level of which is modulated during selection in the thymus, changed in its expression considerably during LIP in response to tonic pMHC signals. Upon extensive proliferation CD5 became down-regulated on a substantial proportion of WT cells, while instead PD-1$^{-/-}$ cells had less down-regulation of CD5 and even a portion of cells expressing more CD5 (Fig. 4-3 B).

By day 7 post-cell transfer, the majority of cells within the Rag$^{-/-}$ female recipients had proliferated extensively and diluted their CTV labeling beyond detection limits, although more proliferation had occurred in the PD-1$^{-/-}$ cell population in these recipients as judged by MFI comparison which was statistically significant (Fig. 4-2 A, B). Furthermore, the ratio of PD-1$^{-/-}$:WT cells in these animals increased approximately 2-fold relative to the input cell ratio which was significantly different from the CiiTA$^{-/-}$ x Rag$^{-/-}$ recipients (Fig. 4-2 C) in which the PD-1:WT ratio did not change and also in which the majority of the T cell population still had not proliferated (Fig. 4-2 A, B).

Importantly this alteration in ratio was not affected by anti-IL-7Rα treatment of the hosts. We also performed a similar experiment using transfer of highly purified mixed Marilyn WT and PD-1$^{-/-}$ T cells sourced from the spleen, where we saw an even more dramatic MHC-II-dependent increase in the ratio of PD-1$^{-/-}$:WT T cells (Appendix 4). No difference in the proportion of WT vs. PD-1$^{-/-}$ T cells producing IFN-γ in response to *in vitro* restimulation was seen at the d7 time point in the MHC-II sufficient female hosts, and in the absence MHC-II it was essentially undetectable (Fig. 4-3 C). In the presence of HY antigen in male Rag$^{-/-}$ recipients, by d7 the ratio of PD-1$^{-/-}$:WT cells had increased drastically ~6.3 fold relative to the input cell proportions, and this was also not affected by anti-IL-7Rα treatment (Fig. 4-2 C). Interestingly, the ratio of PD-1$^{-/-}$:WT cells did not change by d7 in CiiTA$^{-/-}$ x Rag$^{-/-}$ male recipients despite relatively robust expansion of input populations in these recipients. During this period of LIP at d7, examination of the percentage of all cells that were TCRβ+CD4+ in blood of the recipients by flow cytometry demonstrated that the size of the T cell compartment in Rag$^{-/-}$ female recipients was not significantly affected by anti-IL-7Rα treatment (Fig. 4-2 E). In contrast, in the absence of tonic pMHC in the female CiiTA x Rag$^{-/-}$ recipients, blockade of anti-IL-7Rα significantly decreased T cell compartment size. Examination of the CTV dilution histograms suggested that a major effect of anti-IL-7Rα treatment in the MHC-II sufficient female Rag$^{-/-}$ recipients was to decrease the size of the unproliferated cell population (Fig. 4-2 A). Male Rag$^{-/-}$ recipients had a much larger T cell compartment compared to females, although again the absence of MHC-II in male recipients led to a significant reduction in the total T cell numbers (Fig. 4-2 E). Unexpectedly, anti-IL-7Rα

treatment was associated with a small increase in T cell compartment size at this time point in male $Rag^{-/-}$ recipients.

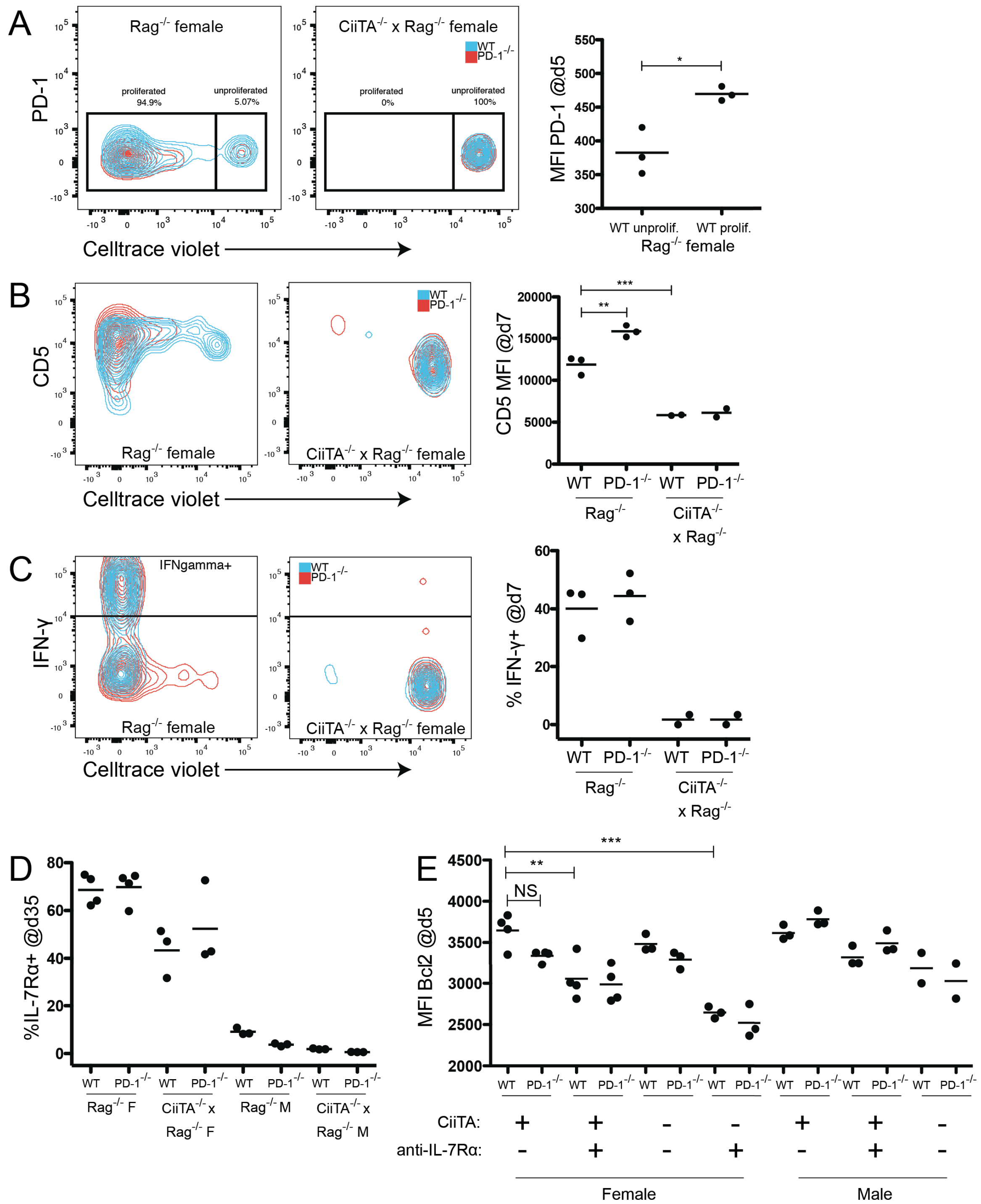


Figure 4-3. Expression of PD-1, cytokines, and survival-associated molecules by mixed Marilyn WT and PD-1$^{-/-}$ T cells undergoing LIP

A) (Left and center panels) Representative flow cytometry overlay contour plots showing PD-1 expression vs. Celltrace violet staining within the indicated recipients of 40 x 10^6 mixed Marilyn-WT and PD-1$^{-/-}$ thymocytes at day 5 post-cell transfer (p.t.) in blood.

(Right panel) mean fluorescence intensity of PD-1 staining within the WT unproliferated or proliferated cells. *$p<0.05$, Student's T test. B) (Left and center panels) Representative flow cytometry overlay contour plots of CD5 expression vs Celltrace violet staining in indicated mixed Marilyn WT and PD-1$^{-/-}$ cell recipients at d7 p.t. in splenocytes. (Right panel) mean fluorescence intensity of CD5 staining within WT and PD-1$^{-/-}$ populations in the indicated recipients. **$p<0.01$, ***$p<0.001$, One-way ANOVA with Tukey's multiple comparison test. C) (Left and center panels) Representative flow cytometry overlay contour plots of IFN-γ expression vs Celltrace violet staining in indicated recipients at d7 p.t. in splenocytes. (Right panel) % IFN-γ+ cells within WT and PD-1$^{-/-}$ T cell populations in the indicated recipients. D) Percent IL-7Rα+ cells within the WT and PD-1$^{-/-}$ CD4+ T cell populations in the indicated recipients' splenocytes at d35 p.t. E) Mean fluorescence intensity of Bcl2 staining in WT and PD-1$^{-/-}$ CD4+ T cell populations in the blood at d5 p.t. within the indicated recipients. **$p<0.01$, One way ANOVA with Tukey's Multiple comparison test. Data in this figure are from two independent experiments.

At the later time point of 35 days post-transfer, in the splenocyte population CTV labeling was virtually undetectable in all cells within the $Rag^{-/-}$ female recipients (Fig. 4-2 A, B). The mean fluorescence intensity of CD44 staining did not significantly vary between WT and PD-$1^{-/-}$ cells within any of the recipient groups. We did however note a statistically significant decrease in CD44 expression between cells in untreated vs. anti-IL-7Rα treated $Rag^{-/-}$ female hosts (WT cells vs. WT cells, $p<0.01$) as well as the anti-IL-7Rα treated $Rag^{-/-}$ female hosts vs. $CiiTA^{-/-}$ x $Rag^{-/-}$ female hosts (WT vs WT, $p<0.001$) (Fig. 4-2 A). In MHC-II deficient female hosts not treated with anti-IL-7Rα, although most cells appeared to have undergone proliferation by d35 a significant proportion remained undivided. In contrast, the few cells detected within anti-IL-7Rα-treated MHC-II deficient females were almost completely proliferated cells, consistent with the previously noted decrease in the undivided cell population upon IL-7Rα blockade. Examination of the ratios of PD-1:WT cells in the spleen showed that in $Rag^{-/-}$ female hosts the ratio had decreased to a level similar to the original input ratio (0.64:1 at d35 vs. 0.76:1), while those receiving IL-7Rα blockade maintained a slight elevation of PD-$1^{-/-}$ relative to WT cells (1.15:1, Fig. 4-2 D). This did not appear to correlate with differential expression of IL-7Rα between WT and PD-$1^{-/-}$ cells at this time point (Fig. 4-3 D). By d35 the ratio of PD-$1^{-/-}$:WT cells in MHC-II deficient female hosts had not changed appreciably from d7 (Fig. 4-2 D), while very few cells overall were detectable in the spleen of MHC-II deficient anti-IL-7Rα-treated females (see Fig. 4-2 F). Furthermore the proportion of T cells that were PD-$1^{-/-}$ in the inguinal lymph node (LN) versus the spleen was similar in all female recipients where sufficient cell numbers were detectable to facilitate comparison. In male MHC-II sufficient recipients, the ratio of PD-1:WT

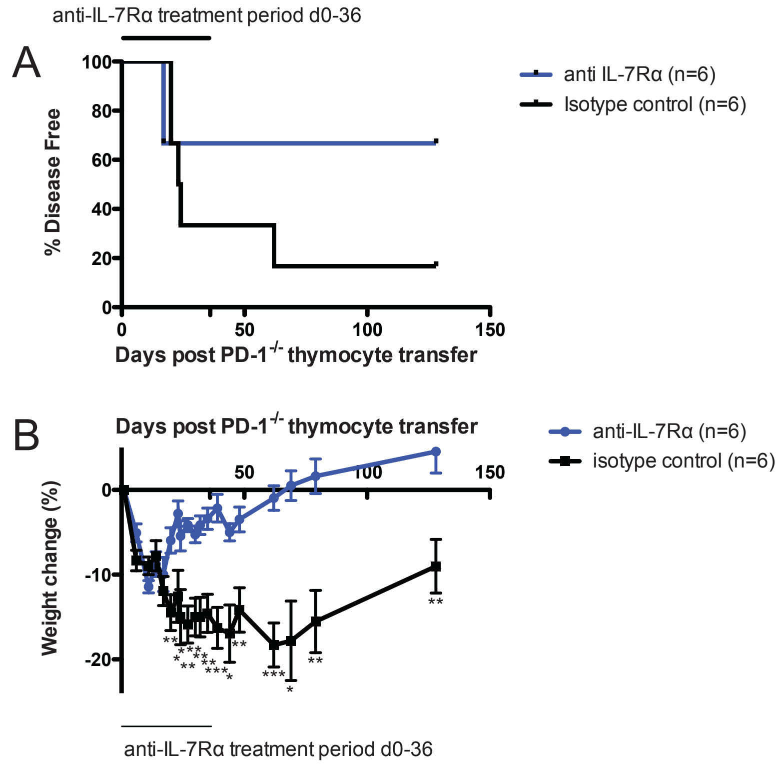
cells remained high at d35 in the spleen regardless of anti-IL-7Rα treatment but we noted that in the inguinal LN the ratio of PD-1 to WT cells was much lower (Fig. 4-2 D). In male MHC-II deficient recipients, the ratio of PD-$1^{-/-}$:WT cells had increased to 1.53:1 by d35 compared to 0.79:1 at d7 and in contrast to the male MHC-II sufficient hosts the PD-$1^{-/-}$:WT T cell ratios were similar in spleen vs. LN. In contrast to the lack of an effect of IL-7Rα blockade on T cell survival in MHC-II sufficient hosts seen during LIP at the day 7 time point (Fig. 4-2 E), at d35 in the spleen both MHC-II sufficient female and male recipients receiving anti-IL-7Rα treatment had markedly reduced T cell numbers compared to their untreated counterparts (Fig. 4-2 F) suggesting that during rapid LIP IL-7 was dispensable but at this late time point when the T cell compartment was more replete IL-7 signals were important for survival of the T cell population. Together, these data indicate that PD-1 controls TCR signals in response to tonic pMHC-II as well as cognate pMHC-II in a cell-intrinsic manner, which is independent of effects on IL-7 signaling. These data also suggest that IL-7 signals are not required for LIP in a lymphopenic host but do promote T cell survival as the T cell compartment becomes repopulated and competition for available pMHC resources increases.

