Regulation of Newly Generated T Cells in the Setting of Lymphopenia by Btla and PD-1

by

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Abstract

Recent thymic emigrants (RTE) are newly generated T cells that have just been exported to the periphery, where they continue their maturation to become mature T cells. RTE have only undergone central tolerance in the thymus but not yet undergone peripheral tolerance. As such, it is imperative that these T cell subsets are well regulated to prevent autoreactivity. Having previously shown by our group that coinhibitory T cell receptors such as PD-1 and Btla are required for establishing tolerance in newly generated T cells during lymphopenia, we investigated what stage during T cell development these coinhibitors are required to prevent autoimmunity. Deletion of the Btla gene in hematopoietic stem cells that otherwise had the potential to express this coinhibitor generated autoimmunity in lymphopenic recipient mice (p=0.02) once RTE began to seed the periphery. Likewise, deletion of PD-1 or Btla in the RTE that otherwise expressed these coinhibitors resulted in autoimmunity, p=0.02 and p = 0.0002, respectively, in lymphopenic recipients. Thus, our data showed that these coinhibitors are required to block autoimmunity at the RTE stage. Given the abundance of RTE in neonatal mice, we hypothesized that, unlike adult splenocytes that contain a fraction of RTE, neonatal splenocytes would induce autoimmunity during lymphopenia in the absence of PD-1 signaling. Supporting our hypothesis, our data showed that PD-$1^{-/-}$ neonatal splenocytes induced autoimmunity in adult lymphopenic mice. We further examined what T cell subsets are required for disease generation in the absence of Btla signaling and found the CD4 T cells and MHCII are required for disease generation.

Since Btla is constitutively expressed in T cells, we examined whether Btla signaling influences other coinhibitors in regulating T cell functions. In this study, we explored

coinhibitory receptors that regulate T cell function. We found an inverse relationship between CD5, another coinhibitor that is constitutively expressed on T cells, and Btla in T cell ontogeny.

To model the disease relapse that occurs in some multiple sclerosis (MS) patients treated with lymphopenia-inducing antibody (anti-CD52) and given our data that PD-1 is required to prevent autoimmunity by RTE during lymphopenia, we asked if deficiency of PD-1 signaling prevented treatment of experimental autoimmune encephalomyelitis [(EAE), a mouse model of MS] by anti-CD52. Induction of EAE and subsequent treatment with anti-CD52 antibody once the mice became sick resulted in an increased proportion of newly generated T and B cells and double negative T cells. To our surprise, PD-1 signaling was dispensable for recovery from EAE following anti-CD52 antibody treatment. The rapid repopulation of RTE in the anti-CD52-treated mice prompted us to ask if the thymus played a role in the recovery. Interestingly, when the thymus was removed in the EAE-induced mice, two-thirds (p=0.001) of these mice had a relapse following anti-CD52 antibody treatment. We concluded that a functional thymus is required to prevent relapse in EAE-induced mice treated with anti-CD52. As such, continuous monitoring of thymic functions and lymphocyte counts in the clinic would benefit multiple sclerosis patients treated with anti-CD52.

Overall, this work showed the benefit of continuous thymic export of RTE and the important role Btla and PD-1 plays in blocking autoimmunity in these RTE under lymphopenic settings.

Preface

This thesis titled “Regulation of Newly Generated T Cells in the Setting of Lymphopenia by Btla and PD-1” is an original work by Adeolu O. Adegoke. This research project received research ethics approval from the University of Alberta Research Ethics Board, projects title ‘Natural self-tolerance and transplantation tolerance in T-Cells and Breeding Colony; AUP00000215’ and ‘EAE protocol; AUP00000369’.

Some of the studies in chapter 4 have been published as two separate manuscripts: ***Adegoke A**, *Haile Y, Laribi B, Lin J, Anderson CC. Anti-CD52 blocks EAE independent of PD-1 signals and promotes repopulation dominated by double negative T cells and newly generated T and B cells. (*co-first authors) Eur J Immunol. 2020 Sep;50(9):1362-1373. doi: 10.1002/eji.201948288. A.A. designed and performed experiments, analyzed data, and wrote the manuscript; Y.H. designed and performed experiments, analyzed data, and wrote the manuscript; B.L. performed experiments and edited the manuscript; J.L. performed experiments and edited the manuscript; C.C.A. designed experiments, analyzed data, and wrote the manuscript.

Adegoke AO., Lin J., Anderson CC. Loss of thymic function promotes EAE relapse in anti-CD52-treated mice. Curr Res Immunol. 2022 Mar 8;3:37-41. doi: 10.1016/j.crimmu.2022.03.001 A.O.A. designed and performed experiments, analyzed data, and wrote the manuscript; J.L. performed experiments and edited the manuscript; C.C.A. designed experiments, analyzed data, and edited the manuscript.

Appendix 3 has been published as Dong M, Audiger C, Adegoke A, Lebel MÈ, Valbon SF, Anderson CC, Melichar HJ, and Lesage S. CD5 levels reveal distinct basal T-cell receptor signals in T cells from non-obese diabetic mice. Immunol Cell Biol. 2021 Jul;99(6):656–67. doi:

10.1111/imcb.12443. M.D. designed and performed experiments, analyzed data, and wrote the manuscript; A.C. designed and performed experiments, analyzed data, and wrote the manuscript; A.A. designed and performed experiments, analyzed data, and edited the manuscript; M.E.L. designed experiments and edited the manuscript; S.F.V. designed experiments, analyzed data, and edited the manuscript; C.C.A. designed experiments, analyzed data, and edited the manuscript; H.J.M. designed experiments, analyzed data, and wrote the manuscript. S.L. designed experiments, analyzed data, and wrote the manuscript.

Dedication

To myself: thank you Adeolu for forging ahead and sailing through, despite all odds.

To my wife, Abby: thank you for your sacrifices, support, and encouragement.

To my unborn triplets: thank you for the “hugs” that kept daddy going while writing this thesis.

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List of abbreviations

AD	Autoimmune diseases
AICD	Activation-induced cell death
APC	Antigen-presenting cell
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS-1	Autoimmune polyendocrine syndrome type 1
AUC	Area under the curve
BBB	Blood-brain barrier
BCR	B cell receptor
Breg	Regulatory B cells
Btla	B and T lymphocyte attenuator
CFA	Complete Freund's adjuvant
CIS	Clinically isolated syndrome
CNS	Central nervous system
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DC	Dendritic cells
DMT	Disease-modifying therapies
DN	Double negative
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
FBS	Fetal bovine serum
FLC	Fetal liver cells
FVD	Fixable viability dye
GC	Germinal center
GFP	Green fluorescent protein
GM	Grey matter

Grb-2	Growth factor receptor bound protein-2
H&E	Hematoxylin and eosin
HAART	Highly active antiretroviral therapy
HBSS	Hanks' Balanced Salt Solution
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
HSLAS	Health Sciences Laboratory Animal Services
HVEM	Herpes virus entry mediator
Ig	Immunoglobulins
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITP	Immune thrombocytopenic purpura
ITSM	Immunoreceptor tyrosine-based switch motif
LAG-3	Lymphocyte activation gene-3
LIGHT	Lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes
LIP	Lymphopenia-induced proliferation
MBP	Myelin basic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOG	Myelin-oligodendrocyte glycoprotein
MS	Multiple sclerosis
NIAID	National Institute of Allergy and Infectious Diseases
NK	Natural killer
NOD	Non-obese diabetic
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline

PD-1	Programmed death-1
PRR	Pattern recognition receptors
PT	Pertussis toxin
pTreg	Peripheral regulatory T cells
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
RFI	Relative fluorescent intensities
RRMS	Relapsing-remitting MS
RTE	Recent thymic emigrants
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SP	Single-positive
SPMS	Secondary progressive multiple sclerosis
SRCR	Scavenger receptor cysteine-rich
TAT	Tunable activation threshold
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TEC	Thymic epithelial cells
TGF-β	Transforming growth factor-β
TNF	Tumour necrosis factor
TREC	TCR rearrangement excision circles
Treg	Regulatory T cells
tTreg	Thymic regulatory T cells
WM	White matter
WT	Wild type

Chapter 1

Introduction

1.0 Introduction

The vertebrate immune system consists of the innate (lacks fine specificity) and adaptive/acquired (specific) components that work together to protect the host from a "limitless" number of pathogenic microbes and/or toxins while avoiding excessive or misguided immune responses that pose a danger to the host. The innate immune response provides the first line of defense against invading pathogens in a generic manner through the recognition of evolutionary conserved pathogen-associated molecular patterns (PAMPs) by the pattern recognition receptors (PRRs) of innate immune cells (1). On the contrary, the adaptive arm of the immune system is orchestrated by thymus-derived lymphocytes (T cells) and bone-marrow-derived lymphocytes (B cells), whose antigen receptors are products of the recombination-activating gene (RAG)-mediated random rearrangement of T cell receptor (TCR) and B cell receptor (BCR) gene segments (2). These gene segments rearrangement produces a diverse clonotypic repertoire of antigen-specific receptors recognizing both foreign and self-antigens. Therefore, the ability of the immune system to discriminate between foreign and self-antigens is crucial, as a failure of which may trigger unwanted immune responses, such as autoimmunity. Thus, to avoid autoimmunity, the immune system must achieve a state of active, highly regulated unresponsiveness to self-antigens, known as immune tolerance.

1.1 T cell development

1.1.1 The thymus: where it all begins

The thymus is a bilobed primary lymphoid organ where T cell development occurs, and it is located directly on top of the heart in the thoracic cavity. The importance of the thymus was demonstrated in 1961 when J.F.A.P. Miller reported that most of the mice thymectomized in the

first 16 hours of life died from common laboratory infections, unlike their euthymic counterparts (3). In addition, lymphocyte counts were significantly reduced in the thymectomized mice relative to their euthymic counterparts, and allogeneic graft survival was also enhanced in the thymectomized mice relative to the euthymic mice, suggesting that the thymus participates in immune reactions (3). Furthermore, the importance of the thymus is underscored by patients with DiGeorge syndrome, or chromosome 22q11.2 deletion syndrome, who lack the genes required for thymic development (4). These patients do not develop T cells and hence develop profound immunodeficiency. Anatomically, the thymus is organized into a distinct outer cortical region and inner medulla region, each containing various functionally different thymic epithelial cells (TECs) (5). As such, the TECs are divided into cortical (cTECs) and medulla (mTECs) based on their localization within the thymic cortex or medulla, respectively (6,7). In humans, the thymus increases in size from fetal and postnatal life through puberty (8). Thereafter, the size progressively decreases with aging as a consequence of thymic involution (9). A recent study by Donna Farber showed a significant decline in thymic function occurs after the fourth decade of life (10). Males undergo thymic involution more rapidly than females, suggesting that androgens may contribute to thymic atrophy (11,12). Other possible causes of thymic involution are pregnancy, malnutrition, infections, and cancer (13). Likewise, thymic involution is observed in mice around 6 – 8 weeks, as evidenced by decreasing thymus size and cellularity (14). Nonetheless, there is evidence that T lymphocytes continue to develop throughout life, potentially developing in some extrathymic sites (15).

Marrow-derived $CD34^+$ precursor cells enter the thymus via small blood vessels called corticomedullary junctions and migrate into the outer cortex, where they become double negative (DN; $CD4^-$ $CD8^-$) thymocytes (16). These DN thymocytes transit through four developmental

stages, which are characterized by the differential expression of the activation markers CD25 and CD44 (DN1: $CD25^{-}$ $CD44^{+}$; DN2: $CD25^{+}$ $CD44^{+}$; DN3: $CD25^{+}$ $CD44^{-}$; DN4: $CD25^{-}$ $CD44^{-}$) to become double positive (DP; $CD4^{+}$ $CD8^{+}$) thymocytes. Terminal deoxynucleotidyl transferase (TdT), a polymerase enzyme found in immature thymocytes but absent in mature T cells (17), increases the junctional diversity of the TCR (18). The DP thymocytes undergo a positive selection step that is mediated exclusively by cTECs to ensure that developing T cells recognize peptides in the context of self–peptide major histocompatibility complex (self-pMHC) molecules (19). Thymocytes with TCR capable of interacting with a relatively low affinity with self-pMHC molecules expressed by cTECs undergo extensive clonal expansion, whereas thymocytes with defective TCR are neglected, and those with too high an affinity are also deleted (20–22).

Presentation of tissue-restricted antigen- major histocompatibility complex (MHC) complexes (TRA:MHC) by mTECs deletes any thymocytes with high affinities for these complexes (23,24,22) or diverts thymocytes expressing TCR with moderate affinity for the TRA:MHC complex into the regulatory T cell (Treg) lineage (25,26). This ensures that only T cells with TCR that have the appropriate level of affinity for self-pMHC yet are not reactive against self-antigens proceed to the final maturation stages and eventually exit the thymus via efferent lymphatics as functionally competent naïve $CD4^{+}$ and $CD8^{+}$ single-positive (SP) T cells. In patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or autoimmune polyendocrine syndrome type 1 (APS-1), genetic defects in Aire preclude their TECs from expressing the large variety of TRA required for the negative selection of self-reactive thymocytes and the generation of central T cell tolerance (27,28).

The thymic negative selection prevents most but not all potentially autoreactive T cells from exiting the thymus. One such potentially autoreactive T cells that escape thymic negative

selection is the myelin-oligodendrocyte glycoprotein (MOG)-specific T cells, which promotes inducible experimental autoimmune encephalomyelitis (EAE) disease in mice (29). Once T cells complete their maturation in the thymus, they exit the thymus into the peripheral circulation as recent thymic emigrants (RTE) to populate the secondary lymphoid tissues.

1.1.2 Recent thymic emigrants (RTE): the journey continues

RTE are a subset of T cells that recently completed thymic development and egress to the peripheral secondary lymphoid tissues. Thymic export of RTE alongside homeostatic proliferation helps maintain the peripheral T cell pool in healthy individuals. In the neonate, most T cells are considered to be RTE, which also helps maintain TCR repertoire diversity throughout life, despite reduced thymic output in the aged (30–32). The importance of RTE in maintaining TCR repertoire diversity is emphasized in female mice, which gradually lose reactivity to the male H-Y antigen following thymectomy (33), whereas their euthymic littermates do not. In addition, signs of premature immunosenescence, such as naïve T cell lymphopenia, and increased pools of oligoclonal memory T cells, have been reported in young adults who were thymectomized as children during cardiac surgery (34).

Our current understanding of RTE biology has benefited from novel immunological tools that helped improve the identification and isolation of RTE from their more mature but still naïve (MN) T cell counterparts. The development of a transgenic (Tg) mouse model in which live RTE can be accurately identified and isolated from their MN counterparts has allowed for a more thorough analysis of RTE biology. In these mice (Rag2p-GFP Tg), a transgene encoding green fluorescent protein (GFP) is driven under the control of the Rag2 gene promoter (35). Although Rag2 expression is turned off during the DP stage of T cell development, residual GFP signal

temporarily persists in RTE as they populate the lymphoid periphery, marking RTE as GFP+ peripheral T cells (36). Notably, the GFP signal strength correlates inversely with the time since the loss of Rag2 expression, such that the brightest cells for GFP in the periphery are the youngest RTE (37). Analyses of RTE isolated as GFP+ peripheral T cells from Rag2p-GFP mice have revealed that T cell maturation, a process once thought to be completed within the thymic microenvironment, continues for several weeks after cells have left the thymus and entered the lymphoid periphery (36).

In humans, RTE can be identified by the expression of CD31 on $CD4^+$ T cells (38) or by the appearance of TCR rearrangement excision circles (TRECs), which are nonreplicating pieces of extrachromosomal DNA left over from TCR gene rearrangement. Although TRECs are enriched in RTE, the presence of TRECs in the lymphoid periphery years after thymectomy implies that not all TRECs are RTE. Moreover, RTE help reconstitute the peripheral T cell pool following lymphoablative viral infections or therapies, such as HIV infection and chemotherapy (39). Thus, RTE constitutes a clinically important lymphocyte population to study.

1.2 Immune tolerance

Immune tolerance is a continuous state of active unresponsiveness of the immune system in an immunocompetent host to self-antigens or a particular antigen that can induce an immune response. Tolerance is an evolutionary refined mechanism that the immune system uses to prevent the occurrence of self-destruction by self-reactive T and B cells. An important precept of normal function requires that the thymus, in the case of T cells, and bone marrow, in the case of B cells, generate T and B cells capable of recognizing and mounting effective immune responses against foreign antigens but not self-antigens. Prevention or dampening of immune responses to

self-antigens is partly achieved by central tolerance mechanisms in the primary lymphoid organs where developing lymphocytes are subjected to stringent and rigorous 'filtration' processes; while further filtration or immune education continues once these developing lymphocytes seed the periphery and encounter antigens in the secondary lymphoid organs – a process known as peripheral tolerance. To better understand the immune tolerance mechanisms, I will briefly discuss some of the proposed theories of self/non-self discrimination by the immune system.

1.2.1 Theories of self/non-self discrimination

1.2.1.1 Immune tolerance is achieved in early life

The tolerance mechanisms are active processes that begin very early on in life and persist throughout a vertebrate's life. Critical to establishing tolerance is the fetal period, which has been described as an immune “window phase” based on evidence from several experimental findings. The first evidence of fetal tolerance induction was attributed to an American immunogeneticist, Ray D. Owen, who observed in 1945 that dizygotic twin calves, who shared a common placenta *in utero*, became chimeric with respect to their twin’s erythrocytes and were immunologically unresponsive to each other’s cells (40). Subsequently, Macfarlane Burnet and Frank Fenner postulated in 1949 that specific antibodies would not be produced to an antigen a host is exposed to during fetal life before the immune system is formed (41). In other words, the antigens encountered during fetal life are regarded as self, whereas subsequent antigens encountered after the fetal stage are regarded as foreign in their postulate.

The postulate by Burnet and Fenner seemed logical at the time due to the immune system's exposure to most self-antigens before birth and to non-self antigens in post-natal life. Testing this idea, Billingham, Brent, and Medawar, adoptively transferred allogeneic cells into

fetal mice, which resulted in the generation of chimerism and acceptance of a skin allograft from the donor mice in adult life, although tolerance among recipient mice was variable (i.e., not an all-or-nothing phenomenon). Their finding, published in 1953, also demonstrated that tolerance was donor-specific as recipient mice rejected skin grafts from a third-party (42,43). That same year, Milan Hašek also showed that parabiosis of chick embryos prevented the parabionts from generating humoral responses to erythrocyte alloantigens from their partner (44).

One can easily conclude from the findings above that immunological immaturity of the animals in the perinatal period skews the education of immune cells towards a tolerant phenotype when exposed to antigens during the development of the immune system – a process referred to as "actively acquired tolerance" by Medawar *et al*. (42). In 1960, Medawar and Burnet were awarded the Nobel Prize "for the discovery of acquired immunologic tolerance."