4.3.3. Blockade of IL-7Rα ameliorates weight loss post-transfer of high- but not low-dose PD-$1^{-/-}$ thymocytes to $Rag^{-/-}$ recipients.

TCR signals can downregulate IL-7Rα expression and have been reported to block, in an affinity-dependent manner, the response to IL-7 mediated survival signals[265,381]. Because we noted that IL-7Rα blockade had a marked effect on the size of the T cell compartment 5 weeks post-mixed Marilyn T cell transfer (Fig. 4-2 F) but not

after only 7 days (Fig. 4-2 E), we reasoned that IL-7 signals became more important to T cell survival as competition for available pMHC became more intense (i.e. as the T cell compartment became more replete, and fewer cells could access pMHC and receive a TCR signal). In order to explore this concept and examine whether constraints on LIP potential in high vs. low competition settings would influence the development of autoimmunity, we used our model of disease following transfer of polyclonal PD-1$^{-/-}$ thymocytes to Rag$^{-/-}$ animals, which results most commonly in weight loss, kyphosis, diarrhea, dermatitis, and ocular lesions. We transferred either a low dose (10 x 10^6) or high dose (30 x 10^6) of PD-1$^{-/-}$ thymocytes to NK depleted recipient animals (to maximize cell survival as even syngeneic cells can be targets of NK cell killing[367-369]). The recipients were thereafter treated bi-weekly with monoclonal anti-IL-7Rα or isotype control antibodies and monitored for weight changes and disease symptoms. Approximately 80% of the isotype control treated animals in both high and low-dose thymocyte experiments developed at least two symptoms of autoimmunity (Figures 4-4 A, C). Anti-IL-7Rα treatment in both experiments showed a trend toward reducing the incidence of autoimmunity although comparison of the survival curves demonstrated no statistically significant difference between the groups. However, using the more precise quantitative measure of weight change, we did note a striking effect of anti-IL-7Rα treatment on loss of weight in the high-dose thymocyte recipient group, which was statistically significant from d20 through to the termination of the experiment even though anti-IL-7Rα treatment was withdrawn at d36 (Fig. 4-4 B). On the other hand, anti-IL-7Rα treatment of the low-dose thymocyte recipient group did not have any significant effect on the loss of weight (Fig. 4-4 D). Taken together, these data indicate

that limitation of LIP potential by inhibiting the ability of T cells to respond to IL-7-mediated signals in situations of high but not low competition for finite pMHC can inhibit the loss of weight associated with the pathology of LIP-driven autoimmunity.



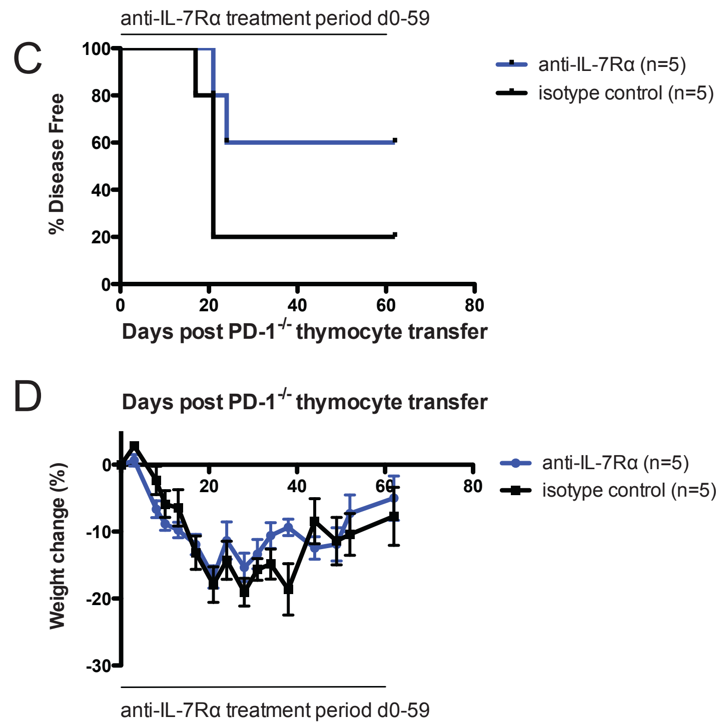


Figure 4-4. IL-7Rα blockade ameliorates high- but not low-dose PD-1$^{-/-}$ thymocyte-mediated weight loss in lymphopenic recipients.

Either (A, B) high dose (30 x 10^6 total cells) or (C, D) low dose (10 x 10^6 total cells) PD-$1^{-/-}$ thymocytes were transferred to NK-depleted $Rag^{-/-}$ recipient animals with i.p. injection of 0.5 mg per mouse anti-IL-7Rα or isotype control twice weekly for the time periods indicated. Mice were monitored for disease symptoms and weight loss. A) Kaplan-Meier survival curve analysis of disease incidence in high dose thymocyte recipients. Differences between disease curves did not reach statistical significance. B) Weight change in high dose thymocyte recipients over the course of the experiment. *p<0.05, **p<0.01, ***p<0.001, Student's T test. C) Kaplan-Meier survival curve analysis of disease incidence in low dose thymocyte recipients. Differences between disease curves did not reach statistical significance. D) Weight change in low dose thymocyte recipients over the course of the experiment.

4.4. Discussion

The potential for LIP in a host depends on the resources available for T cells (tonic or cognate pMHC, homeostatic cytokines) relative to the level of competition for those resources[216]. Importantly, LIP can drive inflammatory and autoimmune disease[186,214,298,303,309-316,373], and thus modulation of LIP can increase or decrease its potential to result in pathologic T cell activation. Several molecules with co-inhibitory function such as BTLA, LAG3, and TGFBRII have been shown to modulate T cell homeostasis and LIP[298-300,360], although to our knowledge none have demonstrated that the control of LIP *in vivo* is via modulating the TCR signal in response to pMHC. Recently we described a severe systemic autoimmune disease mediated by newly

generated PD-1$^{-/-}$ T cells in lymphopenic animals which could be blocked by the addition of competitors or reducing lymph node stroma[214], and which was not due to a deficiency in the generation or function of PD-1$^{-/-}$ Treg[374], suggesting that the co-inhibitory molecule PD-1 also plays an important role in the establishment of immune tolerance through control of LIP. This gave rise to our hypothesis that the disease in lymphopenic recipients of PD-1$^{-/-}$ newly generated T cells is mediated by inappropriate activation of T cells in response to normally tonic self pMHC in the context of LIP. However, the possibility exists that the T cell response in our disease model is directed toward tissue restricted antigens that were not appropriately negatively selected against in the thymus in the context of PD-1 deficiency, or due to altered population dynamics because of changes in positive selection[382]. Furthermore, it is unclear whether the relatively low-affinity interactions with tonic pMHC can promote up-regulation of PD-1 on T cells for it to mediate its inhibitory function. To address this and the question of whether PD-1 can, in a cell-intrinsic fashion, modulate TCR signals in response to tonic pMHC, herein we used the Marilyn CD4+ TCR transgenic system[331] in which T cells bear a TCR specific for the male antigen HY in the context of I-A^b and thus in female B6-Rag$^{-/-}$ recipients only tonic pMHC-II is available to mediate their LIP-driven expansion. We used Marilyn x PD-1$^{-/-}$ mice and a competitive *in vivo* assay with mixed Marilyn-PD-1$^{-/-}$ (CD45.2) and Marilyn WT (CD45.1+) thymocytes for this examination. We also generated CiiTA$^{-/-}$ x Rag$^{-/-}$ hosts, which are deficient in MHC-II expression, to explicitly test the role of pMHC *in vivo* in PD-1's control of LIP, and used IL-7Rα blockade to interrogate potential effects of PD-1 deficiency on IL-7 signaling *in vivo*. We found that during LIP in female hosts, at least up to 7 days post transfer, PD-1$^{-/-}$ Marilyn T cells showed greater

proliferation (Fig. 4-2 A, B) and came to significantly outnumber the Marilyn WT cells by ~1.7 fold and this was not affected by IL-7Rα blockade but was completely dependent on host MHC-II expression (Fig. 4-2 C). When we examined the expression of the anti-apoptotic molecule Bcl2 at 5 days post-cell transfer (a time during which rapid LIP was occurring), the PD-1$^{-/-}$ cells in female Rag$^{-/-}$ hosts showed a trend toward expressing less of this molecule (Fig. 4-3 E). Although we did not examine other IL-7 / TCR signaling-associated anti-apoptotic molecules such as Bcl-Xl and Mcl-1[383], this suggests that any potential increased survival in the PD-1$^{-/-}$ population was not dependent on Bcl2. Importantly, examination of Bcl2 expression in T cells within all the recipient groups clearly indicated that Bcl2 expression was depressed in groups treated with anti-IL-7Rα and particularly in combination with pMHC deprivation in the CIITA deficient hosts (Fig. 4-3 E). We also noted that at day 7, at which time cells were presumably still undergoing substantial LIP, IL-7Rα blockade did not negatively impact T cell survival in MHC-II sufficient hosts (Fig. 4-2 E). It is important to note that in contrast to studies that suggested that IL-7 signals were critical for the LIP of T cells[245,246], anti-IL-7R treatment did not significantly inhibit proliferation in MHC-II sufficient hosts during the first week after thymocyte transfer when competition for pMHC was presumably low (Fig. 4-2 B). The failure of T cells to undergo LIP in IL-7$^{-/-}$ x Rag$^{-/-}$ hosts[245,246] is likely attributable to defects in lymph node structure and function as IL-7 is important for lymph node development[31].

Despite its now clear role in controlling LIP in response to tonic pMHC-II signals, it was unclear whether PD-1 would be up-regulated during LIP stably and sufficiently such that it would be detectable by flow cytometric staining. Indeed at 5 d post-transfer

PD-1 expression was moderately increased in the WT T cell population but only among the highly proliferated cells (Fig. 4-3 A). The lack of obvious PD-1 expression on non- or intermediately-proliferated cells (i.e. mean fluorescence intensities >~10^3, Fig. 4-3 A) might suggest that regulation of LIP (and even perhaps normal primary T cell activation in a lymphoreplete host) by PD-1 may not require its high-level surface expression on T cells, or might occur primarily after many rounds of division in the highly proliferated cells.

CD5 is a negative TCR signaling regulator commonly considered as a marker of TCR affinity set during thymic T cell selection processes[38,384]. CD5 expression has been correlated with the propensity for T cells from various TCR transgenic backgrounds to undergo LIP in a lymphopenic host[236,286-288]. We examined CD5 expression at d7 post transfer in female hosts with or without MHC-II expression, and found that highly proliferated cells in both the WT and PD-$1^{-/-}$ Marilyn T cell populations contained subpopulations that had either up- or down-regulated CD5 expression – with significantly higher overall CD5 expression in the PD-$1^{-/-}$ group based on mean fluorescence intensity of staining (Fig. 4-3 B). Expression of CD5 was identical between WT and PD-$1^{-/-}$ cells within CiiTA$^{-/-}$ hosts. One could speculate that the PD-$1^{-/-}$ T cells are under pressure to maintain or increase CD5 expression due to a lack of co-inhibition from PD-1. However, pragmatically speaking these findings suggest that CD5 expression can change considerably in a monoclonal T cell population during LIP, which raises important questions about the validity of its use as a marker of T cell affinity for thymic pMHC.