1.2.1.2 Immune tolerance is achieved early in lymphocyte ontogeny

The discovery that lymphocytes are generated throughout the life of an animal, and somatic mutation generates new B cell specificities after antigenic stimulation implies that the key factors in determining whether tolerance or immunity results cannot uniquely be the development stage of the individual. Joshua Lederberg proposed in 1959 that tolerance is induced early on in the ontogeny of lymphocytes, regardless of the age of the animal (45). According to Lederberg, "*If an antigen is introduced prior to the maturation of any antibody-forming cell, the hypersensitivity of such cells, while still immature, to an antigen-antibody reaction will eliminate specific cell types as they arise by mutation, thereby inducing apparent tolerance to that antigen. After the dissipation of the antigen, reactivity should return as soon as one new mutant cell has arisen and matured*." (45). Lederberg's proposal, however, did not

consider the need for tolerance in lymphocytes beyond their early life, especially because not all self-antigens are present in primary lymphoid organs.

1.2.1.3 Antigen-specific lymphocytes are tolerized in the absence of costimulation

Bretscher and Cohn later proposed a 2-signal model of lymphocyte activation/inactivation in 1970, where signal 1 is generated through an antigen-specific receptor, and signal 2 is delivered by an antigen-specific helper cell (46). According to the 2-signal model, the presence of signal 1 without signal 2 will result in the inactivation or death of the lymphocytes. Lafferty and Cunningham proposed a revised 2-signal model where signal 1 is the antigen-specific receptor and signal 2 is a costimulatory (positive signal) delivered by an antigen-presenting cell (APC) rather than an antigen-specific helper cell (47). In this model, lymphocytes receiving signal 1 without signal 2 become inactivated, but this model failed to explain how signal 2 could help discriminate self from non-self.

1.2.1.4 The immune system responds to pathogens or dangerous stimuli

To further elucidate how the immune system discriminates between self and non-self, Charles Janeway argued that a costimulation signal is not generated in the presence of "non-infectious self-antigen." However, the presence of "infectious non-self," which through evolution, has not only antigenic epitopes but also highly conserved PAMPs, e.g., lipopolysaccharide (LPS), would generate antigen-specific signal 1 and costimulatory signal 2, thereby ensuring immune responses to pathogenic microbes (1,48). In contrast to Janeway's proposal, Polly Matzinger argued that immune responses are not triggered by non-self but by danger/alarm signals from distressed or injured cells (49–51). Matzinger further argued that 'the

"foreignness" of a pathogen is not the important feature that triggers a response, and "self-ness" is no guarantee of tolerance' (50).

1.2.1.5 Inhibitory signals and tuning

A growing body of evidence supports the concept of negative coreceptors, which was part of the Tripartite Inactivation model earlier proposed by Sinclair and Chan (52). These negative receptors, which promote tolerogenic signals, were later termed 'co-inhibitors' by Sinclair and Anderson (53). Some of these coinhibitors are discussed below in section 1.2.3.1.

Also, the tunable activation threshold (TAT) postulated by Grossman and Paul in 1992 states that "the activation threshold is dynamically modulated by the cell's environment, and it is geared to the recent excitation history of the cell" (54). As such, "tuning" is a dynamic process that allows lymphocytes to continuously tune (i.e., calibrate) their activation threshold to avoid unwanted immune responses.

1.2.1.6 The Quorum Hypothesis

Bretscher expanded on their 1970 model and proposed in the late 90s a 2-step 2-signal model, later termed "Quorum Hypothesis" (55), with step 1 involving the two signals proposed by Lafferty and Cunningham to generate a minimum number (i.e., quorum) of helper cells that will be required in step 2 (56,57). In step 1, naïve CD4 T cells are activated when they receive signal 1 upon recognition of their cognate pMHCII complexes (e.g., antigen A) on the surface of an APC such as dendritic cells or macrophages, followed by the delivery of signal 2 upon binding of costimulatory molecules on the surface of APC to their receptors on the surface of CD4 T cells. Although the activated CD4 T cells in step 1 do not fully attain effector function,

these cells undergo clonal expansion to generate a quorum of antigen-specific cells needed in step 2 (Fig. 1.1). However, a single CD4 T cell cannot undergo sufficient clonal expansion in step 1 to achieve quorum in step two.

In step 2, the CD4 T cells primed in step 1 receive signal 1 upon recognizing antigen A on the surface of antigen A-specific B cells. Signal 2 is generated via a linked recognition of antigen A present on the surface of antigen A-specific B cell by the expanded step-1-primed antigen A-specific CD4 T cells (Fig. 1.1). The activation of step-1-primed CD4 T cells is abrogated, and these cells are deleted or inactivated should they fail to complete step 2. Thus, the requirement of an antigen-specific B cell and linked recognition of a specific antigen by the corresponding anti-specific lymphocytes in step 2 of the Quorum Hypothesis minimizes unwanted immune responses by [1] minimizing antigen A-specific CD4 T cell activation with the help provided by antigen B-specific T cells [2] blocking antigen A-specific CD4 T cell activation in the absence of antigen A-specific B cells. If, for example, antigen A above is a self-antigen and given that dendritic cells and macrophages present both self and foreign antigens on their surface, the absence or paucity of antigen A-specific B cells will reduce the chances of activating antigen A-specific CD4 T cells. Even if the A-specific B cell exists, if there are too few antigen A-specific CD4 T cells generated from step 1, then step 2 activation will not occur, instead, it will result in tolerance.

So how does the Quorum Hypothesis account for self/non-self discrimination? Given the paucity of lymphocytes in the first few days of life, antigen A-specific lymphocytes will be deleted or inactivated due to the generation of signal 1 (from self-antigen A) without signal 2. Thus, the absence of antigen-A-specific helper lymphocytes to deliver signal 2 makes it impossible to maintain antigen-A-specific CD4 T cells in the periphery. Since antigen-A is

chronically present in the host, there is a paucity of antigen-A-specific CD4 T cells to take part in step 1 of the Quorum Hypothesis. This same process governs the generation of antigen-A-specific B cells that is later required in step 2 of the Quorum Hypothesis. In contrast, the absence of foreign antigens allows the accumulation of lymphocytes specific for foreign antigens, which can form a quorum and be activated.

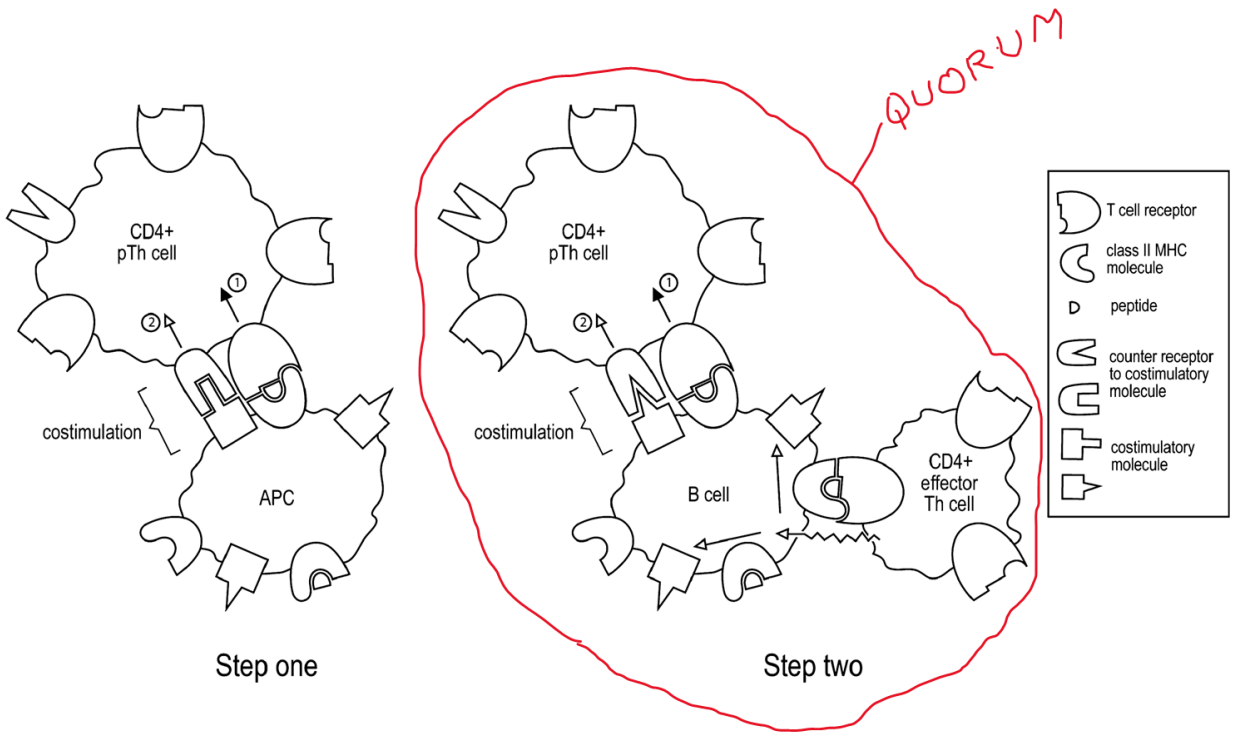


Figure 1.1. *The Two Step, Two Signal Model of CD4 T cell activation.* See the text in section 1.2.1.6 for a detailed explanation. Figure adapted from Bretscher P.A. (The activation and inactivation of mature CD4 T cells: a case for peripheral self-nonself discrimination. Scand J Immunol. 2014) (57)

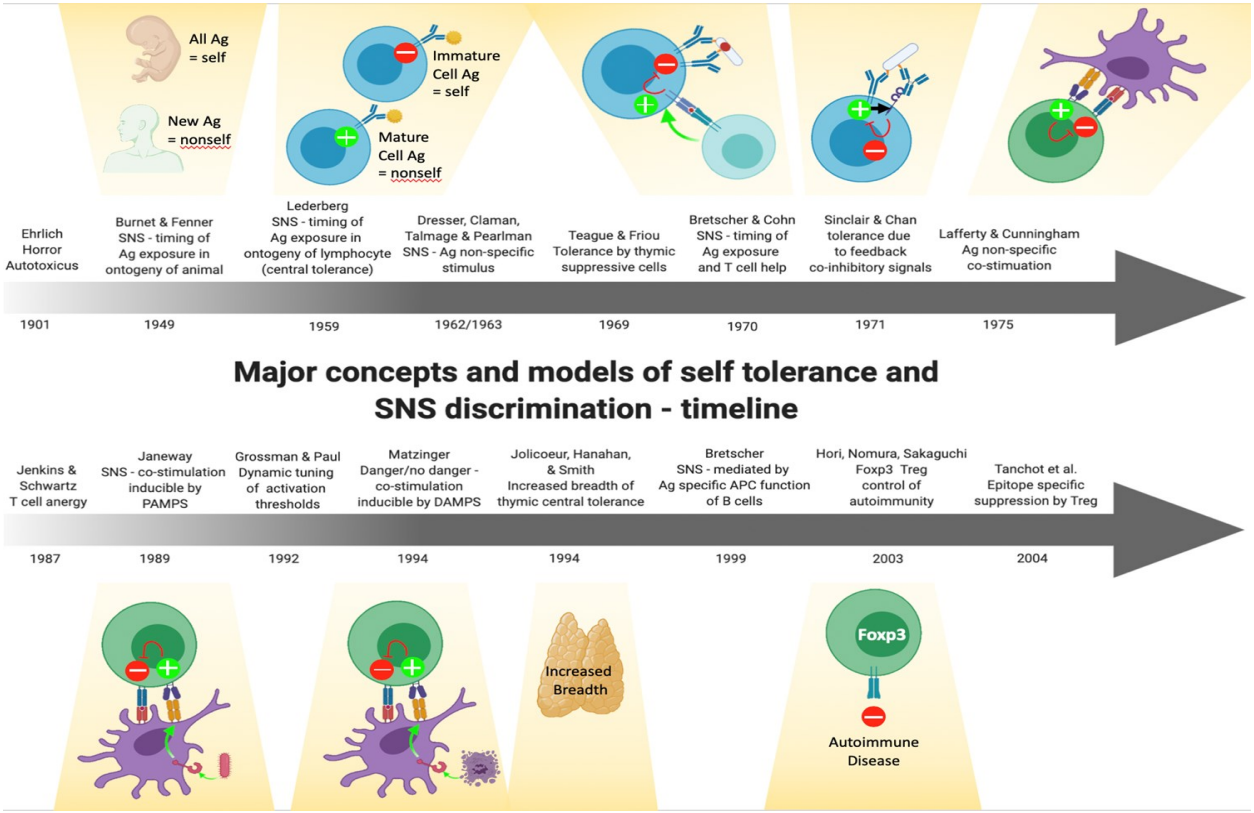


Figure 1.2. *A timeline of the origins of selected major concepts and models of tolerance and self/non-self discrimination.* See the text in section 1.2.1 for a discussion on selected models. Figure adapted from Immunologic Tolerance Chapter 32 of Paul's Fundamental Immunology 8th Ed., 2022, Wolters Kluwer Publisher.

As interesting as all the above postulates and experimental findings may sound, induction of tolerance is still an ongoing puzzle for immunologists due to an incomplete understanding of the factors contributing to immune tolerance and autoimmunity. This incomplete knowledge necessitates using several conditioning regimens prior to transplantation and life-long use of immunosuppressants post-transplantation. The development of tolerance is a complex process that includes central and peripheral mechanisms acting together to eliminate or suppress self-reactive lymphocytes.

1.2.2 Central tolerance mechanisms

The high specificity of antigen recognition is one of the attributes of the adaptive immune system that is mediated by antigen receptors expressed on the surface of T and B cells. These lymphocytes test their receptors for antigenic recognition during ontogeny in the thymus and bone marrow for T and B cells, respectively. Lymphocytes bearing self-reactive receptors are filtered through clonal deletion, inactivation, and diversion in the case of T cells; deletion and receptor editing in the case of B cells (58,59). Central tolerance is based on these filtration mechanisms in the primary lymphoid organs. Clonal deletion involves physically removing a particular T cell clone from the repertoire. Clonal deletion is mostly mediated by thymic negative selection, as discussed below. Inactivation does not eliminate the cells, but they become anergic or unresponsive in the periphery, hence unable to induce immune responses. Such is the basis of the functional impairments in transgenic CD8 T cells with TCR specific for the SIINFEKL peptide of ovalbumin in the context of H-2K^{b}, lacking the proapoptotic protein Bim. These OT-I Bim$^{-/-}$ T cells that escaped thymic clonal deletion in transgenic mice expressing ovalbumin in the pancreas (RIP-mOVA), in theory, should generate autoimmunity but did not go on to cause

autoimmunity (60). In this model, functional impairments are characterized by defects in activation, proliferation, and cytokine production, which implies anergy (60). Evidence showing that anergy can be reversed in T cells (61,62) indicates that clonally inactivated T cells could be a time bomb waiting to explode. Contrary to clonal deletion and inactivation, clonal diversion occurs when thymocytes bearing high-affinity autoreactive TCR differentiate from a conventional T cell phenotype to Treg cells (63,64). Treg cells are a subset of $CD4^+$ T cells that mediate suppression of immune responses, thereby preventing or reducing the onset of autoimmunity.

Similar to the T cells, studies showed that potentially autoreactive B cells are clonally deleted in the bone marrow once they encounter their cognate antigen during development (65,66). In addition to clonal deletion, B cell receptor editing occurs in the bone marrow (59). Unlike the soluble immunoglobulins (Ig) secreted by plasma cells, membrane-bound Ig molecules expressed on the B-cell surface function as antigen receptors. During ontogeny, B cells bearing BCR with rearranged Ig variable (V) region genes specific for autoantigens can "edit" or express alternative receptors rather than die (59). Subsequent discussion will focus on T cell tolerance because B cell activation/effector function largely depends on T cell help. However, regardless of the lead role T cells play in orchestrating immune responses, preventing the generation of autoreactive B cells should not be ignored as B cells function as APC, hence they could activate autoreactive T cells bearing TCR specific for the same autoantigen as their BCR (67,68).

Although central tolerance is highly effective in filtering out potentially autoreactive T cells, it is not an absolute checkpoint. Some potentially dangerous autoreactive T cells escape into the periphery to form part of the normal repertoire (69,70). A mouse model for multiple

sclerosis (MS), known as EAE, takes advantage of the presence of potentially autoreactive T cells in the periphery. Inoculation of mice with MOG_{35-55} emulsified in complete Freund's adjuvant drives the clonal expansion of myelin-specific T cells that traffick to and attack myelin sheath insulating neurons of the central nervous system (29). This necessitates the need for a second checkpoint, the peripheral tolerance mechanisms, to prevent autoreactivity in T cells that have escaped central tolerance filtration.

1.2.3 Peripheral tolerance mechanisms

1.2.3.1 The role of coinhibitors

1.2.3.1.1 B and T lymphocyte attenuator (Btla; CD272)

As the name denotes, B and T lymphocyte attenuator (Btla) is a member of the CD28 superfamily that is expressed on B cells, T cells, and also on natural killer (NK) cells, macrophages, and dendritic cells (DCs) (71–74). Btla gene is located in the q13.2 region of chromosome 3 and consists of 5 exons and the protein shares structural and functional similarities with coinhibitors like programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (75). The cytoplasmic domain of Btla protein contains growth factor receptor-bound protein-2 (Grb-2) association motif, immunoreceptor tyrosine-based switch motif (ITSM), and immunoreceptor tyrosine-based inhibitory motif (ITIM) (75). The tumour necrosis factor (TNF) receptor family member, herpes virus entry mediator (HVEM), which is broadly expressed on hematopoietic cells, including T cells, macrophages, and DCs, is the Btla ligand (76–78). Ligation of Btla with HVEM, following TCR signaling, induces tyrosine phosphorylation of its ITIM and recruitment of the Src homology domain 2 (SH2)-containing protein tyrosine phosphatases, SHP-1/SHP-2, which mediate immunosuppressive effects (75,79).

Recently, Btla signaling was shown to regulate germinal center (GC) formation, Treg homeostasis, and gut microbial homeostasis (80).

Although HVEM is the only identified ligand for Btla, HVEM also interacts with CD160, LIGHT (TNFSF14), lymphotoxin-a, and synaptic adhesion-like molecule 5 (SALM5) (81–83). Interestingly, CD160 and Btla compete for the same binding site within the CRD1/CRD2 region of HVEM, while LIGHT independently binds the opposite side of HVEM within the CRD2/CRD3 region (76,82,84). In naïve T cells where Btla and HVEM are coexpressed, Btla can interact with HVEM in cis to form a heterodimeric complex, thereby preventing other co-signaling molecules from binding to HVEM and stimulating the NF-kB signaling pathway, thus maintaining the tolerance of T cells (85). Alternatively, when Btla and HVEM are expressed on different cells, Btla can interact with HVEM in trans (85).

Germline Btla knockout mice go on to develop spontaneous autoimmune hepatitis (AIH)-like disease in adult life, which is characterized by elevated transaminase levels, interface hepatitis, and hepatic spotty necrosis (86). In a follow-up study, Btla was shown to play a protective role in autoimmune diseases. Whereas AIH-like disease developed in $Btla^{-/-}$ MRL-lpr/lpr (lupus-prone) mice in the absence of Fas-dependent signaling, the $Btla^{+/+}$ MRL-lpr/lpr mice were spared of disease (87). Further supporting a protective role of Btla in autoimmunity, $Btla^{-/-}$ mice exhibit enhanced antigen-specific IgG responses and sensitivity to EAE (75,88). In addition, anti-Btla mAb has been shown to delay the onset of autoimmune diabetes in non-obese diabetic (NOD) mice, increasing the proportion of $Foxp3^{+}PD\text{-}1^{+}CD4^{+}$ Tregs, and enhancing the production of anti-inflammatory cytokines (89).

The role of the Btla-HVEM pathway in the pathogenesis of autoimmune diseases in humans is not fully understood, but single nucleotide polymorphisms (SNPs) C+800T SNP, and

590C in the human Btla gene have been associated with rheumatoid arthritis (RA) susceptibility in Chinese and Japanese populations, respectively (90,91). Also, it was reported that the percentage of $CD19^+/Btla^+/IL\text{-}10^+$ cells was significantly reduced in MS patients relative to healthy controls. In line with this finding, fingolimod-induced disease remission was associated with a significant increase in regulatory B cells (Bregs), $CD19^+/Btla^+$, and $CD19^+/Btla^+/IL\text{-}10^+$ B lymphocytes (92). Similarly, a significant decrease in the percentages of peripheral unstimulated $Btla^+$ $CD4^+$ T cells was reported in patients with active systemic lupus erythematosus (SLE) (93). Furthermore, the capacity of Btla to inhibit T cell activation in SLE was impaired due to a poor Btla recruitment to the immunological synapse following T cell stimulation (94).

1.2.3.1.2 Programmed death-1 [PD-1 (CD279)]

PD-1 is a member of the CD28/B7 superfamily originally cloned from a T cell hybridoma line as an apoptosis-associated gene (95). PD-1 is a 55-kDa type 1 transmembrane protein with a cytoplasmic tail containing ITIM and ITSM. In humans, PD-1 is encoded for by the *Pdcd1* gene consisting of 5 exons located on chromosome 2 and by the *Pdcd1* gene on chromosome 1 in mice (96). Its expression on naïve T cells is extremely low, but it is expressed on activated T cells, B cells, and myeloid cells (96). PD-1 interaction with any of its ligands PD-L1 (B7-H1 or CD274) or PD-L2 (B7-DC or CD273) dampens T cell responses by inhibiting membrane-proximal TCR signaling, downstream T cell proliferation, cytokine production, and CD28 costimulation (97,98). However, PD-1-mediated inhibition can be overcome by strong TCR stimulation and CD28 costimulation (99,100). PD-L1 is constitutively expressed in hematopoietic and non-hematopoietic cells, whereas PD-L2 expression is limited to hematopoietic cells (96). The tyrosine motifs in the cytoplasmic tail of PD-1 become

phosphorylated upon T cell activation and ligation with its ligands, followed by the binding of the SH2 domain-containing tyrosine phosphatase 1 (SHP-1) and SHP-2 to the ITIM and ITSM motifs of PD-1. This interaction downregulates TCR signaling and dephosphorylates other signaling intermediates (101). However, a mutation in the ITSM motif of PD-1 abrogates the inhibitory function of PD-1, contrary to the negligible effects of mutations in the ITIM, indicating that the ITSM alone plays a significant role in PD-1-mediated inhibition (101). PD-1 interaction with SHP-2 appears to be stronger than with SHP-1, suggesting that PD-1 functions by recruiting SHP-2 and perhaps SHP-1 to the TCR complex (102). Downstream of TCR signaling, PD-1 signaling inhibits Akt activation, PI3K activity, and phosphorylation of ZAP-70, CD3ζ, and PKCθ (103). Upon TCR ligation and T cell activation, PD-1 molecules are redistributed from uniform cell surface expression to concentrate at the immunological synapse upon TCR ligation (104)

The inhibitory function of PD-1 was discovered from the finding that *Pdcd1*$^{-/-}$ mice developed autoimmunity in late life (105). Disease phenotype in the *Pdcd1*$^{-/-}$ mice depends on mouse strain. The BALB/c mice develop lethal dilated cardiomyopathy, C57BL/6 mice develop lupus-like autoimmune disease, and exacerbated diabetes was seen in the NOD mice (105–107). Also, mice develop an aggressive and accelerated incidence of EAE when PD-1 signaling is blocked *in vivo* (108). Likewise, SNPs in *Pdcd1* in humans have been associated with autoimmune diseases, including SLE, type 1 diabetes (T1D), ankylosing spondylitis, and RA (109–112). Our group and others have shown the importance of PD-1 signaling in establishing tolerance in newly generated T cells (113,114). Similarly, PD-1 was shown to maintain tolerance in the periphery by silencing the activation of high-affinity autoreactive MOG-specific CD4 T cells in the periphery (115). T cells exposed to chronic antigen stimulation such as in

human immunodeficiency virus (HIV) infection or tumour microenvironment undergo a progressive loss of effector function and become “exhausted” (116). Chronic expression of PD-1 on these viral-specific and tumour-specific T cells is associated with exhaustion (116). As such, blockade of the PD-1 signaling in tumour-specific T cells was shown to improve anti-tumour immune responses (117) and is now used clinically in the tumour therapy.

1.2.3.1.3 CD5

CD5 (Leu-1 in humans and Lyt-1 in mice) is a 67-kDa type I transmembrane glycoprotein and a member of the highly conserved group B scavenger receptor cysteine-rich (SRCR) protein superfamily (118). CD5 is expressed early in T cell ontogeny and is detectable at the pre-TCR complex stage (119). CD5 expression levels increase with progression in development, correlating with that of CD3 expression (119). Interestingly, Lyt-1 (i.e., CD5) was initially considered a marker to distinguish the Treg cell population before the FoxP3 discovery (120). Subsequently, it was reported that $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells expressed higher levels of CD5 than $CD4^+$ $CD25^+$ $Foxp3^-$ conventional T cells (121). Although B cells do not typically express CD5, a small subset of B cells, B1a cells ($IgM^{hi}IgD^{lo}CD23^-CD5^+$), expresses CD5 (122,123). These B1a cells are typically localized in the mouse peritoneum and human bone marrow (124). Also, CD5 is highly expressed in the regulatory B cells (Breg) (125,126). As a negative regulator of T/B cell receptor signaling, CD5 expression is often associated with preventing non-desirable autoreactivity (124).

The proposed ligands for CD5 include CD72 (127), IL-6 (128,129), gp40–80 (130–132), gp150 (133), gp200 (134), IgV_H framework region (135), and the CD5 itself (136); however, their physiological relevance in interacting with CD5 is not clearly understood. CD5 is recruited

to the immune synapse between T cells and APCs, where it co-localizes with TCR/CD3 complexes to dampen TCR signals, such as Ca^{2+} mobilization induced by antigen recognition and the extent of tyrosine phosphorylation, without affecting the formation and stability of conjugates (137,138). The cytoplasmic domain of CD5 has four tyrosine residues at positions 378, 429, 441, and 463, including several putative sites for serine/threonine phosphorylation (139,140). CD5 clustering with the TCR may be essential to trigger the phosphorylation of the cytoplasmic tyrosine residues. However, how phosphorylation of the cytoplasmic tyrosine residues leads to the inhibition of TCR signaling is not entirely understood. Effector molecules positively or negatively regulating TCR signaling, such as SHP-1, rasGAP, CBL, CK2, ZAP70, and PI3K, have been reported to associate with tyrosine phosphorylated CD5 (141–143). This suggests that CD5 phosphorylation could be necessary to recruit inhibitory signaling molecules in the proximity of the TCR and/or to sequester activation kinases away from the TCR complex, thereby reducing the TCR signaling strength.

In contrast to its cytoplasmic tail (144,145), where the pseudo-ITAM domain is likely to play an essential role (138,142), it appears that the extracellular domain of CD5 is dispensable for CD5-mediated TCR signaling inhibition (146). However, a role for the extracellular domain of CD5 in coinhibition cannot be totally excluded since soluble CD5-Fc molecules (147) or neutralizing monoclonal antibodies against the extracellular domain (148) can block the inhibitory effect of CD5 in the EAE-induced mice or the tumour microenvironment. This also suggests the participation of a putative ligand in these effects.

Although CD5 is a coinhibitory receptor, the germline $CD5^{-/-}$ C57BL/6 (B6) mice are free of autoimmunity, unlike their PD-$1^{-/-}$ and $Btla^{-/-}$ counterparts (86,105,149). Interestingly, Schuster *et al*. recently showed that transiently silencing the CD5 gene in the NOD background

resulted in T cell-dependent wasting disease driven by chronic gut immune dysregulation. These CD5 knockdown NOD mice also had aggressive acute experimental colitis with elevated IL-17A secretion. However, the disease was reversed in these NOD mice once the CD5 expression was restored, supporting the role of CD5 in tuning (i.e., calibrating) the T cell activation signal (150). Also, we recently showed that NOD mice have an MHC-dependent increase in $CD4^+$ thymocytes and mature T cells that express lower levels of CD5 relative to the B6 mice. In contrast, T cell-intrinsic mechanisms lead to higher levels of CD5 on peripheral $CD8^+$ T cells from NOD relative to B6 mice, suggesting survival advantages of peripheral $CD8^+$ T cells in NOD mice (151). CD5 has also been implicated in the pathogenesis of MS, although studies on the role of CD5 in MS have been inconsistent, probably due to the complexity of MS. Studies showed that an increased percentage of $CD5^+$ B cells in cerebrospinal fluid (152,153) and blood (154) has been associated with increased risk of early conversion to MS in clinically isolated syndrome patients (CIS) with oligoclonal IgG bands (OCGB). In contrast, Niino *et al*. reported a significantly lower percentage of $CD5^+$ B cells in the blood of secondary progressive multiple sclerosis (SPMS) patients relative to the healthy control (155). Also, the emergence of $CD5^+$ B cells has been associated with a lower prevalence of anti-myelin antibody production (156). Meanwhile, in the mouse model of MS, EAE, evidence showed a protective role for CD5 or $CD5^+$ B cells (157–159). Surprisingly, when EAE was induced in the $CD5^{-/-}$ mice, they exhibited significantly delayed EAE onset and decreased disease severity (147). It was speculated in this study that protection from EAE in the $CD5^{-/-}$ mice was due to the prosurvival activity of CD5 in T cells via engagement of the serine/threonine kinase CK2 that binds to the receptor (147). Confirming this, mice expressing CD5 unable to bind and activate CK2 were also resistant to EAE, which was attributed to

elevated activation-induced cell death (AICD), and decreased populations of cells co-expressing IFN-γ and IL-17 (160).

1.2.3.2 The role of regulatory T cells (Tregs)

Seminal studies demonstrated that thymectomy of otherwise healthy neonatal mice led to inflammation and severe organ-specific autoimmune pathologies such as oophoritis (161–163), gastritis with megaloblastic anemia (164), thyroiditis (165), and orchitis (166). This indicated the involvement of a thymus-derived population in the control of self tolerance, and disease was also reproduced in rats thymectomized in early life (167). Adoptive transfer of cell suspensions from the spleen of BALB/c mice depleted of $CD25^+$ cells into athymic nude mice induced autoimmunity that affected several organs, and reconstitution of $CD4^+CD25^+$ cells within a limited period after transfer of $CD4^+CD25^-$ cells prevented these autoimmune developments in a dose-dependent fashion (70). These experiments demonstrated that T cells are capable of generating autoimmune disease and can also inhibit it. That led to the hypothesis that in the periphery of normal mice, there are two populations of $CD4^+$ T cells: one potentially capable of inducing autoimmunity and a second population of 'suppressor' cells that would inhibit autoreactive T cells.

Constitutive expression of IL-2 receptor α -chain (CD25) and the transcription factor forkhead box protein 3 (Foxp3) are used to identify Treg cells in mice and humans (70,168–170). However, because CD25 is also expressed by activated $CD4^+$ T cells, the absence of the IL-7 receptor α -chain (CD127) is sometimes used in place of CD25 to identify Treg cells in humans (171). Based on the site of development, Treg cells are classified into two major subsets: [1] thymic Treg (tTreg) cells, previously called "natural" Treg (nTreg) cells, that develop in the

thymus, and "peripheral" Treg (pTreg) cells that arise in the periphery from $CD4^+$ $Foxp3^-$ conventional T cells (172). Currently, no definitive markers distinguish these two Treg cell populations from each other. Treg cells function by cell-contact mechanisms involving specific cell-surface receptors and by secretion of inhibitory cytokines such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and IL-35 (173).

Mutations in Foxp3 result in the spontaneous development of severe autoimmunity with a scurfy phenotype in mice (174) and immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans (175,176). Indeed, Foxp3 is necessary for the development, maintenance, and function of tTreg cells (168–170). Interestingly, depletion of Treg cells has been reported to cause acute T cell lymphopenia in secondary lymphoid organs and non-lymphoid tissues (177), which may contribute to T cell-mediated autoimmune disorders. Treg cell impairment has been associated with autoimmune diseases, such as T1D (178–180), MS (181,182), SLE (183), myasthenia gravis (184), and RA (185).

1.2.3.3 Other factors that contribute to tolerance induction and maintenance

1.2.3.3.1 Autoimmune regulator (Aire)

Aire was discovered as a result of intensive research into a human disease called APS-1 or APECED. Individuals with this disease usually have severe candidiasis, infection of the mucosal surfaces, and multiorgan autoimmune attack (186). Mutations in the gene encoding a transcription factor, Aire, was identified in 1997 as responsible for this multiorgan autoimmunity (187,188). Aire is expressed primarily in mTECs, and it promotes ectopic expression and presentation of TRAs in the thymus (189). The level of TRA expression in Aire knockout mice is significantly reduced, and these mice show signs of multiorgan autoimmunity similar to APS-1

(23,190). Insulin hormone normally secreted by the β-cells of the pancreatic islets is also expressed in the thymus as a result of Aire. Studies show that the loss of one allele of Aire reduced thymic expression of the endogenous insulin gene and ultimately resulted in diabetes due to a significant increase in the frequency of islet-reactive CD4 T cells that escaped thymic deletion in TCR transgenic mice (191,192). While this is true for insulin, the absence of Aire does not significantly alter the expression levels of autoantigens such as glutamic acid decarboxylase, C-reactive protein, and α-fodrin (23,193). Worthy of note is the observation that disease phenotype is altered by the genetic background of mice with Aire mutation. Pancreatitis and thyroiditis were exclusively seen in the NOD.Aire$^{-/-}$ and SJL.Aire$^{-/-}$ mice, whereas gastritis was observed in BALB/c.Aire$^{-/-}$ mice, but the incidence was rare in the B6.Aire$^{-/-}$ mice (194). Providing evidence for the role of Aire in the neonatal period, Guerau-de-Arellano *et al.* used a doxycycline-regulated transgene to control Aire expression and found that Aire expression during the neonatal period is essential for self tolerance but that it is dispensable beyond weaning age. The autoimmunity triggered by Aire deficiency was attenuated by the transfer of previously tolerized T cells. Also, lethal irradiation (to mimic physiological lymphopenia in neonatal life) during Aire turn-off recreated the disease in adult mice (195).

1.2.3.3.2 Terminal Deoxynucleotidyl Transferase (TdT)

TdT directs the addition of nontemplated-coded nucleotides (N additions) between the recombining ends of immunoglobulin and TCR genes, thereby significantly increasing the junctional diversity of the TCR and BCR repertoires (196,197). However, lymphocytes generated in neonatal mice express receptors with significantly less diversity than the adult lymphocyte repertoires due to the delayed expression of TdT during neonatal life (198). While

TdT-mediated lymphocyte receptor diversity improves antigenic recognition and neutralization of foreign antigens, studies suggest it also plays a role in generating autoimmunity. Using the lupus-prone (NZB×NZW)F_1 mice, Conde *et al*. observed that, although TdT deficiency did not reduce autoantibody production and hypergammaglobulinemia in (NZB × NZW)F_1 $TdT^{-/-}$ mice compared to $TdT^{+/+}$ wild type (WT) control mice, the absence of TdT abrogated the occurrence of autoimmune nephritis in the (NZB × NZW)F_1 $TdT^{-/-}$ mice and also improved their survival (199). To examine whether the reduction of disease in the (NZB × NZW)F_1 as a result of TdT deficiency could be extended to a different mouse strain, Feeney *et al*. generated another lupus-prone mouse model (MRL-Fas^{lpr}) that is deficient in TdT. Surprisingly, MRL-Fas^{lpr} $TdT^{-/-}$ mice had increased lifespan, decreased incidence of skin lesions, and much lower serum levels of anti-dsDNA, anti-chromatin, and IgM rheumatoid factors. Also, the generalized hypergammaglobulinemia characteristic of MRL-Fas^{lpr} mice was significantly reduced in the absence of TdT (200). In the C57BL/6 (B6) H-2^b genetic background, Molano *et al*. showed that the absence of TdT reduced the production of anti-DNA antibodies and rheumatoid factors in the normally autoimmune-prone B6-Fas^{lpr} mice (201). In the NOD mice, TdT deficiency in the NOD $TdT^{-/-}$ mice prevented diabetes and reduced insulitis compared to NOD $TdT^{+/+}$ WT control mice (202). Although Robey *et al*. observed no increase in $CD4^+CD25^+$ regulatory T cells present in the spleen and thymus of age-matched NOD $TdT^{-/-}$ and $TdT^{+/+}$ mice, the knowledge that TdT expression is absent in the neonatal mice suggests that the diversity and function of Treg cells generated during this period are limited (202).

All the above findings indicate that the absence of TdT prevents or reduces the incidence of autoimmune diseases regardless of genetic background. However, it appears that the expression of TdT could also limit the generation of autoimmunity. Motheaten mice (me/me), a

model for autoimmune and inflammatory diseases, develop chronic dermatitis, hypergammaglobulinemia, and produce autoantibodies. Immune complexes are also deposited in the thymus, skin, lungs, and kidneys of these mice. Landreth *et al*. discovered a complete absence of TdT^+ cells in the bone marrow, thymus, and spleen of C3HeB/FeJ me/me mice compared to C3HeB/FeJ WT mice (203).

1.3 Autoimmunity: from non-self to self recognition

Developing T cells are positively selected in the thymus upon their TCR interaction with low-affinity self-pMHC, which are also required for their maintenance in the periphery. This suggests that all T cells are moderately "autoreactive." To maintain homeostasis, the immune system is tightly regulated such that it allows immune responses to foreign antigens (non-self) and neoantigens while maintaining tolerance to self-antigens. Despite this tight regulation, a break in tolerance can generate autoimmunity, where the host's immune system recognizes and attacks self-antigens as foreign, a phenomenon originally called "horror autotoxicus" by Paul Ehrlich (204). Autoimmune diseases (AD) are largely mediated by T or B cells, although a broad spectrum of immune cells, cytokines, chemokines, and other factors are involved in the autoimmune process. There is a wide array of AD broadly classified as systemic (e.g., RA, SLE, scleroderma, and dermatomyositis) or organ-specific (Graves' disease, T1D, and MS) (Fig. 1.3). Although most AD have an idiopathic (unknown) origin, genetic predisposition [e.g., expression of class II gene products, human leukocyte antigen (HLA)-DR3 and DR4, in T1D (205)], environmental stimuli [e.g., Epstein-Barr virus (EBV) infection in MS (206,207)], and defective immune regulation [e.g., defects in Treg phenotype and suppressive capacity in T1D (208)], generally contribute to AD incidences (209). In addition, AD appear to be gender biased as the

female-to-male ratio of AD incidences ranges from 10:1 to 1:1 (210,211). Although the underlying factors for the increased AD predisposition in females is not clearly understood, sex hormones are thought to play a role in the gender bias (211). Like many complex diseases, AD vary significantly in their clinical manifestations but are generally believed to transition through sequential phases of initiation, propagation, and resolution (209). Despite recent advances in AD treatment (212), these treatments are not curative, and prevalence of AD continues to be on the rise (213,214). Susceptibility to AD is high among first-degree relatives with known genetic predisposition, and higher in monozygotic twins (215).