Compared to its effects on LIP in response to tonic pMHC signals, the effect of PD-1 deficiency on LIP of Marilyn T cells was even more pronounced in response to

cognate pMHC in male Rag$^{-/-}$ hosts, with a PD-1$^{-/-}$:WT T cell ratio of ~5:1 at d7 post transfer. Similar to female Rag$^{-/-}$ hosts, there was no effect of anti-IL-7Rα on the establishment of this elevated PD-1$^{-/-}$:WT ratio. In CiiTA$^{-/-}$ hosts, APC within the eye and brain can use a CiiTA-independent pathway to express MHC-II in response to IFN-γ and TNF-α[379], and it is possible that low numbers of MHC-II sufficient thymic APC co-transferred with the Marilyn thymocytes. While proliferation occurred in male CiiTA$^{-/-}$ x Rag$^{-/-}$ hosts, there was a much more gradual increase in PD-1$^{-/-}$:WT cell ratio over time, with a ~2 fold increase relative to the input ratios by d35 (Fig. 4-2 D). This may reflect infrequent contact of these cells with sparse APC in these hosts that were capable of presenting antigen. Such occult MHC-II expression or transfer of low numbers of thymic DC might also explain the expansion of significant numbers of CD4+ T cells seen in the mixed WT and PD-1$^{-/-}$ polyclonal thymocyte transfer experiment (Fig. 4-1), although the CD4+ T cell proliferation seen here in CiiTA$^{-/-}$ hosts may also reflect LIP of CD1d-restricted NKT cell populations.

In contrast to the female Rag$^{-/-}$ hosts we did note that at the late time point of d35 post-transfer, the PD-1$^{-/-}$:WT T cell ratio in male Rag$^{-/-}$ recipients remained elevated, while in female Rag$^{-/-}$ hosts it had returned to a level similar to the input cell mixture (Fig. 4-2 D). Furthermore, at this time point, unlike what was seen at d7 post-transfer, treatment of all recipient animals with anti-IL-7Rα lead to robust and significant decreases in the overall size of the T cell compartment (Fig. 4-2 F). Rag$^{-/-}$ females at d35 contained approximately 1 x 10^6 total splenic CD4+ T cells, and we typically yield ~3-5 x 10^6 CD4+ T cells from an adult Marilyn x PD-1$^{-/-}$ spleen. Together this may suggest that at d35, the recipients had become largely lymphoreplete and due to competition, tonic

pMHC signals were no longer the most significant source for survival signals for the T cells. Thus we speculated that the return of the ratio of PD-1$^{-/-}$:WT cells in female Rag$^{-/-}$ hosts at d35 back to the original input ratio might be due to lower IL-7Rα expression or a muted signaling response to IL-7 within the PD-1$^{-/-}$ T cells due to their prior experience of increased TCR signals relative to WT in response to pMHC. This might also help explain why the PD-1$^{-/-}$:WT ratio remained slightly elevated in female Rag$^{-/-}$ hosts treated with anti-IL-7Rα: in this circumstance, PD-1$^{-/-}$ cells may have been better able to compete for limited pMHC signals and thus avoid dying like WT cells in the absence of IL-7 signals. However, examination of IL-7Rα expression in T cells within the recipients at d35 did not reveal any significant differences in the proportion of T cells that were IL-7Rα+ between WT and PD-1$^{-/-}$ groups, with the caveat that the cells analyzed in this experiment were, necessarily, selected for survival (Fig. 4-3 D). We did note that compared to female hosts, the IL-7Rα-expressing T cell populations in male recipients were far less numerous, which is consistent with these cells constantly receiving strong TCR signals from high-affinity cognate pMHC interactions and downregulating IL-7Rα. The lack of greater attrition of the much larger PD-1$^{-/-}$ T cell population by d35 in the Rag$^{-/-}$ male recipients might be related to the strength of these TCR signals. Indeed, the much larger size of the T cell compartment within male vs female hosts (Fig. 4-2 F) stems from the presence of pMHC that can provide a higher affinity signal (ie: cognate pMHC) and could be viewed as a richer source of resources. Thus a HY containing host was able to support a much larger population of T cells.

Given our interpretation that as the T cell compartment became more replete, IL-7Rα signals became more important for dictating survival and the size of the T cell

compartment, we hypothesized that IL-7Rα blockade might prove effective at inhibiting LIP-driven autoimmunity following transfer of PD-1$^{-/-}$ polyclonal thymocytes to Rag$^{-/-}$ recipient animals, and that this might depend on the level of competition for pMHC between T cells during LIP. In recipients of both high (30 x 10^6) and low (10 x 10^6) doses of PD-1$^{-/-}$ thymocytes, blockade of IL-7Rα had the modest but statistically insignificant effect of reducing overt symptoms of autoimmunity (Fig. 4-4 A, C). However, a striking difference was seen in terms of autoimmunity-associated loss of weight. While IL-7Rα blockade had a robust effect on preventing and reversing weight loss beginning ~d 20 post-cell transfer in the high-dose thymocyte recipient group (Fig. 4-4 B), no such effect was seen in the low-dose thymocyte recipient group and weight changes were virtually identical between the recipients (Fig. 4-4 D). IL-7 has previously been associated with the promotion of IL-3 and GM-CSF expression in human T cells[385]. Very recently it was reported that IL-7 could promote the development of a unique subset of GM-CSF and IL-3-producing T cells ("Th-GM") in mice and this was associated with encephalitogenicity in the experimental autoimmune encephalomyelitis model[386]. Based on these reports, we considered that the effects of IL-7Rα blockade might be attributable to inhibition of GM-CSF expression and therefore we explored this *in vivo* via administration of anti-GM-CSF or isotype control antibody to lymphopenic recipients of PD-1$^{-/-}$ HSC. We found a small, statistically insignificant effect of anti-GM-CSF blockade in this experiment on disease incidence with no significant effect on weight loss (Appendix 5). These data suggest that the effect of blockade of IL-7Rα is unlikely to be mediated through effects on GM-CSF. Our findings support the concept that in situations of high competition for pMHC, limiting LIP potential by blocking the response to

homeostatic cytokines like IL-7 can help to ameliorate the wasting aspect of systemic autoimmunity.

Our finding that the co-inhibitory molecule PD-1 controls LIP by modulating the response to tonic self pMHC signals *in vivo* lends support to our hypothesis that the disease in lymphopenic recipients of PD-1$^{-/-}$ HSC or newly generated T cells results from exaggerated responses to normally tonic self pMHC signals. Furthermore, the effects of PD-1 deficiency on control of LIP were independent of IL-7Rα mediated signaling, and IL-7-mediated signals were largely irrelevant to the size of the overall T cell compartment while LIP potential was still high and rapid LIP in response to abundant pMHC was occurring within the hosts. These data suggest that in LIP-mediated inflammatory disorders, such as immune reconstitution inflammatory syndrome in HIV patients[313,320], therapies aimed at reducing TCR signaling during early phases of reconstitution may be more effective and should take priority over approaches that aim to limit homeostatic cytokine-mediated signals to T cells.

Chapter 5

Discussion and Future Directions

5.1. General remarks on T cell homeostatic mechanisms and potentiation of T cell responses by LIP

Overall, the same mechanisms that control T cell activation in a lymphoreplete state, namely the strength and availability of the TCR:pMHC interaction, co-stimulation/co-inhibition, and cell extrinsic regulation by Treg, also appear to control the process of LIP and homeostasis of T cells. Importantly, among the set of co-inhibitors known to control LIP, we have shown that at least PD-1 can do so by regulating the response to low affinity "tonic" pMHC ligands or high affinity cognate pMHC, independent of IL-7 signals (Chapter 4). Furthermore, PD-1 was not required for the generation of thymic or peripheral Treg during LIP (Chapter 2). We have since asked the question whether absence of another co-inhibitor, BTLA, could affect pTreg generation during LIP and found that it does not (Appendix 6). Clearly, LIP is strongly associated with the promotion of T cell activation and promotion of inflammatory disease. In cases where peripheral tolerance mechanisms are defective and which would normally be associated with some low level of autoimmune pathology (e.g.: PD-1 deficiency), it is becoming clear that the process of LIP can magnify these autoimmune effects and, such as in our PD-1$^{-/-}$ HSC transfer model (see overview in Figure 5-1), make the difference between a mild pathology and a devastating systemic autoimmune disease[214]. These findings suggest an abundance of caution in certain clinical settings involving recovery of the lymphoid compartment from a lymphopenic state such as bone marrow transplantation, particularly in the presence of underlying genetic tolerance defects or simultaneous therapies to block co-inhibitory molecules. Strategic manipulation of factors that regulate LIP holds the promise to limit or avoid autoimmune pathology in such situations. For example, we have demonstrated that provision of polyclonal Treg to lymphopenic PD-1$^{-/-}$

HSC recipients can inhibit disease[374] (Appendix 7), which, given their known functional mechanisms discussed in chapter 1, could be attributed to them acting as "super competitors" for the pMHC that mediate disease or through reducing co-stimulation. On the other hand, while blockade of single or multiple co-inhibitory molecules has brought about a renaissance in cancer immunotherapy in recent years, the path forward to increasing the efficacy of these treatments may lie in harnessing the potential of LIP to enhance T cell responses or expand intratumoural neoantigen specific T cells in conjunction with reduced co-inhibition. I propose that a more quantitative way of viewing lymphopenia based on a mathematical description of available T cell space or "LIP-potential", which is a concept applicable at the level of the host or sub-compartments within, may serve to guide such approaches. In the following sections, I will first discuss an important gap in our knowledge regarding the function of Treg. These cells are of obvious interest for use in controlling LIP (as noted above) and blocking autoimmunity, or as targets for elimination to promote anti-tumour immunity. Yet, with our current understanding, it is unclear how one of the most important mechanisms by which they appear to function, namely the modulation of co-stimulatory molecules on APC, does not lead to systemic immune suppression. Then, a mathematical tool for estimating "LIP potential" will be defined and subsequently used to outline some unanswered questions, potential mechanisms, and future directions for research in our LIP-driven autoimmunity model as well as some potential approaches to control autoimmunity or anti-tumoural immune responses through modulation of T cell homeostasis.

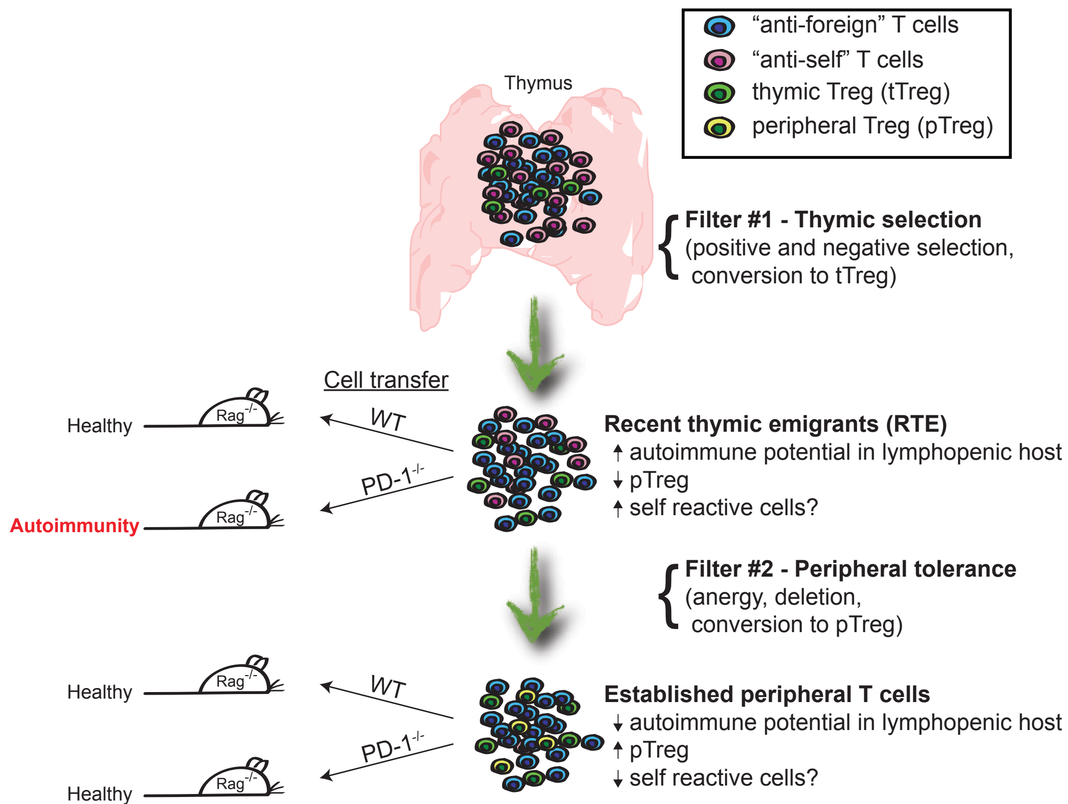


Figure 5-1. Overview of tolerance mechanisms acting on the T cell repertoire in the thymus and periphery with characteristics of corresponding stages of T cell maturation. Early T cell development gives rise to precursors with anti-self and anti-foreign potential. Thymic positive selection ensures that developing T cells can interact at least weakly with self peptide:MHC complexes, while negative selection removes most (but not all) of the cells with high affinity for self from the developing repertoire. Stronger self-reactivity in T cells in the thymus may also result in their development into thymic Treg. After egress from the thymus, recent thymic emigrants (RTE) gradually undergo peripheral tolerance mechanisms which may result in deletion of surviving self-reactive clones or conversion into peripheral Treg. The RTE population would thus be expected to harbour increased self-reactive cells but fewer pTreg compared to established peripheral T cells. pTreg may be needed to control truly self-reactive RTE or dampen the response to tonic self signals that all RTE receive. Accordingly, transfer of PD-1 deficient RTE but not established peripheral T cells to lymphopenic adult Rag$^{-/-}$ recipient

animals yields autoimmunity. Increased self-reactivity within the RTE population, a paucity of pTreg, or both may contribute to their heightened autoimmune potential.