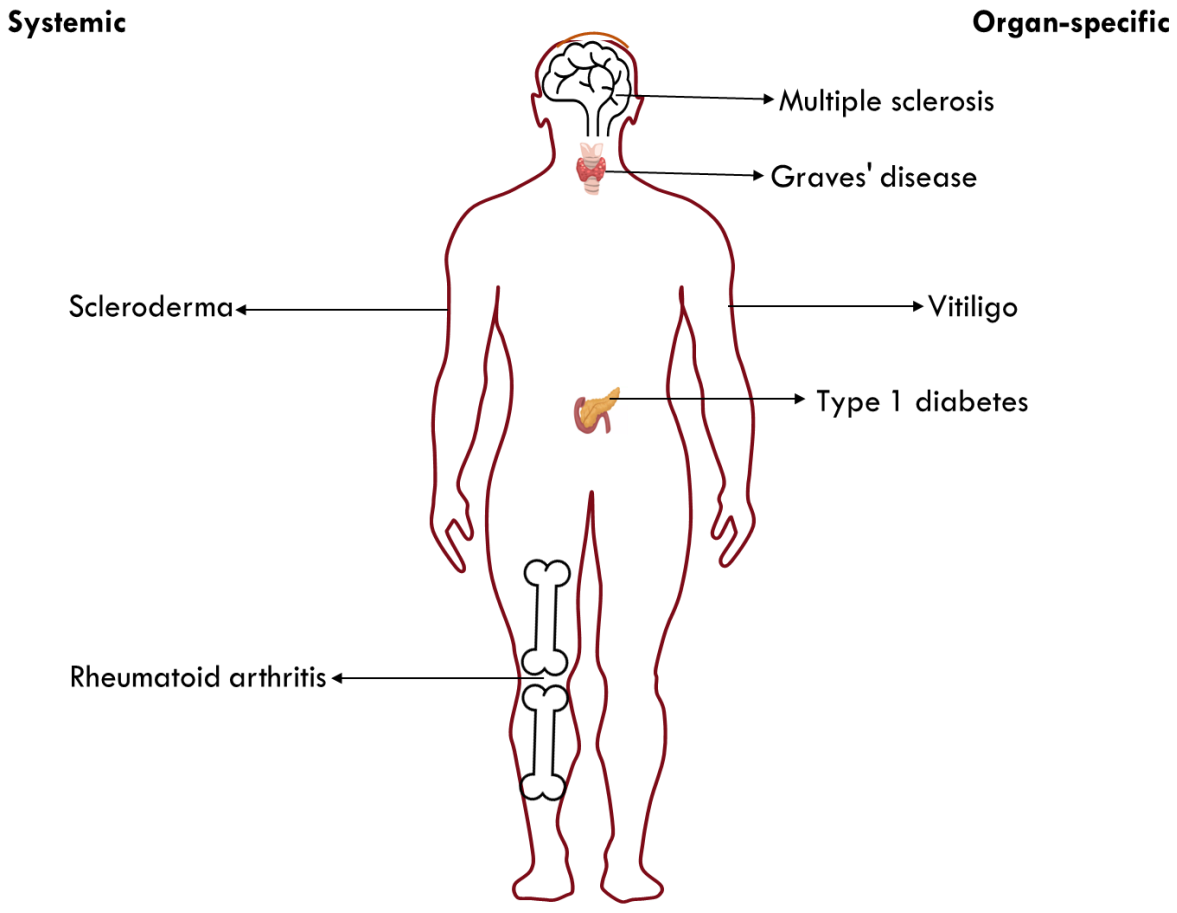


Figure 1.3. *Some of the organs commonly targeted by autoimmune disease*

1.3.1 Multiple sclerosis (MS)

MS is a chronic, immune-mediated, inflammatory demyelinating, and neurodegenerative disease of the human central nervous system (CNS). MS disease is characterized by inflammatory infiltrates, demyelinating plaques, and axonal damage (216). A recent report shows 2.8 million people live with MS worldwide (217). MS subtypes include [1] relapsing-remitting MS (RRMS), which is the most common subtype, characterized by flare-ups (relapses) of disease interrupted by intervals of partial or complete neurological recovery (remission); [2] secondary progressive MS (SPMS), which is the eventual outcome of RRMS is characterized by gradual but steady neurological deterioration (progression) without remissions; [3] primary progressive MS (PPMS), which is characterized by continuous neurological deterioration right from disease onset, most frequently without superimposed relapses; [4] progressive-relapsing MS (PRMS) is a rare MS subtype that is characterized by an early stage progression similar to the PPMS but superimposed with acute clinical attacks with or without full recovery (218,219). As with most AD, MS is more prevalent in females than males (about 3:1) (220).

MS was considered an autoimmune disease primarily affecting the white matter (WM) (221). In active lesions, MS is characterized by the presence of T cells, activated macrophages, microglia, compromised blood-brain barrier (BBB) function, and concomitant levels of cytokines and chemokines that drive demyelination and axonal injury. However, it has become increasingly evident that grey matter (GM) pathology is also an important aspect of the disease. In fact, GM pathology is now believed to begin early and even precede WM damage (222,223). GM pathology is characterized by severe demyelination, axonal transections, neuronal, glial, and synaptic loss, but without significant lymphocyte infiltration, complement deposition, and BBB

disruption (223). In addition, GM damage in MS increases with disease duration and may overlap with processes secondary to WM disease (216).

In both MS and EAE, B cells also contribute to disease pathogenesis through the production of myelin-specific autoantibody, and antigen presentation to myelin-specific T cells (224). In addition, B cells serves as a reservoir for EBV, which promotes MS (225). In contrast, B cells play a protective role in MS pathogenesis via their capacity to produce anti-inflammatory cytokines such as TGF-β1, IL-35, and IL-10 (226).

Despite several years of research, the aetiology of the disease is still unknown, but genetic susceptibility [e.g., expression of HLA-DR15 (227)] and environmental factors such as vitamin D deficiency (228), viral infections [e.g., EBV in humans (206,207), and gamma herpesvirus 68 (γHV-68) in mice (229–231)] play a significant role in disease initiation (232). Current management approaches for MS are focused on treating acute attacks, ameliorating symptoms, and reducing biologic activity through disease-modifying therapies (DMTs), which modify the course of MS through suppression or modulation of immune function. DMTs act by [1] exerting anti-inflammatory activity primarily in the relapsing phase of MS; [2] reducing relapse rate; [3] reducing the accumulation of lesions as seen in MS magnetic resonance imaging (MRI); [4] stabilizing, delaying, and in some cases modestly improving, disability (233). Interferon beta (IFNβ) and glatiramer acetate were the first approved MS therapies, and these well-tolerated medications modestly reduce the frequency of MS relapses (234,235). Meanwhile, DMTs with higher-efficacy used as second-line treatment in the clinic include fingolimod (FTY720; sphingosine-1-phosphate inhibitor), alemtuzumab (anti-CD52 mAb), ocrelizumab (anti-CD20 mAb), natalizumab (α4β1 integrin inhibitor), among others (233).

1.3.2 Experimental Autoimmune Encephalomyelitis (EAE): an animal model of MS

The use of human tissues for studying MS has been severely hampered by limited access to post-mortem tissues and ethical issues affecting the collection of CNS tissue biopsies, hence the need for developing appropriate MS animal models. However, given the complexity of MS and its' unclear aetiology, no animal model recapitulating the exact clinical features together with the pathophysiology of MS has been successfully developed to date. EAE is an animal model of MS currently used in research. EAE was originally described in the 1930s when Rivers and colleagues observed that vaccinating rhesus macaques against rabies with a viral preparation contaminated with rabbit brain proteins occasionally resulted in paralysis (236). Rivers *et al*. later used normal rabbit brain extracts and correlated the disease-inducing capacity of the extract with its amount of myelin (237). Induction of EAE generally requires strong immune adjuvants such as complete Freund's adjuvant (CFA), supplemented with heat-inactivated *Mycobacterium tuberculosis* (M. tuberculosis, strain H37Ra), to act as an antigen depot (238). In addition, purified toxic protein from the *Bordetella pertussis* (B. pertussis) bacterium (PTx) is used as an auxiliary adjuvant to break down the BBB and enhance inflammatory infiltration (239).

Immunization of EAE with spinal cord homogenate or CNS peptides/polypeptides [e.g., MOG] is referred to as 'active EAE,' which generally results in high disease incidence. Disease onset, characterized by weight loss and ascending paralysis or neurological deficit, is observable from about 9 – 12 days post immunization (240). In contrast, EAE immunization through 'adoptive' transfer (AT-EAE) of autoaggressive, myelin-specific T lymphocytes generated in donor animals by active immunization into syngeneic recipients is known as 'passive' or AT-EAE (241). Although active immunization is preferentially used to examine the induction phase of EAE, AT-EAE has facilitated the identification of the key role of myelin-reactive T cells in

the disease pathogenesis and variables underlying the 'effector' phase of the disease. In addition, MOG is commonly used in the induction of EAE due to its ability to elicit encephalitogenic T cell response, severe demyelination associated with the deposition of immunoglobulin (Ig) and complement component 9 (242). In contrast, disease induction with myelin components such as myelin basic protein (MBP) or proteolipid protein (PLP) is characterized by severe inflammation but little or no demyelination (242).

Although immunopathological mechanisms in EAE do provide proof-of-principle for MS studies, EAE itself is very heterogeneous in terms of induction methods, clinical/pathological features, and amenability to treatment, all of which add to its complexity. As such, translating EAE studies to clinical MS is dependent on using appropriate models to answer the specific scientific or clinical questions being addressed. Table 1 below shows a summary of different EAE models induced in mice.

Table 1. EAE induction in different mouse strains

Mouse strain	**Induction regimen**	**Disease phenotype (reference)**
C57BL/6	MOG protein or MOG_{35-55} peptide in CFA + Pertussis toxin (PT)	Progressive or acute (243)
NOD	MOG_{35-55} peptide in CFA + PT	Chronic progressive (244)
Biozzi	MOG protein or MOG_{92-106} peptide in CFA + PT	Chronic relapsing (245)
SJL	PLP or $PLP_{p-139-151}$ peptide in CFA + PT	Chronic relapsing (246)
PL/J	MBP or MBP Ac1-11 (Ac1-9) in CFA + PT	Acute monophasic (247)

1.4 T cell lymphopenia and lymphopenia-induced proliferation (LIP)

In addition to mounting protection against infection and tumour antigens, a competent and healthy immune system must also protect against self-reactivity. Therefore, it is imperative that T cell homeostasis is maintained such that the T cell pool is kept at a relatively constant number with a variety of specificities and tolerance to self-antigens (248,249). In steady state, the size and composition of the peripheral T cell pool is tightly regulated partly due to the continuous thymic output of RTE, partly due to the homeostatic proliferation of residual T cells and partly by competition for resources; T cells proliferate in the periphery and replace those that die by apoptosis (248). T cell interaction with 'resources' such as low-affinity self-pMHC, and homeostatic cytokines like IL-7 and IL-15, are essential for T cell survival in the periphery (250,251). The tonic (basal) signals generated from TCR interaction with low-affinity self-pMHC are insufficient to induce overt T cell activation under normal, lymphoreplete conditions. However, during lymphopenia, when the number of lymphocytes is low or reduced due to various triggers and the ratio of resources to available T cells is increased, these same interactions drive extensive lymphocyte division through a process known as LIP, to replenish the available T cell niche.

1.4.1 LIP and autoimmunity

Perturbations of the lymphoid compartment due to viral infections [e.g., HIV and SARS-CoV-2] (252–254), radiotherapy (255), chemotherapy [e.g., Cyclophosphamide treatment] (256,257), stress and physical exertion, promote LIP. Interestingly, LIP also occurs naturally during the neonatal period (258,259). While LIP is a physiological response to restoring depleted lymphoid compartments during lymphopenia, it was postulated that the clonal expansion of high-

affinity, autoreactive T cell clones during lymphopenia might promote autoimmunity in a susceptible genetic background (260,261). The autoimmune diabetes-prone NOD mice have reduced T cell numbers compared to nonautoimmune strains such as WT BALB/c mice (262). However, diabetes was prevented in the NOD mice when T cell numbers were increased either by adoptive transfer of excess T cells from isogeneic donors or by immunization with complete (heat-killed mycobacterium-containing) Freund's adjuvant (CFA) (262). Likewise, the diabetes-prone biobreeding rats, which develop spontaneous autoimmune diabetes, are severely T cell lymphopenic partly due to reduced thymic output and partly due to increased T cell apoptosis in the periphery (263,264). Acquisition of effector memory phenotype is often seen in T cells undergoing LIP, which could also contribute to the induction of autoimmunity (265–267).

Further supporting the role of LIP in the generation of autoimmunity, studies from our group showed that reconstitution of the lymphoid compartment of $Rag^{-/-}$ mice with PD-$1^{-/-}$ hematopoietic stem cells (HSC) induced a spontaneous lymphoproliferative disease with massive lymphocytic infiltrates in multiple non-lymphoid organs shortly after newly generated T cells populated the periphery (113). Massive $CD4^{+}$ and $CD8^{+}$ T cell infiltration were seen in the kidneys, liver, lung, esophagus, pancreas, heart, and eyes of the recipient mice. Also, concentration levels of serum cytokines/chemokines including IFN-γ, IL-13, TNF-α, IP-10, MIG, MCP-1, and VEGF were significantly higher in PD-$1^{-/-}$ HSC relative to WT HSC recipients (113). In stark contrast, $Rag^{-/-}$ mice reconstituted with WT HSC were disease-free (113). Similarly, reconstitution of $Rag^{-/-}$ mice with $Btla^{-/-}$ HSC or thymocytes resulted in autoimmune disease (268). In both the recipients of PD-$1^{-/-}$ HSC or $Btla^{-/-}$ HSC, the disease manifested as hunched appearance, ruffled fur, reduced mobility, diarrhea, dermatitis, and ocular lesions (113,268). Interestingly, recipients of PD-$1^{-/-}$ HSC or $Btla^{-/-}$ HSC were spared of

autoimmune disease when LIP was reduced via the addition of competitor cells or through a paucity of lymph node stroma (113,268).

HIV infection usually results in HIV-mediated $CD4^+$ T cell lymphopenia (269). However, HIV-infected patients experience a rapid rebound of $CD4^+$ T cells following treatment with highly active antiretroviral therapy (HAART), which then induces the generation of inflammatory disorders known as immune reconstitution inflammatory syndromes (IRIS) (270). Also, many type 1 diabetes patients transplanted with islets were no longer insulin-independent after a year and soon developed autoimmune disease post-transplantation (271). T and B cell lymphopenia was observed in these islet transplant recipients as a result of the immunosuppressive drugs given as part of the islet transplantation protocol. T cell LIP in these islet transplant recipients resulted in the accumulation of memory $CD45RO^+$ T cells, highly enriched in autoreactive glutamate acid decarboxylase (GAD)-specific T cell clones (271). In addition, CD4 T cells containing TREC (i.e., newly generated CD4 T cells) are substantially reduced in RA patients (272). Thymic output was observed to be decreased in these RA patients, which resulted in increased LIP of $CD4^+$ and $CD8^+$ T cells in the periphery and the generation of oligoclonal T cell repertoire (272).

1.4.2 LIP and autoimmunity following anti-CD52 mAb treatment

Current management approaches for MS treatment involve the use of DMTs (discussed in section 1.3.1). One of such DMTs is alemtuzumab (anti-CD52), which is a recombinant, humanized IgG1 kappa monoclonal antibody that targets the CD52 antigen on lymphocytes (273,274). CD52 is a cell surface receptor highly expressed on T and B lymphocytes and to a lesser extent on NK cells, monocytes, and macrophages, with little or no expression on

neutrophils, plasma cells, and HSC (216,274). Since B and T lymphocytes are the key drivers of inflammation in MS, treatment with alemtuzumab depletes peripheral B and T lymphocytes, decreasing relapses in relapsing-remitting multiple sclerosis (RRMS) (273). Alemtuzumab is administered by intravenous infusion in 2 treatment cycles, with the first cycle involving administration of 12 mg/day infusion for 5 consecutive days (60 mg total dose); followed by a repeat dosage after 12 months, as in the first cycle, but for 3 consecutive days (36 mg total dose). Following anti-CD52-mediated lymphopenia, T cell recovery is slower relative to B cells, reaching approximately 30 and 45% of baseline at 12 months for CD4 and CD8 T cells, respectively (275–277). Similarly, in mice, B cells recover faster than T cells. However, T cell repopulation post anti-CD52 treatment is quite rapid in mice, taking several weeks in contrast to more than a year in humans (278,279). Lymphocyte repopulation post anti-CD52 therapy involves the homeostatic expansion of mature lymphocytes that escaped depletion and production of RTE and new B cells from precursors (280,281).

Anti-CD52 treatment comes with an associated risk of autoimmune disease, which is hypothesized to be promoted by the LIP of the repopulating lymphocytes. Cases of thyroid disorders such as hyperthyroidism (Graves' disease), hypothyroidism, and thyroiditis are the most frequent secondary autoimmunity reported in patients treated with alemtuzumab. In phase III clinical trial, thyroid-associated adverse events were seen in 18% of patients treated with alemtuzumab (282). Other autoimmune adverse events in phase III clinical trials include neutropenia, hemolytic anemia, pancytopenia, and agranulocytosis (282,283). In the 5-year follow-up of the CAMMS223 study, thyroid dysfunction occurred in 34% (73 of 216) of the alemtuzumab group [39%, 42 of 108 receiving 12 mg and 29%, 31 of 108 receiving 24 mg (Fisher's exact test, p=0.015)] (284). As a precautionary measure, thyroid function tests are

recommended before anti-CD52 treatment, every 3 months post treatment, and up to 48 months after the last infusion of alemtuzumab (285).

1.4.3 Regulatory T cells control LIP of T cells

Although LIP promotes autoimmunity, the paucity of autoimmunity in lymphopenic mice and humans suggests that other factors in concert with lymphopenia promote autoimmunity development. Krupica *et al.* proposed a two-hit model of lymphopenia-potentiated autoimmunity, where they described lymphopenia as factor 1 and 'other insults' as factor 2 (270). An example of the 'other insults' is the paucity of $CD4^+CD25^+$ Treg cells as seen in thymectomized 3-day-old (3dTx) mice, which is associated with the development of multiorgan autoimmunity (286). This suggests that the LIP of newly generated T cells or RTE, which are the T cell subset predominant in neonatal mice (31,30), was dysregulated in the 3dTx mice due to a deficiency of Treg cells. Indeed, evidence showed that RTE are the preferential precursors of Treg cells differentiated in the periphery (287,288). The sparse evidence mentioned above indicates that regulation of LIP is important in preventing autoimmunity, especially during the neonatal period, where physiological lymphopenia occurs and RTE predominates (31,258,259).

1.4.4 Coinhibitors negatively regulate the LIP of T cells

Coinhibitory receptors regulate the LIP of T cells. In addition to the deficiency of Treg cells in 3dTx mice, as mentioned previously, loss of coinhibitory molecules like CTLA-4 also results in early-onset autoimmunity and mortality in neonatal mice due to dysregulated LIP (289,290). Indeed, CTLA-4 controls the LIP of conventional T cells and regulatory T cells (291,292). A recently published study from our group showed that PD-1 controls the LIP of T

cells in response to tonic signals generated through the TCR interaction with self-pMHC-II and is independent of IL-7 signals (293). Also, PD-1 was shown to control LIP in RTE. Since LIP is often associated with the acquisition of effector memory phenotype, PD-$1^{-/-}$ RTE mostly became effector memory T cells during LIP relative to their WT counterparts (113). In this study, the frequency of effector memory phenotype in the PD-$1^{-/-}$ RTE was reduced when co-transferred with WT competitor cells to $Rag^{-/-}$ mice. Similarly, Btla was shown to control LIP in RTE as most of the $Btla^{-/-}$ RTE populating the periphery of $Rag^{-/-}$ hosts acquired the effector memory phenotype, unlike their populating WT counterparts (268). Btla signaling also negatively regulates the LIP of $CD8^+$ T cells (294). In addition, lymphocyte activation gene 3 (LAG-3), an MHC class II binding CD4 homologue, negatively regulates T cell LIP by Treg cell-dependent and independent mechanisms (295). An immunomodulatory cytokine, TGF-β, has also been shown to regulate LIP in T cells during the physiological lymphopenia in neonatal mice (296).

1.5 Overview and objectives of my thesis

Studies on PD-1 from our group showed that this coinhibitory receptor serves to restrain RTE responses in the context of LIP to prevent autoimmunity (113). Indeed, adult lymphopenic mice reconstituted with PD-$1^{-/-}$ HSC or thymocytes developed multiorgan autoimmunity, which was absent in the recipients of WT HSC or mature splenic PD-$1^{-/-}$ T cells (113). Of note, autoimmunity mediated by PD-$1^{-/-}$ RTE in lymphopenic mice [1] could be blocked when recipient mice are lymph node deficient or competitor cells are co-transferred (113); [2] is mediated by $CD4^+$ T cells and dependent on MHCII (113,293,297); [3] is independent of IL-7 and IL-17 signaling, and the presence of gut microbiota (288,293); [4] is not due to defective quality or decreased quantity of Tregs (288). The finding that gut microbiota appeared

dispensable in our PD-1 autoimmunity model suggests that other stimuli are involved. A subsequent study from our laboratory demonstrated that PD-1 regulates RTE responses to tonic signaling generated by their interactions with low-affinity self-ligands (293). This was also confirmed in the finding that PD-$1^{-/-}$ RTE but not their established mature splenic T cell counterparts induced autoimmunity during lymphopenia, suggesting the need for PD-1 in establishing tolerance early on in T cell ontogeny (113). The autoimmune potential of newly generated PD-$1^{-/-}$ T cells persists after they are exported to the periphery (297). Similarly, Btla signaling was shown to play an important role in establishing tolerance in newly generated T cells (268). This finding prompted our group to postulate RTE as an important cofactor in the generation of lymphopenia-potentiated autoimmunity (298). In previous studies, germline knockout HSC or thymocytes were used, thus obscuring the interpretation of the data as to which cells needed to lack these coinhibitors to generate autoimmunity. Thus, we asked in **Chapter 2** what cells needed to lack Btla signaling to generate autoimmunity. *We hypothesized that the constitutively expressed Btla is needed during or post thymic selection to establish tolerance in RTE.* We found that the inducible deletion of Btla in RTE generated disease in adult lymphopenic mice, and in contrast, there were no signs of disease in the recipients of WT RTE, suggesting that Btla is required in newly generated T cells at the RTE stage to prevent lymphopenia-induced autoimmunity. Supporting this, we showed that recipients of neonatal PD-$1^{-/-}$ splenocytes, which are mostly RTE, developed autoimmunity that is absent in the recipients of adult PD-$1^{-/-}$ splenocytes, which are mostly matured established T cells. Furthermore, we showed that disease caused by Btla$^{-/-}$ RTE is mediated by CD4 SP T cells and dependent on MHCII but not MHCI.

Since both Btla and CD5 exert their inhibitory function through lymphocyte activation-induced recruitment of SHP-1 to their cytoplasmic tail tyrosine residues, we explored the relationship between these coinhibitors in **Chapter 3**. We found an inverse relationship between CD5 and Btla expression levels in T cell ontogeny and that this reflects a causal relationship and not simply an association between these receptors.

In **Chapter 4**, we asked if RTE only promote lymphopenia-potentiated autoimmunity or if they also control it, given they have the potential to reduce lymphopenia. Since reconstitution of the lymphoid compartments in adult Rag$^{-/-}$ mice with PD-1$^{-/-}$ RTE generates autoimmunity in the recipient mice, *we hypothesized that depleting lymphocytes in the periphery of adult PD-1$^{-/-}$ mice induced with EAE would promote disease relapse when RTE repopulate the periphery.* Surprisingly, EAE-induced PD-1$^{-/-}$ mice did not have disease relapse when treated with the lymphopenia-inducing anti-CD52. Instead, anti-CD52 treatment leads to permanent disease remission. This, we attributed to the rapid repopulation of the peripheral lymphoid niche with contributions from the thymus and the remaining residual lymphocytes that escaped anti-CD52 depletion and are now undergoing LIP to provide sufficient competitors such that new RTE are well controlled. Suggesting a need for input from the thoracic thymus*, we hypothesized that RTE from the thoracic thymus are important in limiting lymphopenia-potentiated autoimmunity.* Consistent with our hypothesis, two-thirds of thymectomized B6 mice had an EAE relapse post anti-CD52 treatment, while no surgery and sham surgery euthymic controls remained relapse-free. We conclude that RTE are an important subset of T cells, and a functional thoracic thymus is important in controlling lymphopenia-potentiated autoimmunity.

Chapter 2

Btla and PD-1 signaling are needed in recent thymic emigrants to block autoimmunity

2.1 Introduction

To generate lymphocyte repertoire diversity, developing T cells in the thymus express receptors generated from random recombination of TCR gene segments (299). The binary outcome of this stochastic TCR gene segment rearrangement is the generation of T cells bearing receptors that recognize foreign antigens or self-antigens, with the latter potentially promoting autoimmunity. Hence, a critical function of the immune system is to discriminate self from non-self, failure of which will result in autoimmunity. Although T cell activation is determined by the interaction of TCR with specific antigenic peptide–MHC complex, the functional outcome of the T cell response is influenced by costimulatory and coinhibitory signals. Such coinhibitory receptors include cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed death-1 (PD-1), B and T lymphocyte attenuator (Btla), and several others (300). Many of these coinhibitory receptors have ITIM and ITSM in their cytoplasmic domain, which they use to recruit protein tyrosine phosphatases to dampen lymphocyte activation (300,301). Indeed, mice deficient in PD-1 develop late-onset autoimmunity similar to lupus (105). Also, polymorphism in the PD-1 gene has been associated with AD such as RA (112), MS (302), type 1 diabetes (110), and SLE (109) in humans. Hence, a balance between costimulation and coinhibition is required to maintain immune homeostasis (53).

In contrast to the late-onset autoimmunity in the PD-$1^{-/-}$ mice (105), adult lymphopenic mice reconstituted with PD-$1^{-/-}$ HSC or thymocytes developed multiorgan autoimmunity, which was absent in the recipients of mature splenic PD-$1^{-/-}$ T cells (113). A subsequent study showed that PD-1 is important in regulating LIP of newly generated T cells (RTE) (293).

Perturbations of the lymphoid compartment due to viral infections [e.g., HIV and SARS-CoV-2] (252–254), radiotherapy (255), chemotherapy [e.g., Cyclophosphamide treatment]

(256,257), stress and physical exertion, promote LIP. Interestingly, LIP also occurs naturally during the neonatal period (258,259). While LIP is a physiological response to restoring depleted lymphoid compartments during lymphopenia, it was postulated that the clonal expansion of high-affinity, autoreactive T cell clones during lymphopenia might promote autoimmunity in a susceptible genetic background (260,261). However, the paucity of autoimmunity in lymphopenic mice and humans suggests that other factors in concert with lymphopenia promote the development of autoimmunity.

Similar to the PD-1 study, an unpublished study from our group showed that another critical coinhibitor, Btla, is essential in controlling lymphopenia-induced autoimmunity by newly generated T cells during lymphopenia (268). Btla (CD272) is broadly expressed on hemopoietic cells such as T cells, B cells, dendritic cells, macrophages, and NK cells (73,75). Herpesvirus entry mediator (HVEM) was identified by two independent groups as the unique ligand for Btla (76,78). Like Btla, HVEM is also broadly expressed on hematopoietic cells such as T and B lymphocytes, NK cells, and dendritic cells (303). Btla contains ITIM and ITSM motifs as well as a Grb-2 association motif in its cytoplasmic domain (75). The tyrosine residues in the ITIM and ITSM can be phosphorylated and recruit SHP1/SHP2 phosphatases to inhibit T cell function when the T cell is activated, and Btla is ligated by HVEM (75,76,79). Studies from our group showed that PD-1 and Btla are needed to establish tolerance in newly generated T cells in the setting of lymphopenia. However, in these studies, HSC or thymocytes from whole body germline PD-1 or Btla knockout mice were used as a source of newly generated T cells, hence making it difficult to ascertain what cells need to express the coinhibitors and at which stage of T cell ontogeny they are important. In this study, we employed the inducible Cre-lox system (304,305) to delete genes for Btla or PD-1 only in HSC or T cells that otherwise had the potential

to express these coinhibitors and showed that Btla and PD-1 are required to block autoimmunity at the recent thymic emigrant stage. In addition, we also asked which cells cause autoimmunity when Btla is lacking and showed that the $CD4^+$ T cells are required for disease.

2.2 Materials and Methods

2.2.1 Animals

Mice used in this study included male and female B6.129S7-*Rag1*^{tm1Mom}/J ($Rag^{-/-}$), B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J ($Foxp3^{EGFP}$) (306,307), B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$/J ($B6^{Cre/ERT2+}$), C57BL/6J (H-2^b; B6), and B6.129S2-Ciita^{tm1Ccum}/J ($CiiTA^{-/-}$) mice that were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The C57BL/6 H-$2K^{btm1}$-H-$2D^{btm1}$N12 ($K^bD^{b-/-}$) were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) Exchange Program (NIH: 004215) (308). We generated the $CiiTA^{-/-}$ $Rag^{-/-}$ mice and $K^bD^{b-/-}$ $Rag^{-/-}$ mice by crossing the $Rag^{-/-}$ mice with $CiiTA^{-/-}$ mice and $K^bD^{b-/-}$ mice, respectively. B6.Rag2p^{GFP} mice (35,36) were kindly provided by Pamela Fink (University of Washington, Seattle, WA), and our lab previously generated B6.Rag2p^{GFP}-PD-$1^{-/-}$ mice (297). C57BL/6-$Btla^{-/-}$ (abbreviated as $Btla^{-/-}$) were originally provided by Kenneth Murphy (Washington University, St. Louis, MO), and C57BL/6-$Pdcd1^{-/-}$ [abbreviated as PD-$1^{-/-}$; backcrossed 11 generations to C57BL/6; originally generated by Prof. T. Honjo and colleagues (105)] mice were bred at the University of Alberta. The B6.$Foxp3^{EGFP}$ x $Btla^{-/-}$ mice were generated by crossing $Foxp3^{EGFP}$ and $Btla^{-/-}$ strains. Similarly, $Foxp3^{EGFP}$ x PD-$1^{-/-}$ mice were generated by crossing $Foxp3^{EGFP}$ and PD-$1^{-/-}$ strains (288). The B6.PD-$1^{fl/fl}$ mice were purchased from Taconic Biosciences (Rensselaer, NY, USA) and B6.$Btla^{fl/fl}$ mice were kindly provided by John R Šedý and Carl Ware (Sanford Burnham Prebys Medical Discovery Institute, La Jolla,

United States). All mice were between 7 – 12 weeks old at the time they were used in experiments unless otherwise indicated. Animal care was in accordance with the Canadian Council on Animal Care guidelines. The studies were performed under Animal Use Protocol 00000215, approved by the Animal Care and Use Committee Health Sciences of the University of Alberta. Mice were housed under clean conventional housing conditions at the University of Alberta Health Sciences Laboratory Animal Services (HSLAS) facilities.

2.2.2 Genotyping

Genomic DNA was extracted from ear notch samples using DNeasy Blood and Tissue Kit (Qiagen), following manufacturer's instruction.

Protocol Name: Cre/ERT2 genotyping

Primer Name	**Sequence 5' --> 3'**	**Primer Type**
21306	CTG GCT TCT GAG GAC CG	Wild type Forward
oIMR9021	CCG AAA ATC TGT GGG AAG TC	Wild type Reverse
oIMR3621	CGT GAT CTG CAA CTC CAG TC	Mutant Forward
oIMR9074	AGG CAA ATT TTG GTG TAC GG	Mutant Reverse

Master mix	**x10 samples**
2.5µL of 10x PCR buffer	25µL
1µL of 50Mm $MgCl_2$	10µL
2µL of 2.5mM dNTPs	20µL
1µL of primer oIMR3621	10µL
1µL of primer oIMR9074	10µL
2.3µL Molecular grade H_2O	23µL
0.2µL Taq polymerase	2µL

Add 10µL of the master mix above to 15µL template DNA for a 25µL reaction

*For WT reaction, replace the primers in the above master mix with 21306 and oIMR9021.

Thermocycler program

STEP	**TEMP °C**	**TIME**	**NOTE**
1	94.0	2min	
2	94.0	20sec	
3	65.0	15sec	-0.5 C per cycle decrease

4	68.0	10sec
5	repeat steps 2 – 4 for 10 cycles (Touchdown)	
6	94.0	15sec
7	60.0	15sec
8	72.0	10sec
9	repeat steps 6 – 8 for 28 cycles	
10	72.0	2min
11	10.0	hold

Expected result
WT = 198bp
Mut = 150bp

Protocol Name: Floxed Btla genotyping

Primer Name	Sequence 5' --> 3'	Primer Type
1R	CAG TGC TCA TTT CCG CCT A	Reverse
10R	TAC CGC TCC ATC ACC ACA	Reverse
13F	TGT GCC TTT GGC TGC TT	Forward

Master mix	x10 samples
2.5µL of 10x PCR buffer	25µL
1µL of 50mM $MgCl_2$	10µL
2µL of 2.5mM dNTPs	20µL
1µL of primer 1R	10µL
1µL of primer 10R	10µL
2µL of primer 13F	20µL
0.3µL Molecular grade H_2O	3µL
0.2µL Taq polymerase	2µL

Add 10µL of the master mix above to 15µL template DNA for a 25µL reaction

Thermocycler program

STEP	TEMP °C	TIME
1	94.0	2min
2	94.0	30sec
3	53.0	30sec
4	72.0	1.5min
5	repeat steps 2 – 4 for 30 cycles	
6	72.0	10mins
7	10.0	hold

Expected result
WT = 290bp
Flox = 189bp
KO = 650bp

Protocol Name: Floxed PDCD1 genotyping

Primer Name	Sequence 5' --> 3'	Primer Type
18323_11115_34	TCCATGGAAACCCTACACTCC	Forward
18323_11115_35	GGCAGAATATCGTAAGCAGTTCC	Reverse
18323_11116_39-NULL	CGTGTCAGGCACTGAAGAGATC	Reverse

Master mix	**x10 samples**
2.5µL of 10x PCR buffer	25µL
1µL of 50mM $MgCl_2$	10µL
2µL of 2.5mM dNTPs	20µL
1µL of primer 34	10µL
1µL of primer 35	10µL
1µL of primer 39-Null	10µL
1.25µL Molecular grade H_2O	12.5µL
0.25µL Taq polymerase	2.5µL

Add 10µL of the master mix above to 15µL template DNA for a 25µL reaction

Thermocycler program

STEP	TEMP °C	TIME
1	95.0	15min
2	94.0	45sec
3	60.0	1min
4	72.0	1min
5	repeat steps 2 – 4 for 35 cycles	
6	72.0	5mins
7	10.0	hold

Expected result
WT = 318bp
Flox = 444bp

2.2.3 Thymocyte and T cell transfer

Thymocytes or splenocytes [containing 3 – 5 x 10^6 SP T cells] from the indicated mice were injected into adult $Rag^{-/-}$ mice. For experiment using neonatal splenocytes, 1 x 10^6 SP T cells pooled from three neonatal mice (n=1; splenocytes from nine neonatal mice pooled in batches of three is n=3) were adoptively transferred to adult $Rag^{-/-}$ mice. Briefly, thymuses and

spleens were removed from the donors and mashed with glass slides on ice to make a single-cell suspension, followed by filtration with a 70 µm cell strainer (Fisherbrand™). CD4 and CD8 SP T cells were isolated from a single cell thymocyte suspension using EasySep™ Mouse Naïve $CD4^+$ T Cell Isolation Kit (# 19765) and EasySep™ Mouse Naïve $CD8^+$ T Cell Isolation Kit (# 19858), respectively, from STEMCELL Technologies (Vancouver, Canada). These kits isolate SP T cells by immunomagnetic negative selection. Cells were isolated according to manufacturer's instructions. The purity of the sorted cell population was > 96%. Viability was assessed by trypan blue exclusion and > 90% viable cells were used for experiments.

2.2.4 Hematopoietic stem cell transfer

Fetal liver cells (FLC), a source of HSC, were harvested from embryonic day 14 – 16 $B6^{Cre/ERT2+/-}$ or $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ fetuses. A single-cell suspension was made on ice by gently pipetting the fetal livers and filtration through a 70 µm cell strainer (Fisherbrand™). Viability was assessed by trypan blue exclusion and > 90% viable cells were used for experiments. Seven-week-old male and female $Rag^{-/-}$ mice were used as recipients, and each recipient received 20 x 10^6 FLC, followed by tamoxifen injection as described below.

2.2.5 Tamoxifen-induced conditional deletion

To induce Btla or PD-1 deletion, adult $Rag^{-/-}$ recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ or $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ FLC or thymocytes were intraperitoneally injected with 1.4 mg tamoxifen (Sigma-Aldrich) in corn oil [+5% (vol/vol) ethanol] on days 0, 1, 3, 5, and 6. Recipients of control $B6^{CreERT2+/-}$ FLC or thymocytes also received tamoxifen injection.

2.2.6 Definition of disease

Signs of disease included loss of weight, hunched appearance, piloerection, diarrhea, and skin or ocular lesions. Recipient mice were no longer considered disease-free when two or more of the above signs were evident or mice had lost ≥ 20% of baseline body weight. In addition, for mice to be classified as diseased, disease signs must persist for at least two weeks. Immunohistochemistry staining was performed on tissues from multiple organs collected from recipient mice.