5.2. Regulatory T cells and the dilemma surrounding their suppressive specificity

As discussed in Chapter 1, deficiency of the *foxp3* gene in humans and mice, which is important for the generation and function of FoxP3+ Treg cells, gives rise to a fatal lymphoproliferative and autoimmune disorder[65,159]. While Treg can mediate tolerance through soluble mediators in some circumstances, the importance of these factors varies considerably among the tissues and they are not in general critical for maintaining systemic self-tolerance. Treg have been shown to make stable contacts with DC bearing cognate antigen[187] and thus presumably can act as “super” competitors preventing Tcon from having access to the relevant pMHC. However, CTLA-4 is critical for the function of Treg as demonstrated by the severe systemic autoimmune phenotype of mice engineered to lack CTLA-4 only within FoxP3+ T cells[194]. Importantly, Treg can use CTLA-4 to modulate co-stimulation by directly removing molecules of B7-1 and B7-2 off of the surface of DC via trans-endocytosis[195] and such modulation of co-stimulation has recently been shown to be important for Treg control of LIP[186]. However, this mechanism of Treg suppression raises a dilemma – if one imagines that MHC-II and peptide complexes float freely about the plasma membrane of DC, stripping the co-

stimulatory molecules from DCs would also raise the issue of nonspecific immune suppression, as DCs presenting pMHC derived from foreign antigens that require a strong response will inevitably also be presenting self pMHC for which many Treg have elevated affinity. So how has the system evolved to avoid systemic immune suppression by Treg? A clue to addressing this problem, and more generally that of maintaining coherence in the response to a given antigen and independence in the response to multiple antigens presented by an APC, may lie in observations that MHC-II/peptide complexes containing the same peptide can be found clustered in cholesterol-rich lipid microdomains in the plasma membrane[387-391] and this helps to facilitate antigen presentation particularly in conditions of low ligand density. Therefore, antigens processed by a DC at different times or in different endosomes may give rise to pMHC-II that remain discretely clustered in such lipid domains, at least for a period of time. Furthermore, it has been reported that in DC, pMHC-II complexes and co-stimulatory molecules are co-transported within the same vesicles to the cell surface and remain clustered there[392]. Perhaps if co-stimulatory molecules remain stably associated with 'patches' of particular pMHC-II, Treg-mediated removal of co-stimulatory molecules may occur in a patch specific manner and be long-lasting, thus facilitating regulation of DC antigen presentation ability in an antigen or pMHC-II-specific manner (Figure 5-2). Furthermore, one could speculate that the level of co-stimulatory molecules exported to the surface with pMHC-II complexes in such circumstances may be related to the concentration of PAMPs/DAMPs associated with a phagocytosed entity in the original phagosome, for example, acting as another mechanism of regulating the level of co-stimulation (or co-inhibition) associated with a specific set of antigens derived from said

entity. While some *in vitro* assays using TCR transgenic Tregs and responders with different peptide specificities have suggested that Treg suppression is non-specific as long as the peptide that stimulates Treg was present in a co-culture of Treg and responders[178], the simultaneous uptake of peptides specific to both the Treg and responder would be expected to lead to this finding by the model just proposed. Other *in vitro* and *in vivo* studies have found that suppression is highly peptide specific[393]. Initial investigation of the proposed model could be done with a relatively simple *in vitro* assay. Dendritic cells could be loaded separately and with temporal separation with different model antigens (eg: HY and OVA), and iTreg derived from HY ($Dby_{608-622}$)-specific (Marilyn) or $OVA_{323-339}$-specific (OT-II) TCR transgenic T cells bearing the $FoxP3^{DTR}$ transgene tested for their ability to specifically suppress proliferation of Marilyn or OT-II Tcon after incubation with the loaded DC and diphtheria toxin-mediated depletion of the Treg. Additional initial imaging studies to examine expression levels and co-localization of co-stimulatory molecules with particular pMHC-II complexes derived from separately-loaded HY or OVA before and after Marilyn or OT-II iTreg treatment would also be of importance.

It should again be noted that the speculative model for antigen-specific co-stimulatory molecule modulation by Treg just described also suggests a mechanism by which simultaneous immune responses to multiple pathogens encountered by an APC at different times could each be controlled separately (i.e. Th1 response for one, Th17 response for the other). It could also facilitate maintenance of coherence within the separate responses, if Th lineage-reinforcing signals/cytokines could also be specifically associated with or directed toward specific "patches" of pMHC-II. Furthermore, similar

mechanisms of clustering pMHC derived from the contents of individual phagosomes could be envisaged to help maintain specificity during CD4+ T helper cell licensing of DC to activate CD8+ CTL, if pMHC-I and pMHC-II remain spatially associated.

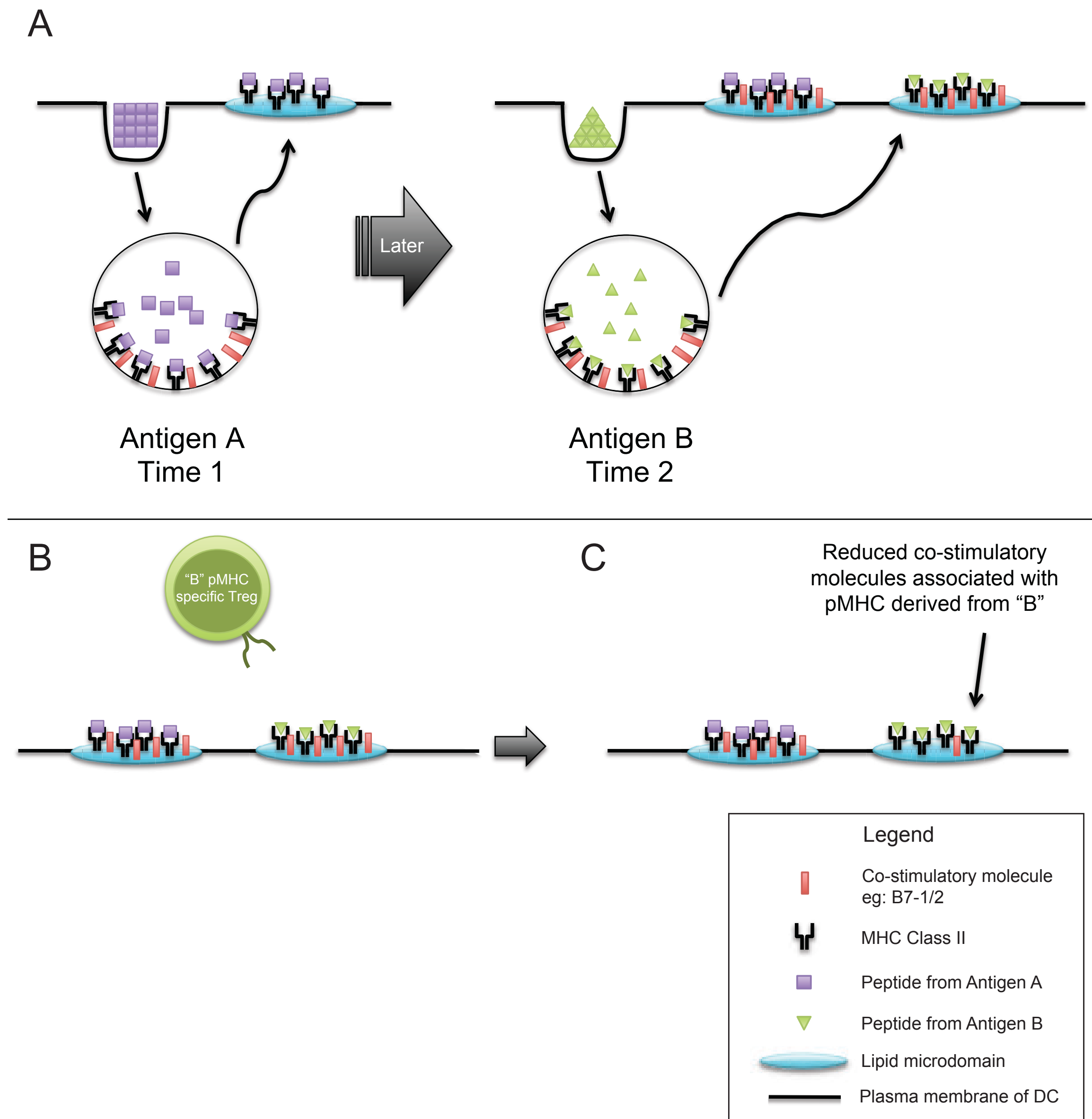


Figure 5-2. A proposed model for antigen-specific modulation of co-stimulation by regulatory T cells

At Time 1, the dendritic cell (DC) takes up Antigen A by phagocytosis. Peptides derived from antigen A during antigen processing are loaded onto MHC Class II molecules in endosomes. Co-stimulatory molecules also present in the endosomes are co-exported with the antigen A-derived peptide-loaded MHC molecules (pMHC) to the cell surface where they remain stably associated in a lipid microdomain. Later, at time 2, the DC takes up Antigen B by phagocytosis and it is similarly processed into peptides which are

loaded onto MHC-II and exported to the surface with co-stimulatory molecules where they remain stably associated in lipid microdomains discrete from those containing pMHC derived from antigen A. B) A Treg cell specific for pMHC derived from antigen B recognizes B-derived pMHC localized to a microdomain, which results in its removal (via CTLA-4) of co-stimulatory molecules (e.g. B7-1, B7-2) from this specific microdomain. C) After encounter with the Treg, pMHC derived from antigen A remain associated with their original concentration of co-stimulatory molecules, while pMHC derived from antigen B are associated with few co-stimulatory molecules, rendering them less competent to stimulate conventional T cells. Figure adapted from Poloso and Roche, 2004 (Ref. 387).

5.3. Towards a more precise and quantitative definition of lymphopenia

In Chapter 1, we considered two criteria, pervasive in the literature, for determining whether a host can be described as lymphopenic: 1) a host is lymphopenic if it has an abnormally low number of circulating lymphocytes, and 2) a host is lymphopenic if it supports LIP of T cells transferred to it. These criteria, however, do not adequately describe all of the factors that we now know to influence whether T cells will undergo LIP when transferred to a host. To illustrate the problem, consider the following question: is an adult BALB/c DO11.10 Rag-2$^{-/-}$ TCR transgenic (Tg) mouse

lymphopenic? Compared to a normal adult BALB/c mouse which contains approximately 46 x 10^6 peripheral naïve T cells, the DO11.10 TCR Tg contains ~25 x 10^6 naïve T cells[234]. Therefore by criterion #1 above, the DO11.10 TCR Tg is lymphopenic. However, transfer of DO11.10 T cells to a DO11.10 TCR Tg host yields no LIP[234], therefore by criterion #2 the DO11.10 TCR Tg mice are not lymphopenic. The input DO11.10 T cells compete for the same self pMHC resources as the endogenous DO11.10 T cells which have already achieved homeostatic balance with the available resources in the host, thus HA Rag2$^{-/-}$ TCR Tg cells transferred to a DO11.10 TCR Tg host undergo efficient LIP[234] as they compete for a discrete set of resources (different pMHC). Similarly, the description of a host as "lymphopenic" lacks the ability to convey differences in the "degree" of lymphopenia in that host. Consider three BALB/c RAG-2$^{-/-}$ mice that have received transfers of 0, 1 x 10^6, or 20 x 10^6 T cells one day previously. All three mice are lymphopenic compared to a WT BALB/c mouse, but subsequent transfer of an equal number of the same T cells to each of these hosts will give rise to different extents of LIP in the secondarily-transferred population. As a final example, consider two adult WT mice, one of which has just received an injection of IL-7. Provision of more of the IL-7 resource will effectively enlarge the niche allowing some T cells to undergo expansion. Was the mouse that received IL-7 then made lymphopenic? In sum, a more informative way to describe the current homeostatic state of the T cell compartment in a host is in terms of "LIP-potential", which will need to take into account the totality of the available resources for a given set of T cells in the host versus the number of competitors for those resources. This concept of LIP-potential is useful because it provides a more inclusive conceptual framework that makes predictions for

how the overall "system" of T cell and resource interactions will respond to perturbations. For example, such a framework could inform strategies to ameliorate autoimmune disease that target the size of the resource or competitor pool specifically for disease-causing T cells in order to have their effect, avoiding the use of broad immunosuppression. The concept of LIP-potential may also be applied to smaller compartments within a host, for example a single draining lymph node or the environment within a tumour or a tissue.