2.2.7 Immunohistochemistry

At the experiment end-point, when mice were euthanized, mice were transcardially perfused with 0.01M (1x) phosphate buffer saline (PBS), followed by 4% paraformaldehyde (PFA) in PBS. Harvested non-lymphoid organs (heart, kidneys, liver, and lungs) were immersed in 4% PFA in PBS overnight at 4°C and then transferred into fresh 30% sucrose in PBS every day for two consecutive days at 4°C. Tissues were embedded in optimum cutting temperature compound (TissueTek OCT, Sakura Finetek, 4583), frozen on liquid nitrogen, and cryosectioned (Leica CM1950) at –20°C with a thickness of 10 µM on glass slides. Following three washes in 1x PBS, tissue sections were blocked in 10% normal goat serum for 1 h at room temperature and incubated in rat anti-mouse CD4 (1:200; MCA2691; Bio-Rad) antibody overnight at 4°C. Slides were washed in PBS-Tween (0.5% Tween 20 in 1x PBS) and incubated in goat anti-rat IgG Alexa Fluor® 488 (1:200; A11006; Life Technologies) antibody for 45 min at room temperature. To visualize cellular nuclei, tissue sections were counterstained with Vectashield mounting medium with DAPI (Vector Laboratories, H-1200) (309,310). Spleens from $Rag^{-/-}$ and WT mice were used as the CD4 negative and positive control, respectively. The negative control for

primary antibody specificity was omitting the primary antibody in the staining. Immunofluorescence images were taken using a Leica DM IRB Microscope and Open Lab software. Confocal microscopy images were taken with a WaveFX microscope from Quorum Technologies with Olympus IX-81 motorized base and Yokogawa CSU 10 spinning disk confocal scan head. An average of three images per section were examined.

2.2.8 Flow cytometry

Fluorochrome conjugated antibodies for flow cytometry staining used in this study were purchased from ThermoFisher Scientific: murine anti-TCRβ (H57-597), CD4 (RM4-5), CD8α (53-6.7), Btla (6F7), and PD-1 (J43). GFP expression was also analyzed in mice expressing the GFP transgene. In the Rag2p^{GFP} mice, GFP expression, under the control of recombination-activating gene 2 (Rag2) gene promoter, is turned on when the Rag proteins are transiently expressed during the late DN and DP stages of T cell development (35). GFP expression persists through T cell egress into the peripheral circulation for up to 3 weeks even though Rag2 expression has been terminated intrathymically (36). This enables a reliable assessment of RTE. Peripheral blood samples, thymocytes, and splenocytes were stained after incubation with FcR block, which was a cocktail of anti-CD16/32 antibody (2.4G2; Bio Express, West Lebanon, NH) and mouse, rat, and hamster sera, to reduce background staining and in turn, increase the specificity of antibody binding. The antibody staining was done at 4°C for 20 minutes, followed by washing and resuspension in Hanks' Balanced Salt Solution (HBSS) supplemented with 2% fetal bovine serum (FBS). A BD LSR II (BD Biosciences) with FlowJo software was used for data acquisition and analyses.

2.2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data of biological replicates are depicted as mean ± standard error. Data were statistically analyzed using Student's t-test with Welch's correction. Disease onset/incidence was compared by the Kaplan–Meier method. Probability values reported for survival curve comparisons were calculated using the Mantel-Cox method.

2.3 Results

2.3.1 Loss of Btla in HSC generates autoimmunity in lymphopenic mice

Previous studies by our group showed that Btla and PD-1 are needed to establish tolerance in newly generated T cells (113,268,288,297). However, in these studies, FLC or thymocytes from germline Btla or PD-1 knockout mice were adoptively transferred to $Rag^{-/-}$ mice and it was determined that these coinhibitory receptors are needed to block autoimmunity in newly generated T cells during lymphopenia (113,288,297). Since Btla expression has been seen on non-hematopoietic cells in some settings (311,312), we reasoned that germline knockout of Btla does not rule out the possibility that Btla is needed in non-hematopoietic cells to generate HSC capable of self tolerance. To address this, we crossed the $B6.Btla^{fl/fl}$ strain to a tamoxifen-inducible Cre recombinase expressing strain, $B6^{Cre/ERT2+/+}$, to generate $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ (Fig. 2.1). FLC from embryonic day 14 – 16 $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ were adoptively transferred to adult $Rag^{-/-}$ mice, followed by five doses of tamoxifen injection intraperitoneally to induce Btla gene deletion in the transferred cells. The wildtype (WT) control group received FLC from $B6^{Cre/ERT2+/-}$ mice (Fig. 2.2 A). FLC was used as a source of HSC in this experiment, allowing

for the deletion of Btla in T cell progenitors pre-thymic selection. T cells were detected in the peripheral blood of FLC recipient mice around four weeks post FLC transfer, which coincides with disease onset in recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC (Fig. 2.2 B). All the recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC (3/3) were diseased before the experiment end-point while their $B6^{Cre/ERT2+/-}$ counterparts (3/3) remained free of disease (Fig. 2.2 B). In addition to the diseased mice having diarrhea, piloerection, hunched appearance, and dermatitis, these mice also had a reduced body weight (Fig. 2.2 B). Flow cytometry evaluation of the lymphocytes populating the periphery of recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC showed that these cells were Btla negative while lymphocytes populating the periphery of $B6^{Cre/ERT2+/-}$ FLC recipients expressed Btla (Fig. 2.2 C). Flow cytometry analysis of the T cells at experiment end-point showed that 83 ± 10% of the CD4 SP T cells in the recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC had acquired the effector memory ($CD44^{hi}$ $CD62^{lo}$) phenotype in comparison to 54 ± 7% of the CD4 SP T cells in the $B6^{Cre/ERT2+/-}$ FLC recipients (Fig. 2.3). On the other hand, a slightly higher proportion of the CD8 SP T cells (88 ± 7%) in the recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC and had acquired the effector memory ($CD44^{hi}$ $CD62^{lo}$) phenotype in comparison to the CD8 SP T cells (78 ± 4%) in the $B6^{Cre/ERT2+/-}$ FLC recipients (Fig. 2.3). These findings are consistent with the findings from the previous study from our lab (313) and it confirmed that Btla signaling is needed in newly generated T cells to block autoimmunity during lymphopenia. Our data also showed that loss of Btla in HSC alone is sufficient for the generation of autoimmunity in the FLC recipient mice.

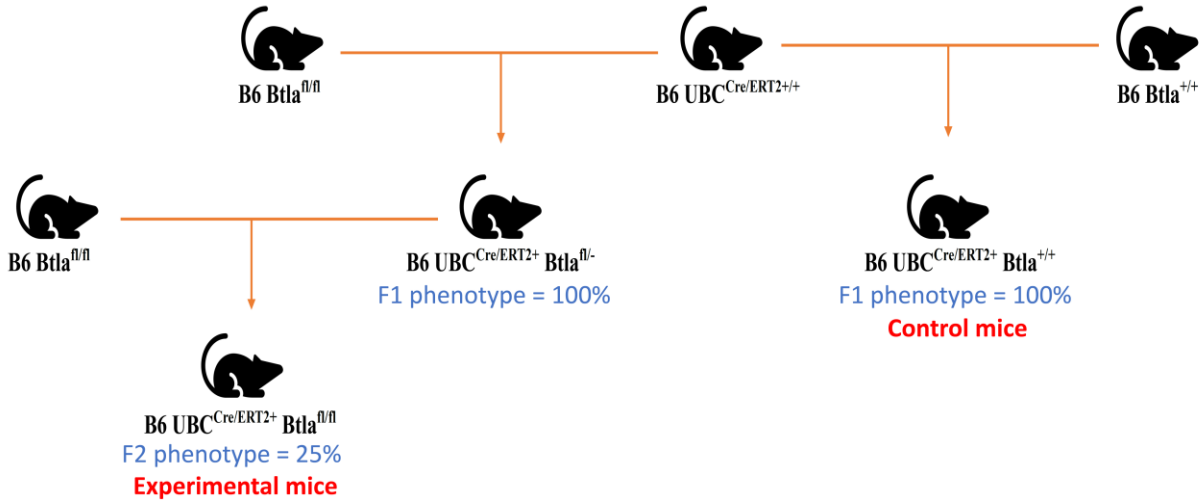


Figure 2.1. *Breeding strategy for the B6 UBC$^{Cre/ERT2+}$Btla$^{fl/fl}$ mice.* The B6 Btla$^{fl/fl}$ strain was crossed to a tamoxifen-inducible Cre recombinase expressing strain, B6 UBC$^{Cre/ERT2+/+}$. The resulting F1 B6 UBC$^{Cre/ERT2+}$Btla$^{fl/-}$ was backcrossed to B6 Btla$^{fl/fl}$ to generate B6 UBC$^{Cre/ERT2+}$Btla$^{fl/fl}$. For the control WT mice, B6 UBC$^{Cre/ERT2+/+}$ mouse was crossed to B6 Btla$^{+/+}$ mice to generate B6 UBC$^{Cre/ERT2+}$Btla$^{+/+}$ mice.

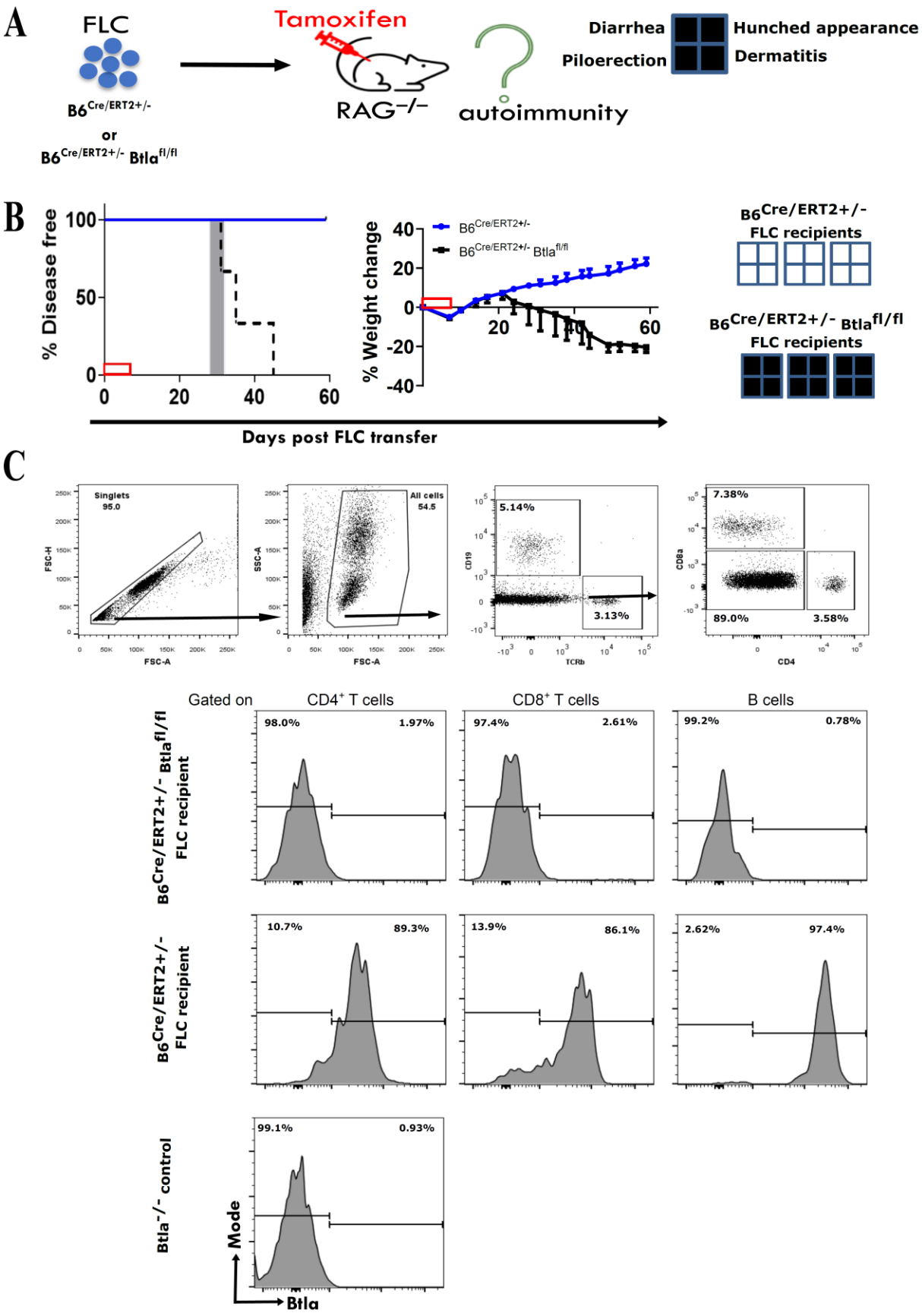

A
FLC
B6Cre/ERT2+/-
or
B6Cre/ERT2+/- Btlafl/fl
Tamoxifen
RAG−/−
autoimmunity
Diarrhea
Piloerection
Hunched appearance
Dermatitis
B
% Disease free
Days post FLC transfer
B6Cre/ERT2+/-
B6Cre/ERT2+/- Btlafl/fl
% Weight change
B6Cre/ERT2+/-
FLC recipients
B6Cre/ERT2+/- Btlafl/fl
FLC recipients
C
Singlets
95.0
All cells
54.5
5.14%
3.13%
7.38%
89.0%
3.58%
Gated on
CD4+ T cells
CD8+ T cells
B cells
B6Cre/ERT2+/- Btlafl/fl
FLC recipient
98.0%
1.97%
97.4%
2.61%
99.2%
0.78%
B6Cre/ERT2+/-
FLC recipient
10.7%
89.3%
13.9%
86.1%
2.62%
97.4%
Btla-/- control
99.1%
0.93%
Mode
Btla

Figure 2.2. *Loss of Btla early in T cell ontogeny generates autoimmunity in lymphopenic mice.* [**A**] We adoptively transferred 20 x 10^6 FLC pooled from 8-10 embryonic days 14 – 16 $B6^{Cre/ERT2+/-}$ or $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ fetuses to 7-wk old $Rag^{-/-}$ mice on day 0 (n=3 recipients per group), followed by tamoxifen injection on days 0, 1, 3, 5, and 6. Recipient mice were monitored for signs of disease for eight weeks post FLC transfer. [**B**] Left panel: disease incidence in recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC. Survival curve comparison demonstrated a significant difference between the two groups with $p = 0.02$. The grey rectangle indicates the range, in days, at which the first T cells were detected in the peripheral blood after FLC transfer. Right panel: weight changes in recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC or $B6^{Cre/ERT2+/-}$ FLC. The presence (shaded) or absence (unshaded) of disease signs is depicted on the far-right panel. The red box on the X-axis indicates the tamoxifen treatment period. Data are from one experiment. [**C**] Flow cytometry gating strategy. Upper panel shows the gate on singlets and then all cells and then T or B cells and then CD4 and CD8 T cells. A representative histogram of Btla expression in the T and B cells populating the periphery of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC recipient mice (middle panel) or $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC recipient mice (lower panel) at four weeks post FLC transfer is shown. Bottom row shows the $Btla^{-/-}$ control.

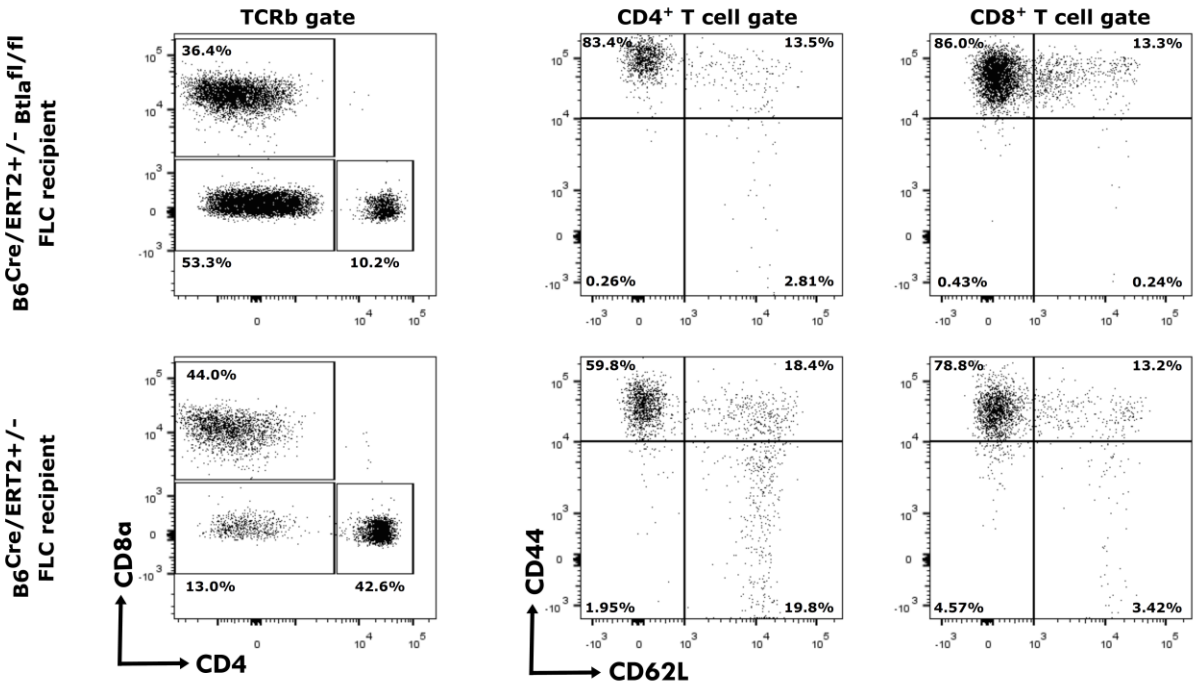


Figure 2.3. *Repopulating T cells in FLC recipients acquired effector memory phenotype.* Representative dot plot of CD44 and CD62L expression in the peripheral blood of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC recipients (upper panel) or $B6^{Cre/ERT2+/-}$ FLC recipient (lower panel) at eight weeks post FLC transfer.