We can use the following points that were reviewed and discussed in Chapter 1 to generate an equation that can make testable predictions about how various perturbations will affect the "LIP potential" of the system.

1. Resources are finite, and consist of homeostatic cytokines and, for a given T cell, the set of pMHC present in the host with which its TCR can interact. The size of the host or compartment in question influences the total available resources.
2. T cells compete for access to homeostatic cytokines and, both intra- and interclonally, for pMHC.
3. Treg can inhibit LIP and may be viewed as "professional competitors", for pMHC and/or other factors.
4. Affinity/avidity for pMHC influences the ability to compete for pMHC and may secondarily influence ability to compete for homeostatic cytokines (by modulation of cytokine receptor expression etc.)
5. Co-stimulation and co-inhibition can influence LIP positively and negatively, respectively. Treg can modulate co-stimulation.

6. Naïve T cells are considered to require tonic TCR/pMHC stimulation as well as homeostatic cytokine signals (chiefly IL-7) for survival. Central memory T cells rely on homeostatic cytokines but do not require TCR/pMHC stimulation for survival.

In general, for a given T cell clone (A):

$$\text{LIP potential } (A) \propto \frac{\text{Resources useable by } (A)}{\text{Competitors for Resources useable by } (A)}$$

Variable definitions:

- Let $[\text{HC}(a)]$ be the concentration of homeostatic cytokines useable by clone A
- Let $[\text{pMHC}(a)]$ be the available concentration of the set of pMHC complexes which can mediate tonic or greater interactions with the TCR of clone A.
- Let $\text{affinity}(a)$ be the affinity of the interaction between the TCR of A and $\text{pMHC}(a)$.
- Let $[\text{Tcon}(a)]$ be the concentration of conventional competitor T cells that can recognize $\text{pMHC}(a)$
- Let $[\text{Treg}(a)]$ be the concentration of Treg that can recognize $\text{pMHC}(a)$
- Let $\frac{\text{costim}}{\text{coinhib}}(a)$ be the basal level of co-stimulation divided by co-inhibition provided by APCs presenting $\text{pMHC}(a)$
- Let $\text{size}(a)$ represent the size of the secondary lymphoid organs of the host or the area (eg. a single lymph node, intratumoural space) accessible to clone A for which LIP-potential is being measured.

Then for **naïve T cells** (which require both IL-7* and pMHC for survival/LIP):

$$\text{LIP potential } (A) \propto \frac{[\text{HC}(a)] * \left(\frac{\text{costim}}{\text{coinhib}}(a)\right) * \text{ affinity}(a) * [\text{pMHC}(a)] * \text{size}(a)}{[\text{Tcon}(a)] + [\text{Treg}(a)]}$$

*Note: in contrast to reports in the literature suggesting that IL-7 has a critical role in mediating LIP of naïve T cells in a lymphopenic host[245,246], with essentially no LIP occurring in IL-7 knockout hosts, in Chapter 4 we reported that blockade of IL-7Rα during early LIP of Marilyn TCR transgenic T cells when relevant pMHC would be expected to be abundant (d7 post cell transfer) did not reduce the proliferation or size of the T cell compartment at that time. Instead anti-IL-7Rα blockade had a dramatic effect on limiting the size of the compartment at d35 when the T cell compartment (with respect to the resources available to support Marilyn T cells) was approaching repleteness and presumably there was high competition for relevant pMHC. In other words, abundant TCR/pMHC signals appear to decrease or obviate the need for IL-7-mediated signals (and possibly other homeostatic cytokines), and thus IL-7-mediated signals seem to become most important when pMHC becomes limiting due to competition. Although we used antibody mediated blockade of IL-7Rα as opposed to a genetic knockout of IL-7, which would be expected to lead to a less complete blockage of IL-7 signals, our findings would not lead us to predict that LIP potential becomes zero in a situation where there is abundant relevant pMHC but no homeostatic cytokines, as the above cited studies would suggest and the equation above would - as is - formally dictate. Indeed, TCR signals have been shown to

directly inhibit IL-7-mediated Bcl2 protein expression in T cells and themselves mediate expression of other anti-apoptotic proteins Bcl-Xl and A1, suggesting that TCR signals can mediate a dominant mechanism of T cell survival[265]. Given the role of IL-7 signaling in lymph node development[31] and the need for lymph nodes in LIP, it seems likely that the lack of/severe decrease in LIP seen in lymphopenic IL-7$^{-/-}$ hosts[245,246] stems from a defect in lymph node structure and function as opposed to a direct effect on the T cells themselves. Thus a speculative condition for the above equation would be that it applies when $[\mathrm{HC}(a)] > 0$, and if the concentration of homeostatic cytokines was zero the $[\mathrm{HC}(a)]$ term would be omitted from the equation, since LIP potential would not likely become zero in the absence of homeostatic cytokines (which might occur in an experimental context) but in the presence of abundant pMHC, for example. Further studies are warranted examining the LIP of T cells with conditional knockouts of IL-7 and/or IL-15 receptors.

For **central memory T cells** (which utilize homeostatic cytokines for survival but do not require pMHC, although pMHC interactions may provide survival and proliferation signals and influence the efficiency of their LIP):

$$\text{LIP potential } (A) \propto \frac{[\mathrm{HC}(a)] + \left(\frac{\mathrm{costim}}{\mathrm{coinhib}}(a)\right) * \text{ affinity}(a) * [\mathrm{pMHC}(a)] * \mathrm{size}(a)}{[\mathrm{Tcon}(a)] + [\mathrm{Treg}(a)]}$$

These equations predict a variety of methods to control LIP potential by decreasing the

numerator or increasing the denominator. It is also important to realize that the set of pMHC recognizable by clone A could overlap with the set of pMHC recognizable by clone B (i.e. $\mathrm{pMHC}(b)$), clone C, and so on, thereby illustrating how interclonal competition could influence LIP potential for all three T cell clones (Figure 5-3).

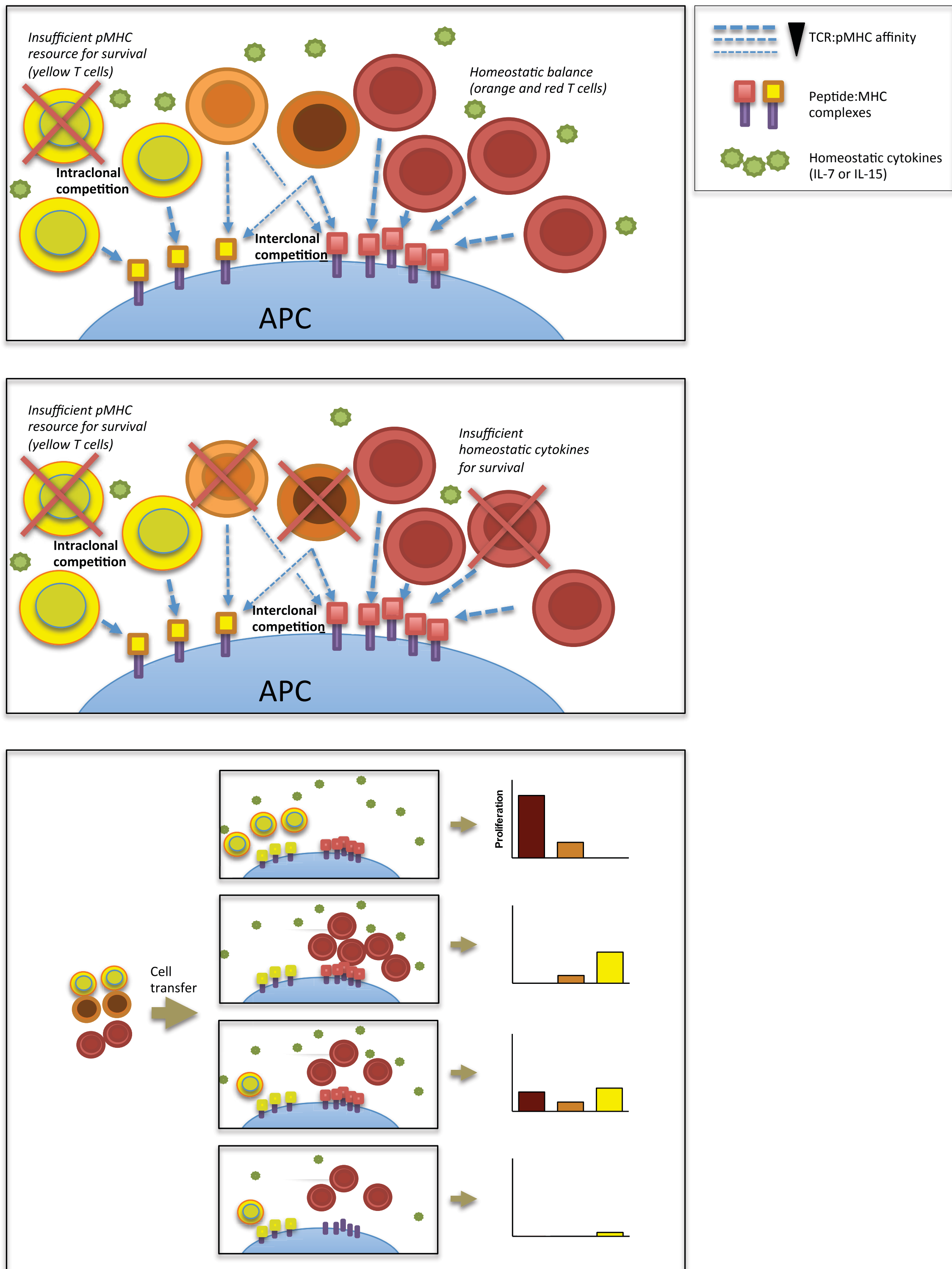


Figure 5-3. Schematic diagram demonstrating competition for homeostatic cytokines and inter- and intraclonal competition for pMHC.

Top panel: IL-7/IL-15 levels are sufficient, but Yellow T cell population has insufficient pMHC resource for survival and undergoes cell death. Orange/Orange and Orange/Brown T cells compete intraclonally for Yellow and Red pMHC with affinities indicated. Red and orange populations are in homeostatic balance. Middle panel: IL-7 and IL-15 are limiting, creating a situation of multiple cells undergoing apoptosis due to lack of resources for survival. Bottom panel: Proliferation behavior of equally mixed Yellow, Orange, and Red T cells transferred into hosts with the indicated resource vs. competition statuses. Figure inspired by Takada and Jameson, 2009 (Ref. 216).

5.4. Defective interclonal competition networks may predispose to autoimmunity or "every T cell is a regulatory cell"

In chapter 4 we showed that PD-1 can control the magnitude of LIP in response to tonic pMHC in lymphopenic hosts, which supports the idea that T cells naturally on the higher end of the self-affinity spectrum may spuriously acquire effector function in response to stimulation by self pMHC, resulting in systemic autoimmunity. We cannot rule out that impaired negative selection to tissue restricted antigens in the thymus in the setting of PD-1 deficiency might contribute to the disease in our model of autoimmunity mediated by LIP of PD-1$^{-/-}$ newly-generated T cells, although one might predict that negative selection might actually be enhanced in the context of stronger TCR signals.

However, there is some evidence that PD-1 deficiency or overexpression of PD-1 may lead to alterations in positive selection and the resulting T cell repertoire[382,394]. Even if the repertoire of T cells emerging from the thymus was identical whether they were PD-1 sufficient or deficient, the effect of PD-1 deficiency on LIP in the periphery might lead to altered T cell repertoire reconstitution during LIP, and this could contribute to disease. As discussed in section 1.3.1.1, a complex network of intra- and interclonal competition between T cells for available self pMHC helps to manage the diversity of the T cell repertoire. Indeed the contraction of clonally-expanded antigen specific CD4+ T cells following an immune response has been suggested to be regulated by their interclonal competition with "neighbouring" T cells that share self- but not foreign pMHC specificity[395]. During LIP, T cells with higher affinity for pMHC have an advantage as they can compete more strongly for the pMHC resource, proliferate faster, and come to predominate, and in WT mice some evidence exists that LIP leads to altered T cell repertoires[396]. In the absence of PD-1, an upward shift in the average TCR signal intensity may exacerbate such an imbalance between lower and higher-affinity T cells that recognize the same self pMHC, and this may be exponentially magnified during LIP in a host with high LIP-potential. One could envision a situation where critical low-affinity "neighbouring" T cells may be outcompeted during LIP to the extent that they are no longer numerous enough to adequately act as relevant competitors of clones with higher self pMHC affinity, paving the way for autoimmunity. Whether PD-1 deficiency exacerbates the disparities in T cell diversity normally associated with reconstitution of the T cell compartment by LIP could be examined by TCR spectratyping or deep sequencing. However, the idea of "neighbouring" T cells acting as *de facto* regulators of

other T cells with overlapping pMHC specificity itself has applications in terms of treating autoimmunity or cancer.