2.3.2 $CD4^+$ T cells and MHC II are required for autoimmunity in $Btla^{-/-}$ thymocytes recipients

Having shown that loss of Btla in HSC generates disease when T cells seed the periphery of FLC recipient mice, we asked what T cell subset(s) is required for disease generation. To examine which T cell subset(s) is involved in the disease generated by $Btla^{-/-}$ thymocytes, we adoptively transferred sorted CD4 or CD8 SP $Btla^{-/-}$ or WT thymocytes into $Rag^{-/-}$ mice (Fig. 2.4 A). We monitored the recipient mice for several weeks or until losing ≥ 20% of baseline body weight, whichever came first. Recipients of $Btla^{-/-}$ CD4 SP T cells started losing weight as early as 21 days post cell transfer, which continued for up to 53 days when they had lost ≥ 20% of their baseline body weight (Fig. 2.4 B). All the $Btla^{-/-}$ CD4 SP T cell recipient mice had a hunched appearance, piloerection, and diarrhea. While the recipient male mice (3/7) had dermatitis, none of the recipient female mice (0/4) had dermatitis (Fig. 2.4 B). Disease occurred in both male and female $Rag^{-/-}$ recipients of $Btla^{-/-}$ CD4 SP T cells (Fig. 2.4 B). In contrast, none of the recipients of $Btla^{-/-}$ CD8 SP T cells (0/10) showed signs of morbidity, and their body weight increased throughout the experiment (Fig. 2.4 B). Although recipients of WT SP T cells had an initial decline in body weight, they quickly recovered and showed an improvement in body weight and no signs of disease (Fig. 2.4 C). Flow cytometry analysis of the T cells at experiment end-point showed that 93 ± 4% of the T cells in the $Btla^{-/-}$ CD4 SP thymocyte recipients had acquired an effector memory ($CD44^{hi}$ $CD62^{lo}$) phenotype in comparison to 15 ± 5% of the T cells in the $Btla^{-/-}$ CD8 SP thymocyte recipients (Fig. 2.5). In contrast, 91 ± 2% of the T cells in the WT CD4 SP thymocyte recipients had acquired effector memory ($CD44^{hi}$ $CD62^{lo}$) phenotype in comparison to 11 ± 4% of the T cells in the WT CD8 SP thymocyte recipients (Fig. 2.6). In addition, the percentage of $Foxp3^+$ regulatory T cells in the WT CD4 SP

thymocyte recipients was significantly higher (p=0.004) at two weeks post thymocyte transfer, relative to the Btla$^{-/-}$ CD4 SP thymocyte recipients (Fig. 2.7).

To examine what MHC molecule is required for this autoimmunity, we adoptively transferred non-sorted Btla$^{-/-}$ SP thymocytes to K^{b}D$^{b-/-}$ Rag$^{-/-}$ or CiiTA$^{-/-}$ Rag$^{-/-}$ mice that lacked both Rag and MHC class I genes or were deficient in both Rag and MHC class II protein expression (314), respectively. All CiiTA$^{-/-}$ Rag$^{-/-}$ mice were free of disease, while 7/7 of their K^{b}D$^{b-/-}$ Rag$^{-/-}$ counterparts had all signs of morbidity within 3 weeks post T cell transfer, regardless of sex (Fig. 2.8 A, B). Thus, MHC class II and MHC class II-restricted CD4^{+} T cells are required for disease. This is consistent with previously published data from our group with newly generated PD-1 deficient T cells, where the disease was also completely dependent on MHC class II (113,297).

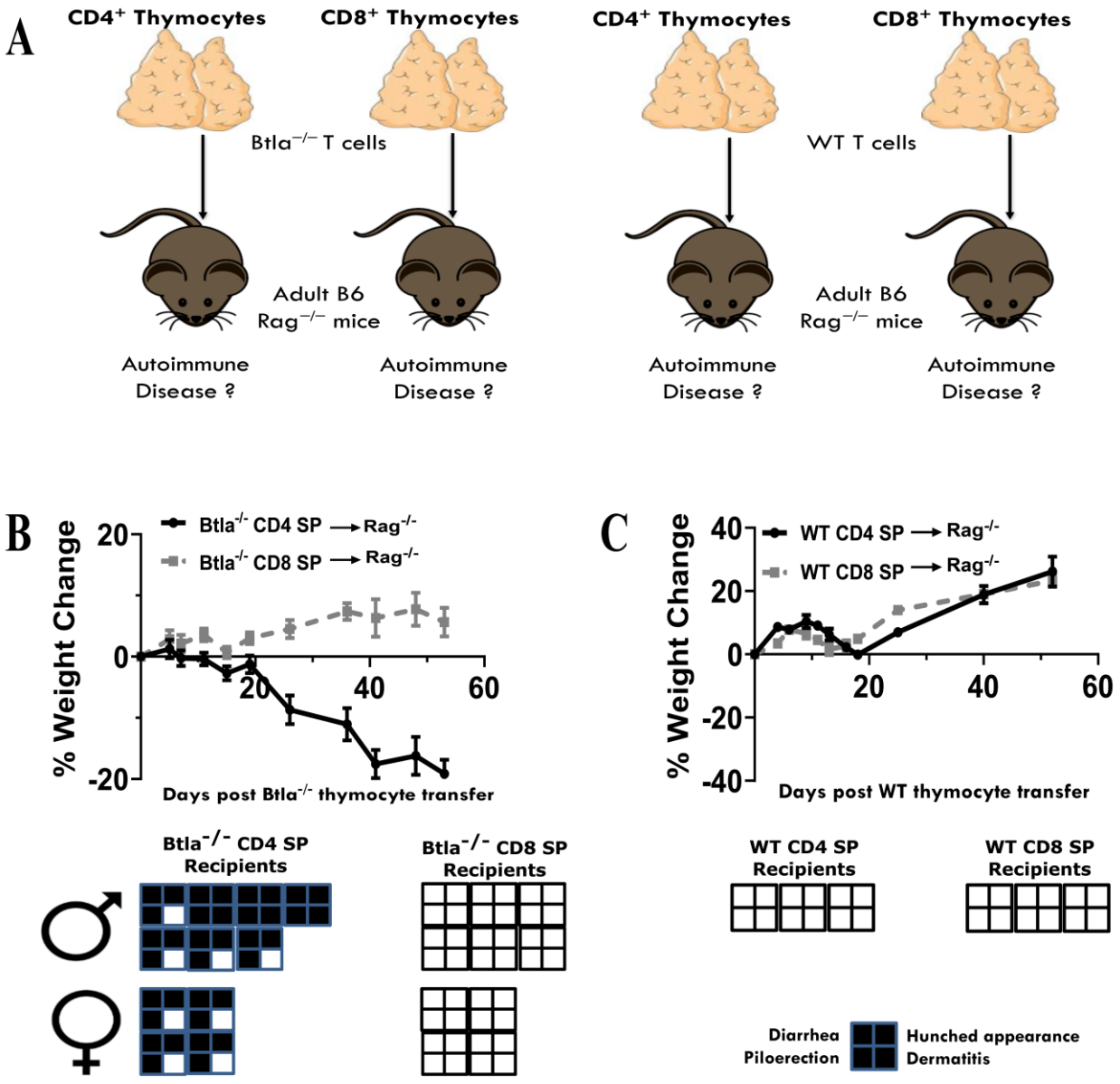

A
CD4+ Thymocytes
CD8+ Thymocytes
Btla−/− T cells
Adult B6
Rag−/− mice
Autoimmune
Disease ?
Autoimmune
Disease ?
CD4+ Thymocytes
CD8+ Thymocytes
WT T cells
Adult B6
Rag−/− mice
Autoimmune
Disease ?
Autoimmune
Disease ?
B
Btla-/- CD4 SP → Rag-/-
Btla-/- CD8 SP → Rag-/-
% Weight Change
20
0
-20
20
40
60
Days post Btla-/- thymocyte transfer
Btla-/- CD4 SP
Recipients
Btla-/- CD8 SP
Recipients
C
WT CD4 SP → Rag-/-
WT CD8 SP → Rag-/-
% Weight Change
40
20
0
-20
-40
20
40
60
Days post WT thymocyte transfer
WT CD4 SP
Recipients
WT CD8 SP
Recipients
Diarrhea
Piloerection
Hunched appearance
Dermatitis

Figure 2.4. *Autoimmunity in Btla$^{-/-}$ thymocyte recipients is associated with CD4^{+} T cells and MHC II.* [**A**] shows the experimental design. We adoptively transferred 3 x 10^6 MACS-sorted CD4 or CD8 SP thymocytes pooled from seven 8 – 10-wk old B6.Foxp3^{EGFP} x Btla$^{-/-}$ (left column) or B6.Foxp3^{EGFP} (right column) mice and were injected via tail vein to 8 – 10-wk old Rag$^{-/-}$ mice (Btla$^{-/-}$ thymocyte recipients, n=3-5 mice/group; WT thymocyte recipients, n=3 mice/group) and monitored for several weeks or after losing ≥ 20% of baseline body weight, whichever came first. Graph of body weight change [**B**] of the Btla$^{-/-}$ SP thymocyte recipients (data was from three independent experiments) or WT SP thymocyte recipients[**C**], the presence (shaded) or absence (unshaded) of disease signs is depicted below the graphs.

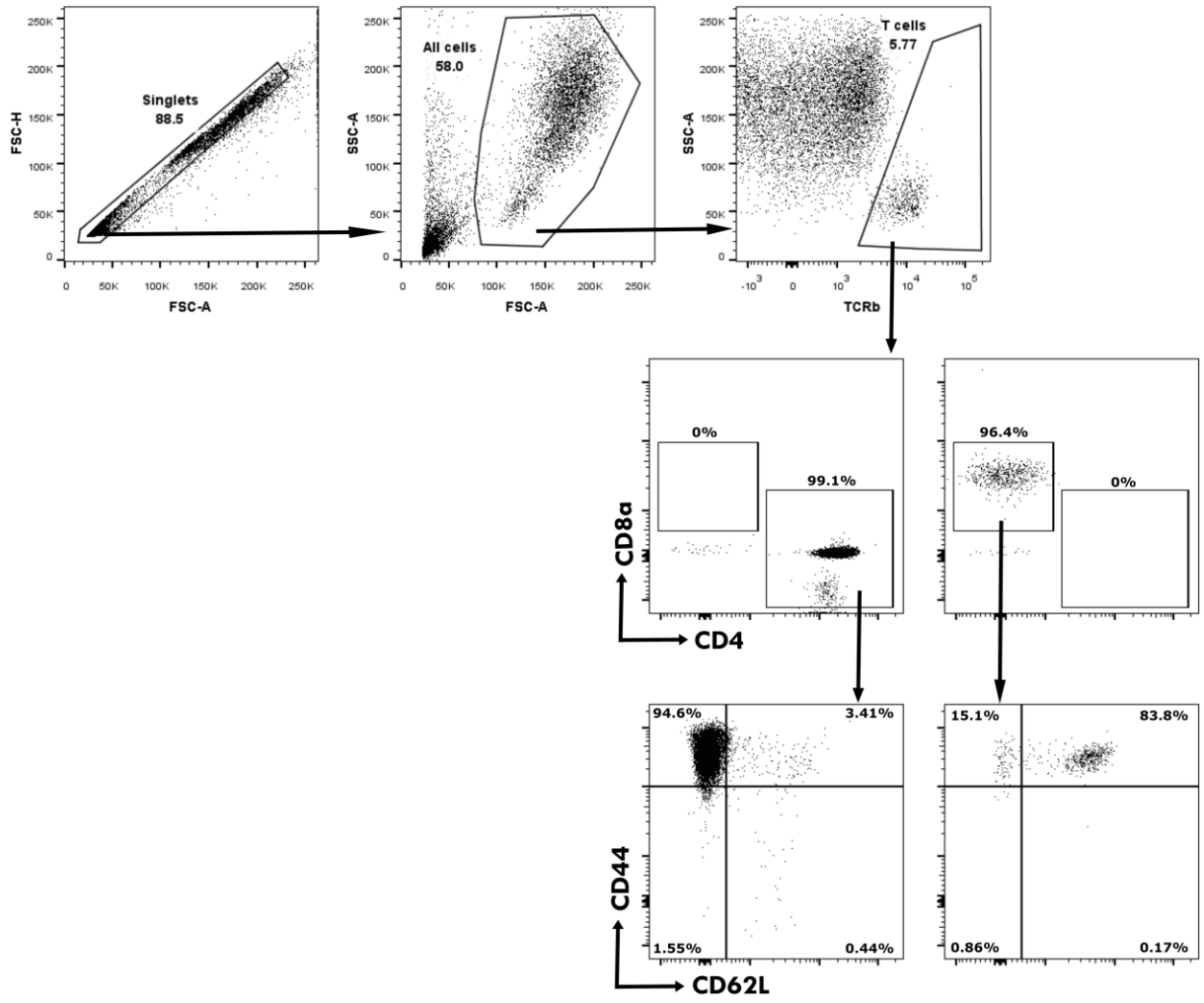


Figure 2.5. *Memory T cell phenotype in Btla$^{-/-}$ thymocyte recipients.* Upper panel shows the gating strategy. We gated on singlets and then all cells and then T cells. Middle panel shows CD4^{+} T cells (left) and CD8^{+} T cells (right) in thymocyte recipients. Lower panel shows a representative dot plot (CD4, n=11; CD8, n=10) of the memory T cell phenotypes in the recipients of Btla$^{-/-}$ CD4 SP thymocytes (left) and Btla$^{-/-}$ CD8 SP thymocytes (right) at seven weeks post thymocyte transfer.

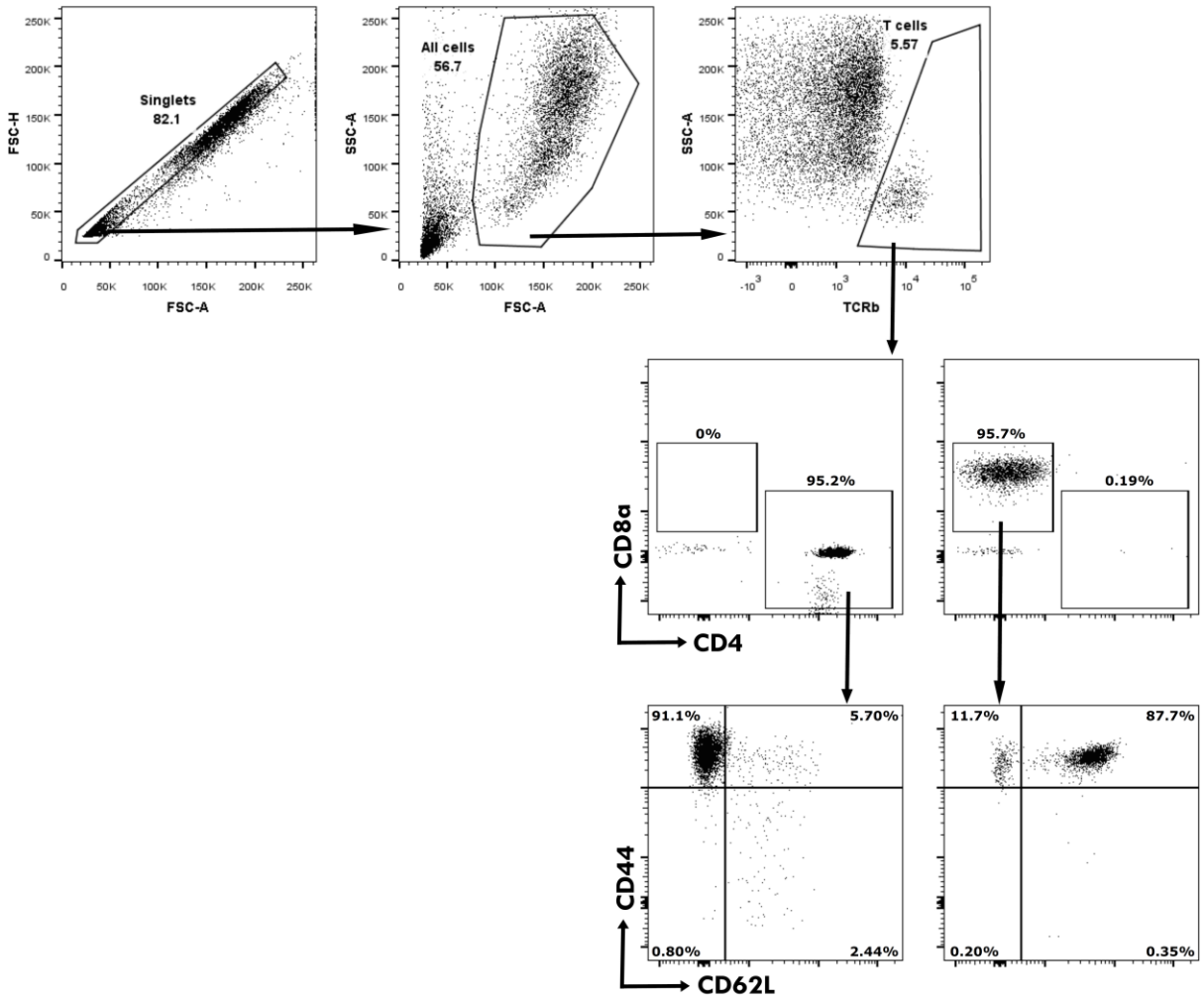


Figure 2.6. *Memory T cell phenotype in WT thymocyte recipients.* Upper panel shows the gating strategy. We gated on singlets and then all cells and then T cells. Middle panel shows $CD4^+$ T cells (left) and $CD8^+$ T cells (right) in thymocyte recipients. Lower panel shows a representative dot plot (CD4, n=3; CD8, n=3) of the memory T cell phenotypes in the recipients of WT CD4 SP thymocytes (left) and WT CD8 SP thymocytes (right) at seven weeks post thymocyte transfer.

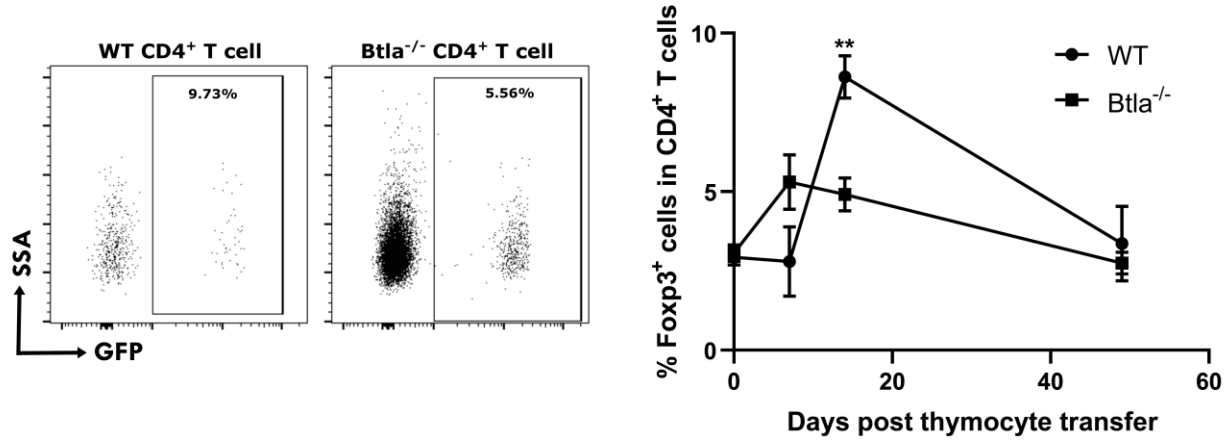


Figure 2.7. *Treg cells in CD4 SP thymocyte recipients.* Representative dot plot of Foxp3^{+} CD4 cells (GFP^{+}) gated on T cells in the WT (left) and Btla$^{-/-}$ (middle) thymocyte recipients at two weeks post thymocyte transfer. Graph on far right show the percentage of regulatory T cells at different intervals during the experiment. Data was from three independent experiments (Btla$^{-/-}$, n=11; WT, n=3). Analysis: T-tests with Holm-Sidak correction for multiple comparisons; ***p* = 0.004.