5.5. Modulating LIP potential to enhance co-stimulatory blockade-mediated tumour immunotherapy approaches or inhibit autoimmunity

The microenvironment within a solid tumour, even within an otherwise lymphoreplete host, might be viewed as having low LIP potential due to the local presence of Treg, limited relevant neoantigen-derived pMHC (neo-self pMHC), and/or lack of availability of homeostatic cytokines to support infiltrating neoantigen-specific Tcon. In recent years, immunotherapy for a number of cancers using anti-PD-1 or anti-PD-L1 blocking antibodies has proven highly effective[135]. One could consider that by blocking PD-1 signals, every neo-self pMHC is being made more "valuable" as a resource because it can effectively supply a larger TCR signal to T cells when the TCR signal-dampening effects of PD-1 are negated. Thus with anti-PD-1 treatment, the available neo-self pMHC within a tumour may either support the survival of increased numbers of neoantigen-specific T cells or promote their activation due to the stronger TCR signals each might receive. Specifically increasing LIP-potential further within this microenvironment might be achievable, for example, by using bi-specific therapeutic approaches such as IL-7 or IL-15 tethered to a tumour antigen-directed monoclonal antibody, further promoting the survival and intratumoural expansion of infiltrating anti-tumour T cells, up-regulation of their effector mechanisms, and subsequent tumour clearance. This might be particularly important in the situation of decreasing antigen

load due to successful tumour clearance, where sufficient neo-self pMHC might not persist to sustain high T cell numbers. By boosting homeostatic cytokines such as IL-7 in the intratumoural environment it may help to maintain sufficient survival signals to sustain an expanded population and effectively "keep the pressure on" the tumour.

One can also apply the concept of modulating "neighbouring" T cells to increase tumour immune responses. Essentially, by eliminating T cells that have overlapping specificity for neo-self pMHC but are not effective in mediating anti-tumoural responses (perhaps due to insufficient affinity), one might promote responses from the remaining neo-self pMHC-specific T cells that can mediate an effective response. While direct and specific modulation of neighbouring T cells would be practically difficult, perhaps involving the use of MHC tetramers as a drug-targeting agent, one strategy to do so through modulation of resources for T cell homeostasis would be as follows: imagine T cell clone A has high affinity for a neo-self pMHC and effectively generates an effector response to it – it is thus a desirable T cell clone in terms of mediating an anti-tumoural response. The set of pMHC that can provide at least tonic survival signals for clone A will be known as pMHC(a). The set of pMHC that can mediate a tonic or greater signal to T cell clone B, pMHC(b), shares some overlap with pMHC(a) but this overlap does not include the desired target neo-self pMHC (Figure 5-4). Thus T cell clone B competes with clone A for resources but cannot mediate any response to the tumour. T cell clone C responds to the set pMHC(c), which shares overlap with pMHC(b) but not pMHC(a), and again, pMHC(c) does not include the neo-self pMHC. A strategy to boost the LIP potential or space for expansion of clone A would be to immunize the host with peptides that will expand clone C. Expansion of clone C would be predicted to compete with and

reduce the resources available for clone B and decrease its survival. With a reduction in clone B, less competition for clone A would be present, yielding increased resources for this desirable tumour-clearing T cell clone (Figure 5-4). A simpler version of this concept has been demonstrated to ameliorate lymphopenia-driven autoimmunity *in vivo* in mice transgenic for pigeon cytochrome c (PCC) that have received TCR transgenic T cells specific for a PCC epitope[395]. In this model, the "deletor" T cell (analogous to clone B above) was isolated from a (restricted) polyclonal T cell population and used to directly compete with disease causing T cells (analogous to clone A). There is no question that considering the current state of the art, application of this principle in humans would be a formidable technical hurdle. However, deep sequencing of TCR repertoires and advances in molecular modeling of TCR/pMHC interactions will likely eventually make such an approach feasible.

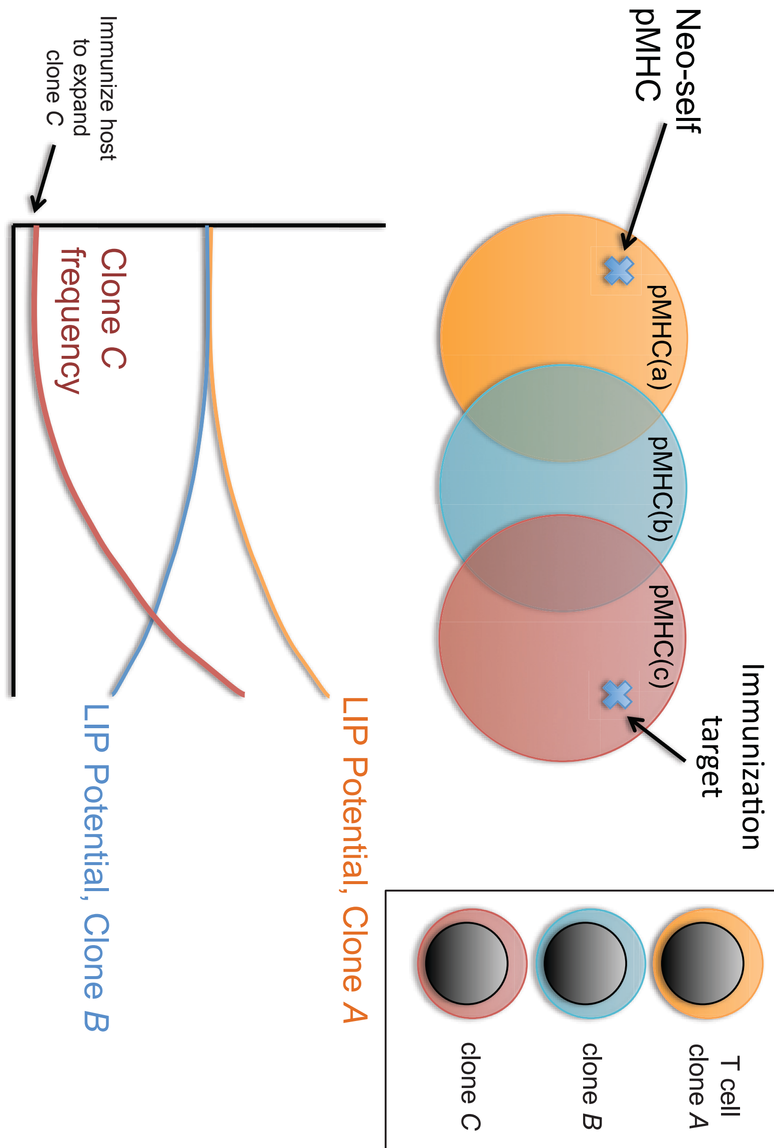


Figure 5-4. Taking advantage of interclonal competition to enhance T cell space or "LIP potential" for a desirable tumour antigen-specific T cell clone

T cell clone A is a tumour-specific T cell clone that is effective and would be desirable to expand. It can receive tonic or greater signals from the set of pMHC denoted as pMHC(a). T cell clone B is irrelevant for tumour-antigen specificity but the set of pMHC it can interact with to receive a tonic or greater signal (pMHC(b)) overlaps with pMHC(a). T cell clone C is also irrelevant for tumour-antigen specificity, and it interacts to receive a tonic or greater signal with pMHC(c), which overlaps with pMHC(b). By

immunizing the host such that clone C is expanded, it will compete with and lower the LIP potential for clone B, which will result in a decrease in clone B survival and thereby increase the LIP potential for the desirable clone A (due to decreased competition from B).

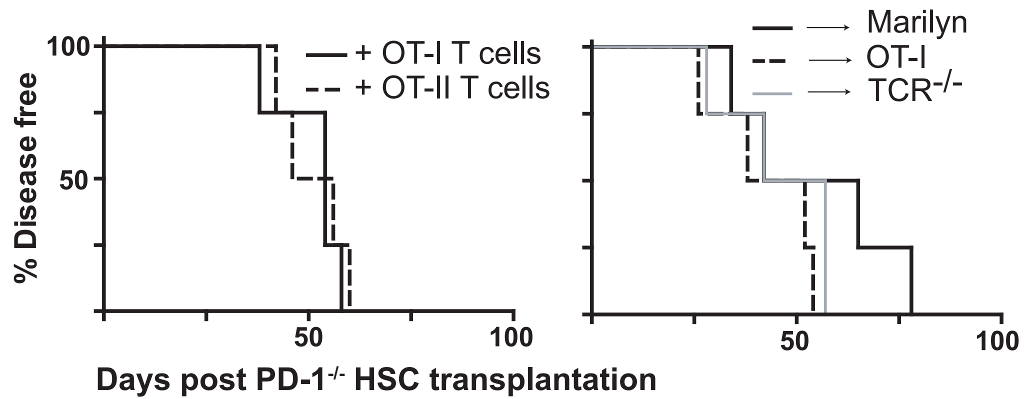
5.6. T cell-intrinsic innate immune control over LIP and LIP-driven autoimmunity

As reviewed in chapter 1, both co-stimulation and co-inhibition can influence the extent to which LIP occurs, and stimulation of DC with DAMPs or PAMPs would be expected to result in modulation of their expression of co-stimulatory molecules. Indeed, LIP of OT-II transgenic T cells was shown to be reduced considerably in germ-free hosts, which was rescued by transfer of cecal bacterial lysate-pulsed bone-marrow derived DC, and this depended on intact MyD88 and IL-6 within the transferred DC[291]. However, influence over LIP by innate stimuli need not only function through APC intermediates. IL-1, originally described as "lymphocyte-activating factor", was described in the early 1970s as a soluble factor which could promote the T cell response[107]. More recently IL-1 was shown, using IL-1R-deficient T cells and hosts, to act directly on T cells to enhance their proliferation and memory formation in response to antigenic stimulation[110]. MyD88, a signaling adapter important for transducing signals downstream of the IL-1R, was also

shown to be important in T cells for mediating their "escape" from suppression by Treg[109]. Furthermore, deficiency of TLR2 in T cells reduced the severity of EAE and negatively regulated the *in vivo* Th17 response[397], further highlighting the ability of PRR ligands to modulate T cell function. As discussed in chapter 1, IL-1β is an end product of inflammasome activation in response to a variety of innate immune stimuli. One might consider that IL-1 acts on T cells as an additional form of co-stimulation that would thus act as a resource for T cells and increase LIP potential. This leads to the hypothesis that MyD88-deficient T cells would proliferate less during LIP compared to WT cells in a competitive assay such as those carried out in Chapter 4. Furthermore, it is possible that reduced LIP potential or modified effector function of the expanded T cells in lymphopenic $Rag^{-/-}$ recipients of $MyD88^{-/-}$ x $PD\text{-}1^{-/-}$ HSC would prevent disease. Although depletion of the intestinal microbiota with antibiotics did not prevent disease in $PD\text{-}1^{-/-}$ HSC recipients[374] (Chapter 2), one might predict that sterile ligands for PRR may be liberated by tissue damage during early stages of LIP-driven autoimmunity which feed back on the expanding T cell population and amplify the inflammation. This largely unexplored line of questioning represents, in my estimation, one of the most important future directions for examining the mechanisms by which LIP can promote autoimmunity and inflammatory disease.

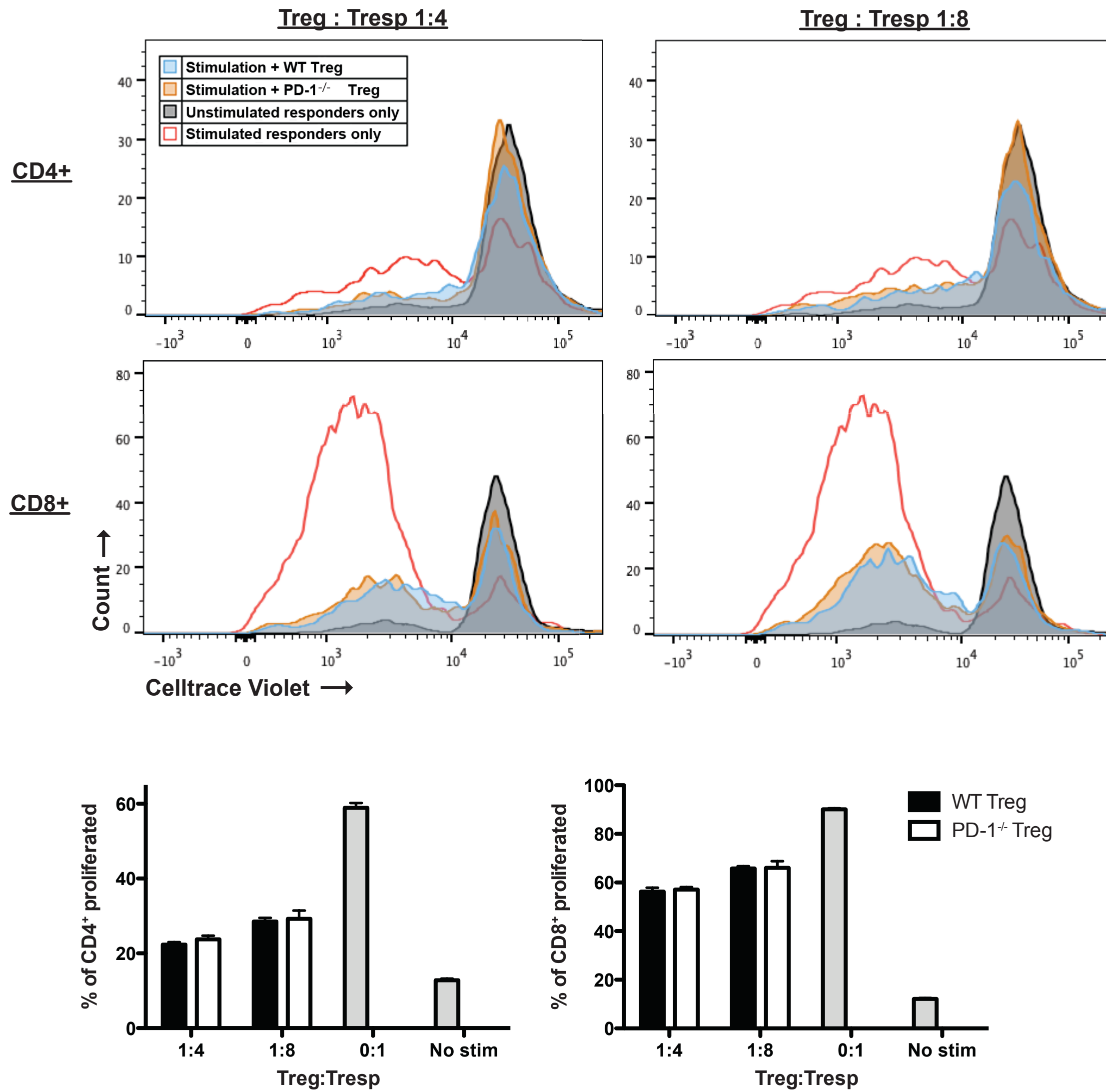
Chapter 6

Appendices



Appendix 1 – Disease in Rag$^{-/-}$ recipients of PD-1$^{-/-}$ HSC cannot be prevented by the presence of TCR transgenic T cells or polyclonal B cells.

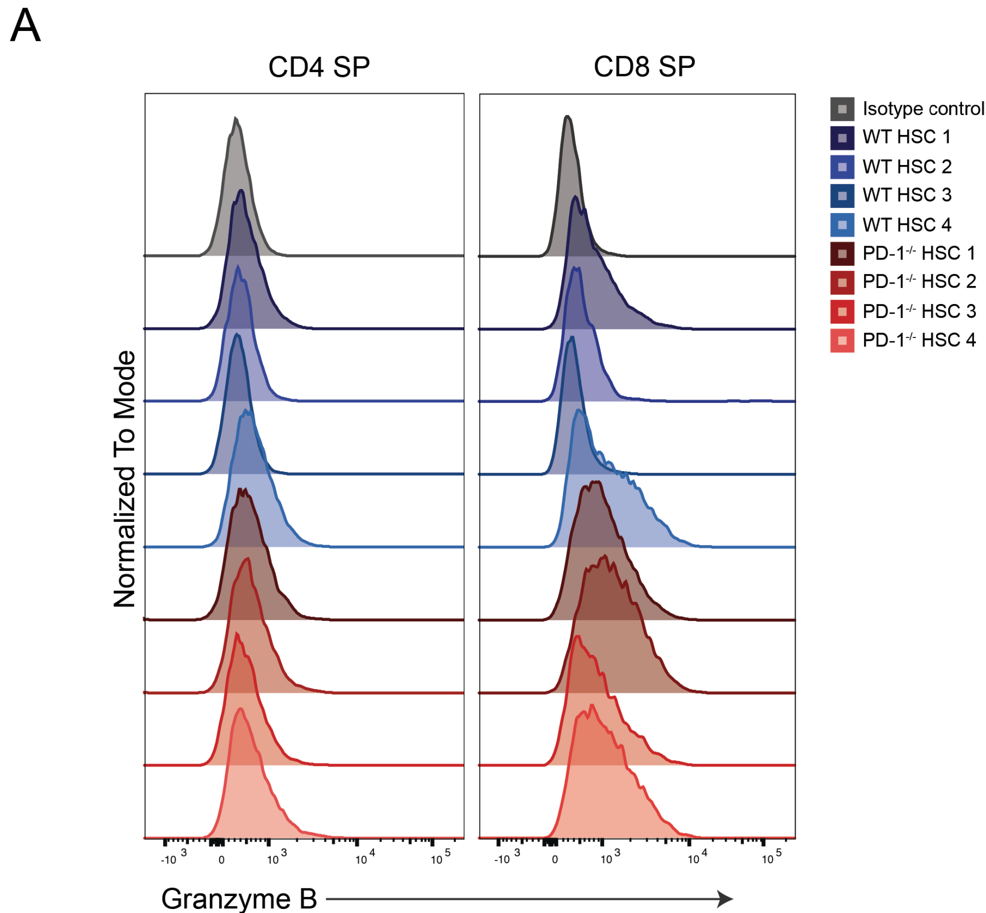
Left: Rag$^{-/-}$ mice (n=4/group) were given PD-1$^{-/-}$ HSC along with 3.5x10^{6} OT-I or OT-II T cells. Right: Female Marilyn mice (CD4+ TCR transgenic, Rag$^{-/-}$, n=4), OT-I mice (Rag$^{-/-}$, n=4), or TCR$^{-/-}$ (n=4) mice were given PD-1$^{-/-}$ HSC. Marilyn recipients received female HSC. All animals were monitored for disease symptoms and scored as described in Chapter 2, materials and Methods.



Appendix 2 - FoxP3+ Treg purified from both diseased PD-1$^{-/-}$ HSC recipients and WT HSC recipients are suppressive.

CD4+FoxP3+ (EGFP+) cells were purified from FoxP3^{EGFP} (WT) and diseased FoxP3^{EGFP} x PD-1$^{-/-}$ HSC recipients by FACS. Suppressive function was assayed *in vitro* using whole Celltrace Violet-labeled B6-CD45.1 splenocytes as responder cells in the presence of 1 µg/mL anti-CD3ε (145-2C11) stimulation. Proliferation of CD45.1+ responder CD4 or CD8 SP T-cells was measured by flow cytometry after 4 days of incubation. Data are representative of 2 experiments using cells sorted from a total of 3 separate HSC recipients per group. For details on mice and culture conditions see methods, chapter 2.

A



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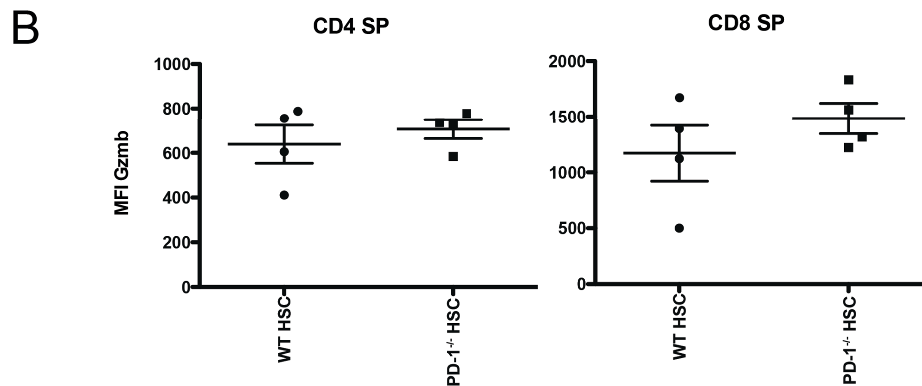
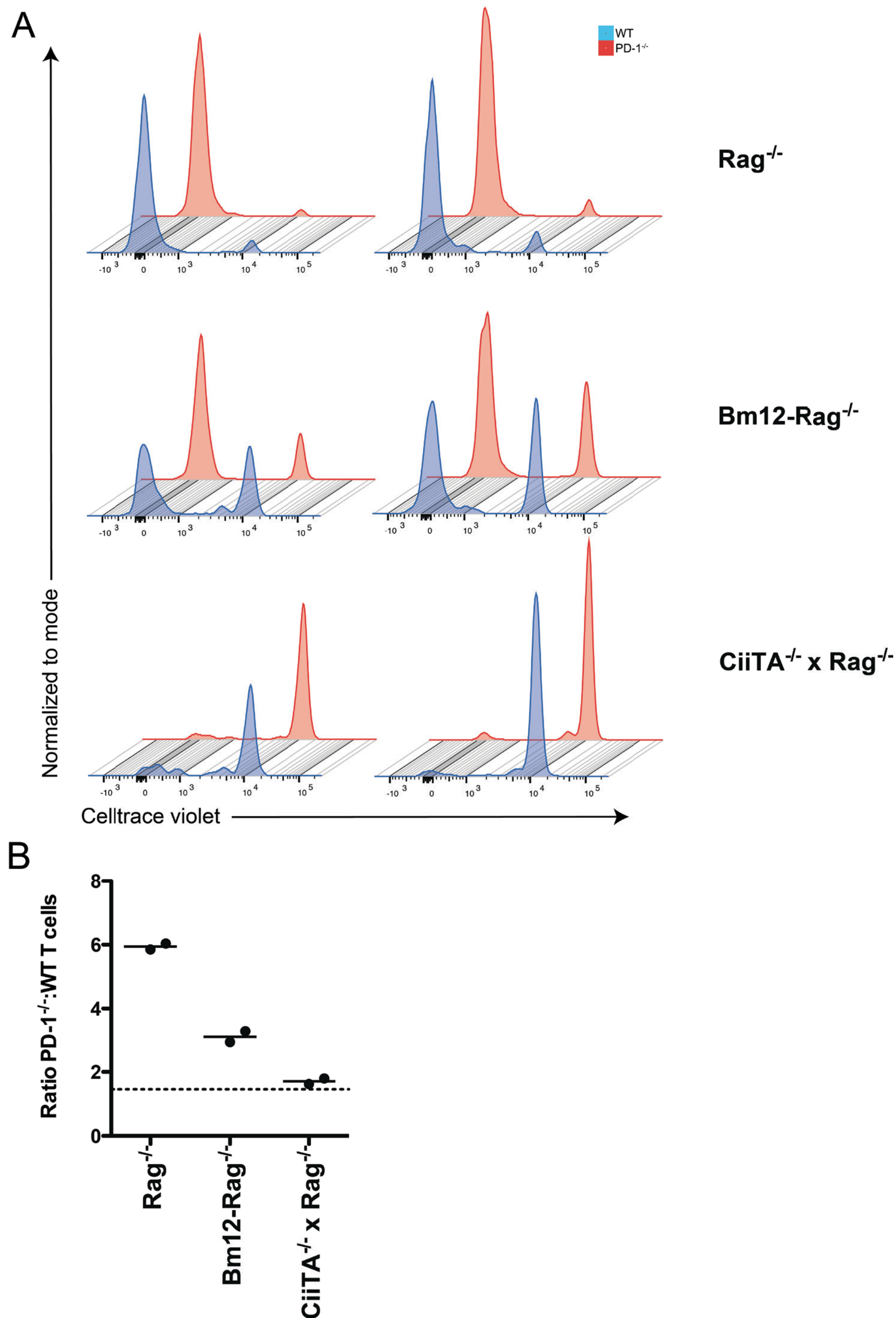


Appendix 3 - Granzyme B expression in peripheral splenic T cells in WT and PD-1$^{-/-}$ HSC recipients at the peak of disease

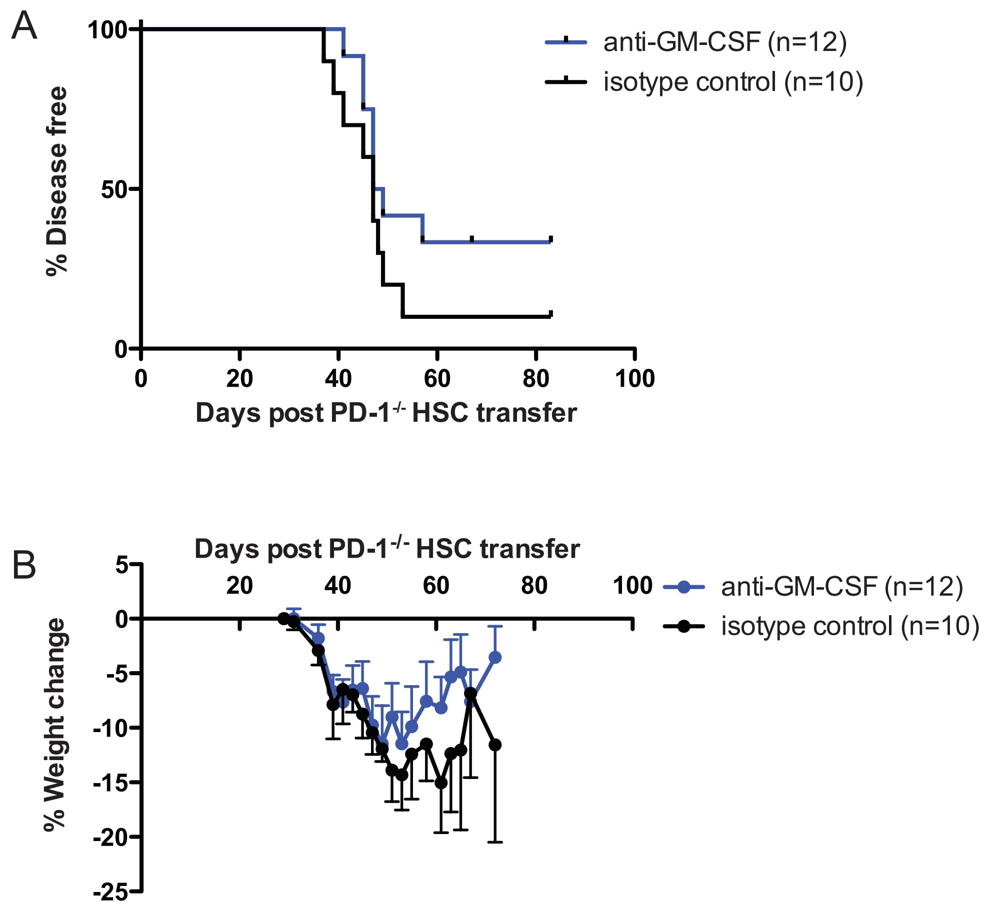
Splenocytes from WT and PD-1$^{-/-}$ HSC recipients were harvested at d45 post-transfer and Granzyme B expression in CD4 and CD8 SP TCRβ+ cells was assayed by flow cytometry. A) Histogram plots of GzmB expression in CD4 and CD8 SP T cells. B) Comparison of mean fluorescence intensity of GzmB staining between groups. Data are from one experiment.



Appendix 4 - PD-1 controls tonic pMHC signals including attenuated signals provided by an MHC-II allele with limited mismatch.

1 x 10^6 Mixed FACS-purified (BD Cytopeia sorter, ~96% pure) CD4+ T cells from Marilyn-WT (CD45.1+) and Marilyn PD-$1^{-/-}$ (CD45.2+) mice were labeled with Celltrace violet (CTV) and injected intraperitoneally into Rag$^{-/-}$, Bm12 x Rag$^{-/-}$, or CiiTA$^{-/-}$ x Rag$^{-/-}$

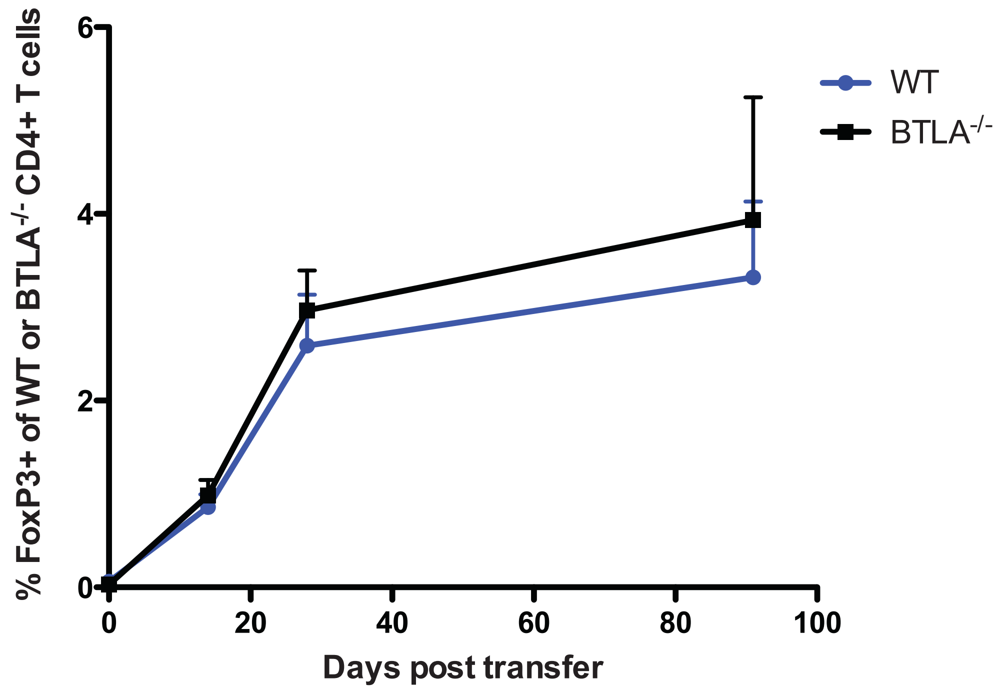
mice (n=2 per group). After 28 days, cells were recovered from the peritoneal cavity by flushing with PBS and analyzed by flow cytometry. A) Histograms depicting CTV dilution (proliferation) in all recipients. B) Ratio of PD-$1^{-/-}$:WT T cells in recovered cells at d28. Initial injected cell mixture ratio is indicated by a dashed line. For descriptions of mice, cell preparation, and cytometry see methods, chapter 4. Bm12 x $Rag^{-/-}$ is described in Thangavelu et al. 2013 (Ref. 119).



Appendix 5 - Blockade of GM-CSF in PD-1$^{-/-}$ HSC recipients does not significantly ameliorate autoimmunity

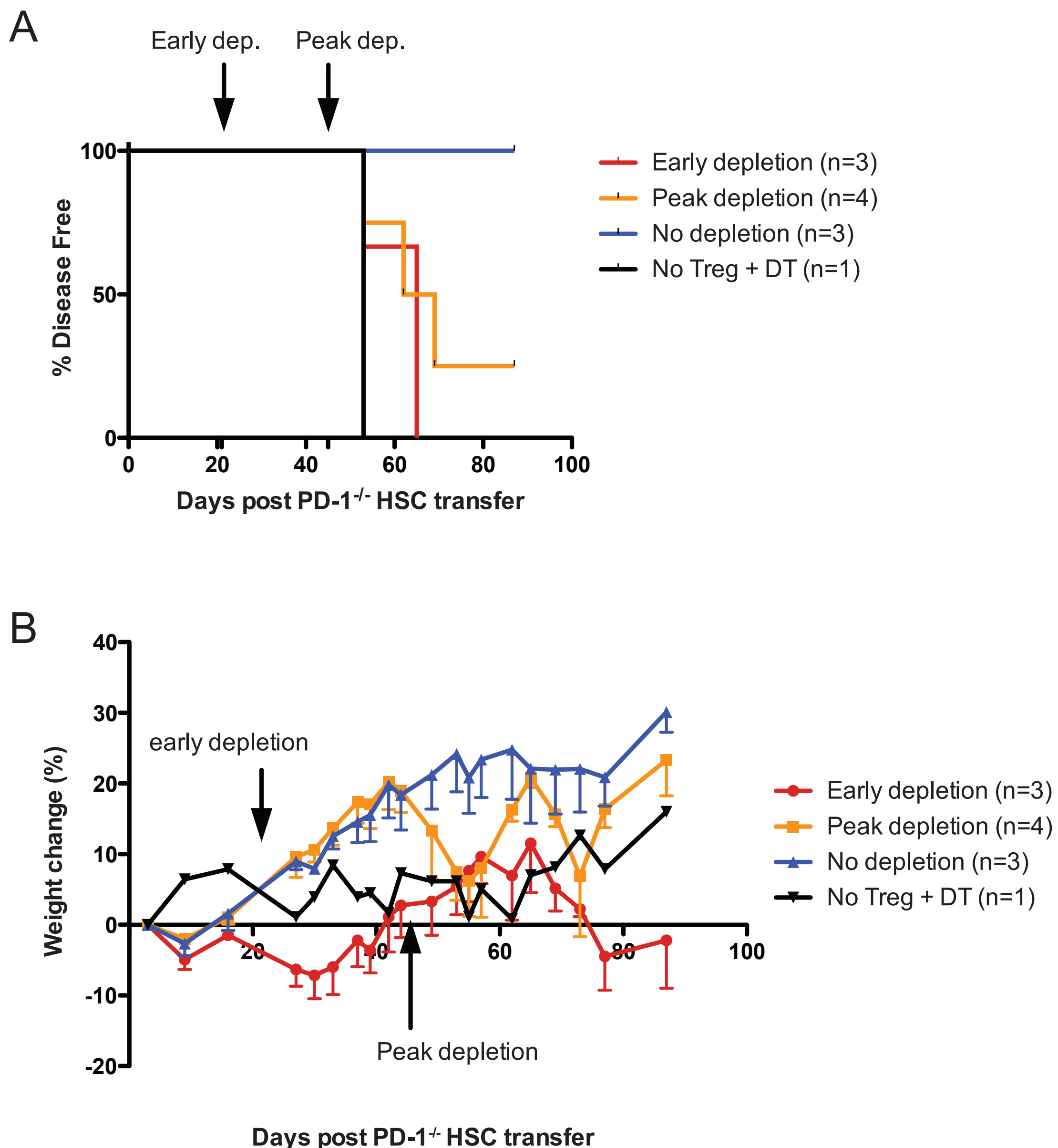
15 x 10^6 PD-1$^{-/-}$ E14-15 fetal liver cells (source of HSC) were transferred i.v. to Rag$^{-/-}$ recipient animals. Mice received intraperitoneal injection of 0.4 mg per mouse of anti-GM-CSF antibody (clone MP3-22E9, BioXCell) or isotype control (Rag IgG2a, clone 2A3, BioXCell) every other day from d25 (trial 1) or d21 (trial 2) until d67. Recipients were monitored for weight changes and other symptoms of autoimmune disease. Mice were considered diseased if two or more of the following symptoms were evident or if mice lost ≥20% body weight: cachexia/weight loss (>15%), kyphosis (hunched appearance), ruffled fur, dermatitis, ocular lesions, and diarrhea. Data are combined from 2 independent trials of n=5-6 per group each. A) Kaplan-Meier survival curve analysis of disease incidence in the indicated recipient groups. No significant difference was

detected using Mantel-Cox analysis. B) Weight changes in the indicated recipient mice. Data were combined from 2 trials, data from non-matching days (± 1d) were combined.



Appendix 6 - BTLA deficiency does not inhibit the in vivo conversion of CD4+ FoxP3- T cells to pTreg

CD4+ FoxP3- (GFP-) thymocytes were sorted from 6-8 week old FoxP3^{EGFP} (CD45.1+CD45.2+) males (referred to here as WT) or FoxP3^{EGFP} x BTLA$^{-/-}$ mice generated by crossing FoxP3^{EGFP} and BTLA$^{-/-}$ strains (BTLA$^{-/-}$, see Chapter 2 methods for mouse description and flow cytometry methods). An approximately equal mixture of WT and BTLA$^{-/-}$ cells was generated (99.5% CD4+FoxP3-, starting FoxP3+ proportions were 0.06% and 0.03% of CD4+ cells in WT and BTLA$^{-/-}$ populations, respectively). 2 x 10^6 mixed cells were transferred into n=6 Rag$^{-/-}$ recipients and the proportion of the CD4+ T cells in the blood that were FoxP3+ was monitored over time.



Appendix 7 - Continued presence of adoptively-transferred Treg in the periphery is required to prevent autoimmunity in lymphopenic recipients of PD-1$^{-/-}$ HSC

15 x 10^6 PD-1$^{-/-}$ fetal liver cells (a source of HSC, see Chapter 3 for mouse descriptions) were transferred to 6-8 week old Rag1$^{-/-}$ recipient animals i.v. On day 8 or 9 post-HSC transfer, recipients received a further adoptive transfer of 1 x 10^6 FACS sorted (BD Cytopeia) CD4 SP FoxP3+ (GFP+) Treg from FoxP3^{DTR} (B6.129(Cg)-FoxP3$^{tm3(DTR/GFP)Ayr}$/J, Jackson Laboratories) mice, i.v. Mice were monitored for A)

symptoms of disease and B) weight changes. At d21 (early Treg depletion group) or d45, which is approximately the usual peak of disease in this model (peak Treg depletion group), mice received 2 consecutive daily doses of 50 ng/g Diphtheria toxin (DT), i.p. in PBS to deplete the transferred Treg population. 1 group received no Treg depletion treatment, and 1 mouse received DT but no Treg. No significant difference between disease curves using the Mantel-Cox test was detected (Graphpad Prism v5.0).

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