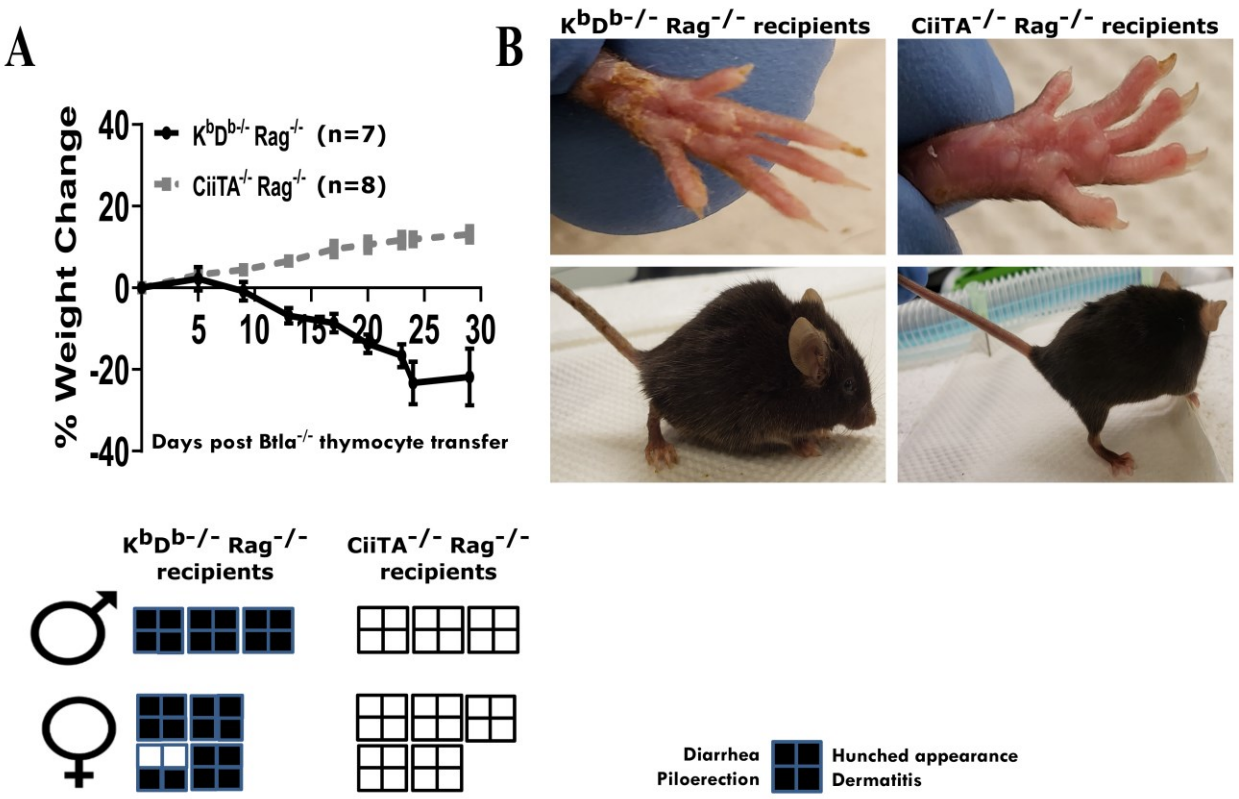


Figure 2.8. *Autoimmunity in Btla$^{-/-}$ thymocyte recipients is associated with CD4^{+} T cells and MHC II.* [**A**] Thymocytes containing 3 x 10^6 SP cells (i.e., non-sorted) pooled from seven 8 – 10-wk old B6.Foxp3^{EGFP} x Btla$^{-/-}$ mice were injected via tail vein to 8 – 10-wk old K^{b}D$^{b-/-}$ Rag$^{-/-}$ mice (n=7) or CiiTA$^{-/-}$ Rag$^{-/-}$ (n=8) mice, which were then monitored for several weeks or after losing ≥ 20% of baseline body weight, whichever came first. Body weight change of recipient mice is shown. Data are from two independent experiments. The presence (shaded) or absence (unshaded) of disease signs is depicted below the graph. [**B**] Representative photos of diseased K^{b}D$^{b-/-}$ Rag$^{-/-}$ mice showing dermatitis (upper left panel) and hunched appearance (lower left panel) and their disease-free CiiTA$^{-/-}$ Rag$^{-/-}$ counterparts (right panel) following Btla$^{-/-}$ thymocyte transfer.

2.3.3 Btla and PD-1 signaling are needed post thymic selection in newly generated T cells to block autoimmunity

The above studies showed that Btla is needed in newly generated T cells for T cell tolerance, but they did not address whether Btla is needed in newly generated T cells during thymic selection or post thymic selection when the newly generated T cells seed the periphery. To examine if autoimmunity occurs when Btla is deleted only at post thymic selection, thymocytes from adult (7 – 9-week-old) $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ mice were adoptively transferred via tail vein to adult $Rag^{-/-}$ mice, followed by five doses of tamoxifen injection intraperitoneally to induce Btla gene deletion in the transferred thymocytes. The control group received $B6^{CreERT2+/-}$ thymocytes and tamoxifen injection (Fig. 2.9 A). There was a near complete deletion of Btla by day 7 post the last dose of tamoxifen in the T cells of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ thymocyte recipients, while this coinhibitor's expression persisted in the $B6^{CreERT2+/-}$ thymocyte recipients (Fig. 2.9 B). The $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ thymocyte recipients but not their $B6^{CreERT2+/-}$ counterparts began to lose body weight around day 7 post thymocyte transfer and also showed signs of disease around day 12 post thymocyte transfer (Fig. 2.9 C). While all recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ thymocytes developed disease, recipients of $B6^{CreERT2+/-}$ thymocytes remained disease-free (Fig. 2.9 C). To examine if PD-1 is also needed at the RTE stage to block autoimmunity, thymocytes from adult (7 – 12-week-old) $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ mice were adoptively transferred to adult $Rag^{-/-}$ mice, followed by five doses of tamoxifen injection intraperitoneally to induce PD-1 gene deletion in the transferred thymocytes. The control group received $B6^{CreERT2+/-}$ thymocytes and tamoxifen injection (Fig. 2.10 A). Similar to the findings above, PD-1 expression was absent by day 7 post the last dose of tamoxifen in the T cells of $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ thymocyte recipients, whereas the $B6^{CreERT2+/-}$ RTE expressed PD-1 (Fig. 2.10 B). The recipients of $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$

thymocyte began to lose body weight around day 14 post thymocyte transfer and also showed signs of morbidity around day 17 post thymocyte transfer (Fig. 2.10 C). Histological examination showed CD4 SP T cell infiltration in the kidneys and livers of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ thymocyte recipients (Fig. 2.11). Similarly, there was CD4 SP T cell infiltration in the kidneys, livers, and lungs of $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ thymocyte recipients (Fig. 2.11). In contrast, no infiltration of CD4 SP T cells was seen in the hearts of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ or $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ thymocyte recipients. Likewise, there was no CD4 SP T cell infiltration in the examined organs of $B6^{Cre/ERT2+/-}$ thymocyte recipients. Since Btla and PD-1 were present during thymic selection but deleted post thymic selection in the RTE, we concluded that both Btla and PD-1 are required post T cell export from the thymus at the RTE stage to establish tolerance.

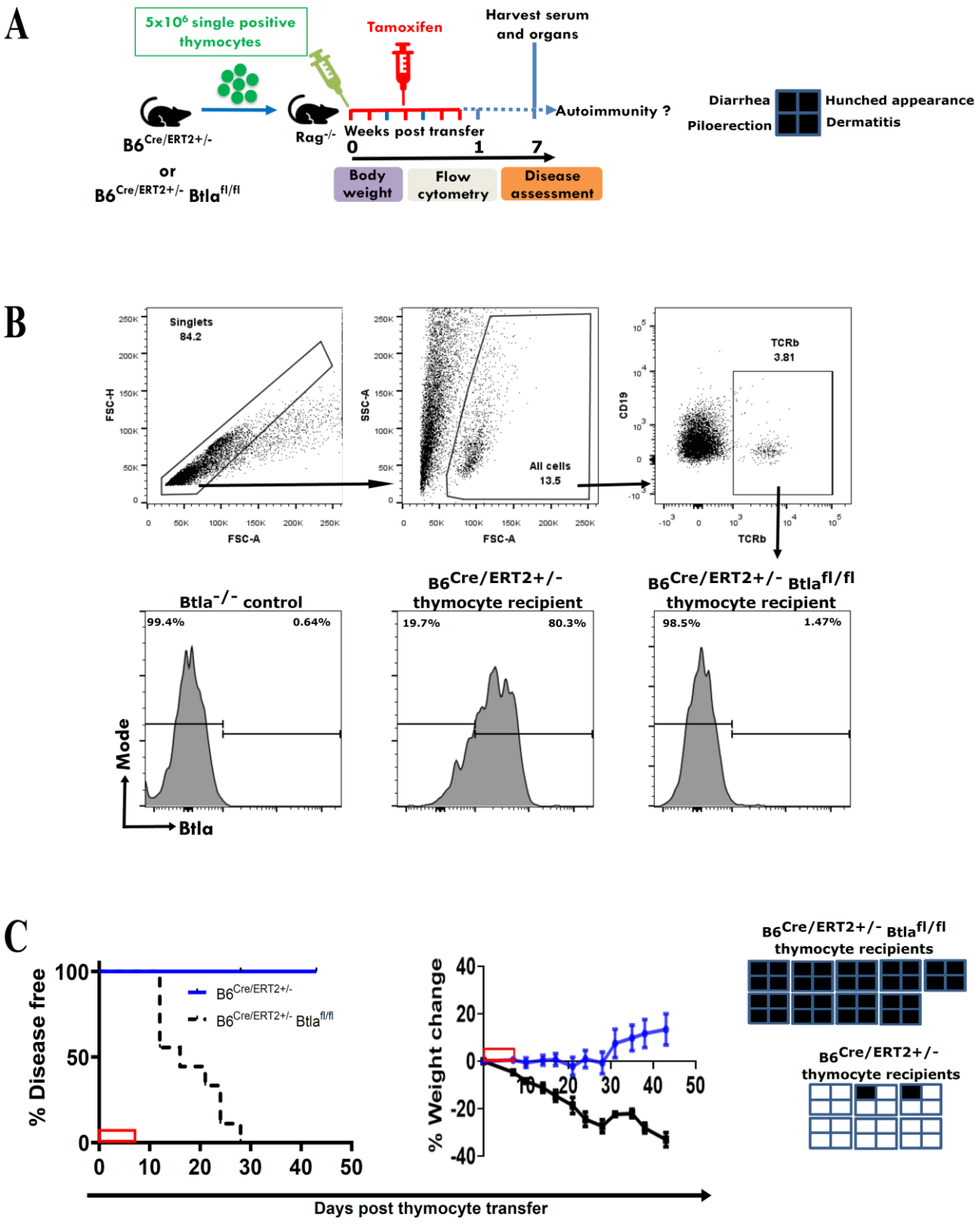

A
5x10⁶ single positive thymocytes
Tamoxifen
Harvest serum and organs
B6Cre/ERT2+/-
or
B6Cre/ERT2+/- Btlafl/fl
Rag-/-
Weeks post transfer
0
1
7
Autoimmunity ?
Body weight
Flow cytometry
Disease assessment
Diarrhea
Piloerection
Hunched appearance
Dermatitis
B
Singlets
84.2
FSC-H
FSC-A
SSC-A
All cells
13.5
CD19
TCRb
3.81
Btla-/- control
99.4%
0.64%
B6Cre/ERT2+/- thymocyte recipient
19.7%
80.3%
B6Cre/ERT2+/- Btlafl/fl thymocyte recipient
98.5%
1.47%
Mode
Btla
C
% Disease free
B6Cre/ERT2+/-
B6Cre/ERT2+/- Btlafl/fl
% Weight change
Days post thymocyte transfer
B6Cre/ERT2+/- Btlafl/fl thymocyte recipients
B6Cre/ERT2+/- thymocyte recipients

Figure 2.9. *Btla signaling is needed post thymic selection in newly generated T cells to block autoimmunity.* [**A**] We adoptively transferred thymocytes containing 5 x 10^6 SP (non-pooled) from 7 – 12-week-old $B6^{Cre/ERT2+/-}$ or $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ mice to 7 – 12-week-old $Rag^{-/-}$ mice on day 0, followed by tamoxifen injection on days 0, 1, 3, 5, and 6. Mice were then monitored for signs of disease for seven weeks [**B**] Flow cytometry gating strategy. Upper panel: we gated on singlets and then all cells and then T cells. Lower panel: representative histogram of Btla expression in the T cells of $B6^{Cre/ERT2+/-}$ or $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ thymocyte recipients at two weeks post thymocyte transfer (i.e., seven days post tamoxifen) is shown. [**C**] Left panel: disease incidence in recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ (n=9) or $B6^{Cre/ERT2+/-}$ (n=6) thymocytes. Survival curve comparison demonstrated a significant difference between the two groups with $p = 0.0002$. Data are from two independent experiments. Right panel: weight changes in recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ or $B6^{Cre/ERT2+/-}$ thymocytes. The presence (shaded) or absence (unshaded) of disease signs is depicted on the far-right panel. The red box on the X-axis indicates the tamoxifen treatment period.

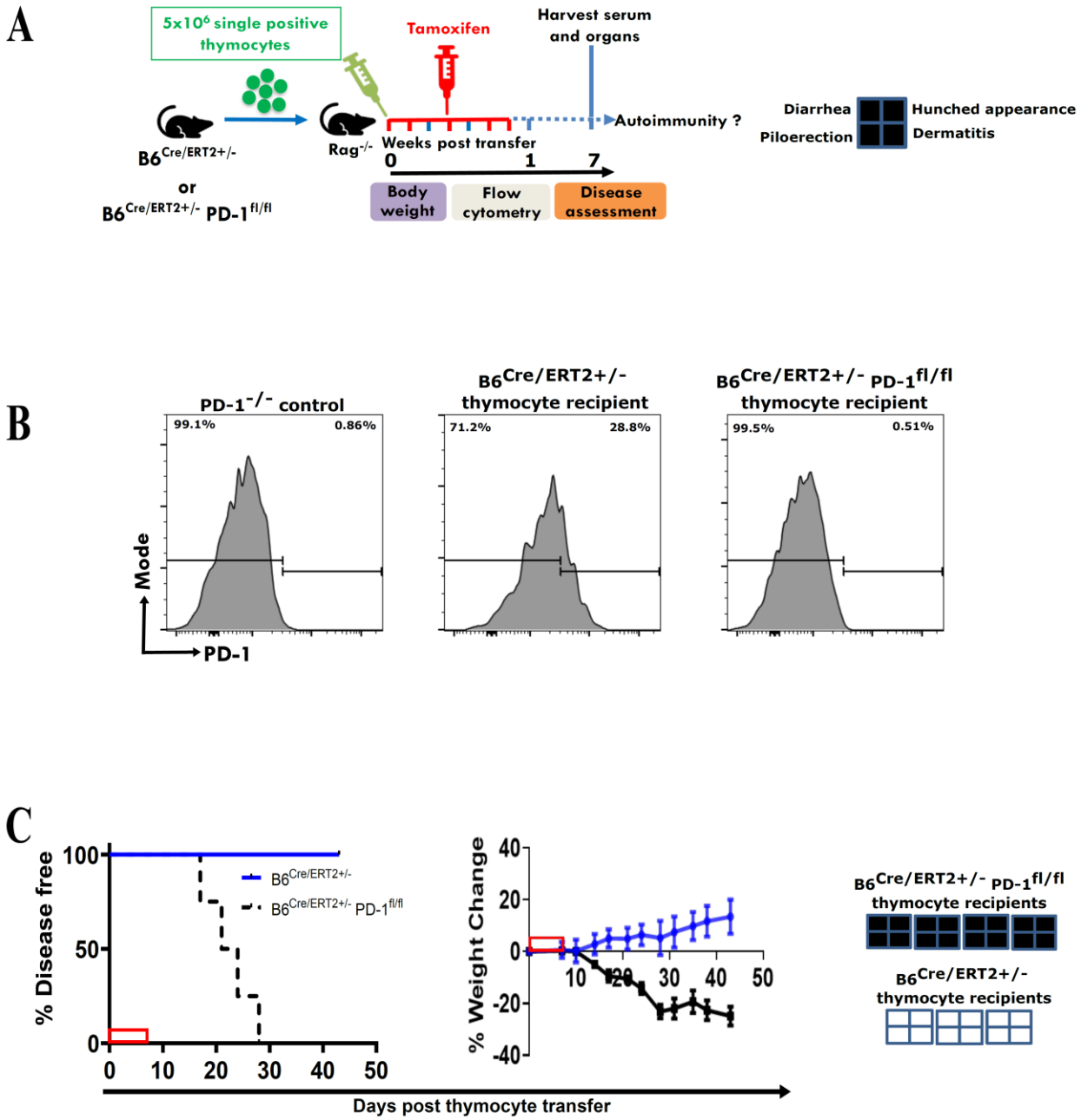

A
5x10⁶ single positive thymocytes
Tamoxifen
Harvest serum and organs
B6Cre/ERT2+/-
or
B6Cre/ERT2+/- PD-1fl/fl
Rag-/-
Weeks post transfer
0
1
7
Autoimmunity ?
Body weight
Flow cytometry
Disease assessment
Diarrhea
Piloerection
Hunched appearance
Dermatitis
B
PD-1-/- control
B6Cre/ERT2+/- thymocyte recipient
B6Cre/ERT2+/- PD-1fl/fl thymocyte recipient
99.1%
0.86%
71.2%
28.8%
99.5%
0.51%
Mode
PD-1
C
% Disease free
100
50
0
B6Cre/ERT2+/-
B6Cre/ERT2+/- PD-1fl/fl
0 10 20 30 40 50
% Weight Change
40
20
0
-20
-40
10 20 30 40 50
Days post thymocyte transfer
B6Cre/ERT2+/- PD-1fl/fl thymocyte recipients
B6Cre/ERT2+/- thymocyte recipients

Figure 2.10. *PD-1 signaling is needed post thymic selection in newly generated T cells to block autoimmunity.* [**A**] We adoptively transferred thymocytes containing 5 x 10^6 SP (non-pooled) from 7 – 12-week-old $B6^{Cre/ERT2+/-}$ or $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ mice to 7 – 12-week-old $Rag^{-/-}$ mice on day 0, followed by tamoxifen injection on days 0, 1, 3, 5, and 6. Mice were then monitored for signs of disease for seven weeks [**B**] Flow cytometry gating strategy is similar to figure 2.4 B. Here is showing a representative histogram of PD-1 expression in the T cells of $B6^{Cre/ERT2+/-}$ or $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ thymocyte recipients at two weeks post thymocyte transfer (i.e., seven days post tamoxifen). [**C**]Left panel: disease incidence in recipients of $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ (n=4) or $B6^{Cre/ERT2+/-}$ (n=3) thymocytes. Survival curve comparison demonstrated a significant difference between the two groups with $p = 0.02$. Data are from one experiment. Right panel: weight changes in recipients of $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ or $B6^{Cre/ERT2+/-}$ thymocytes. The presence (shaded) or absence (unshaded) of disease signs is depicted on the far-right panel. The red box on the X-axis indicates the tamoxifen treatment period.

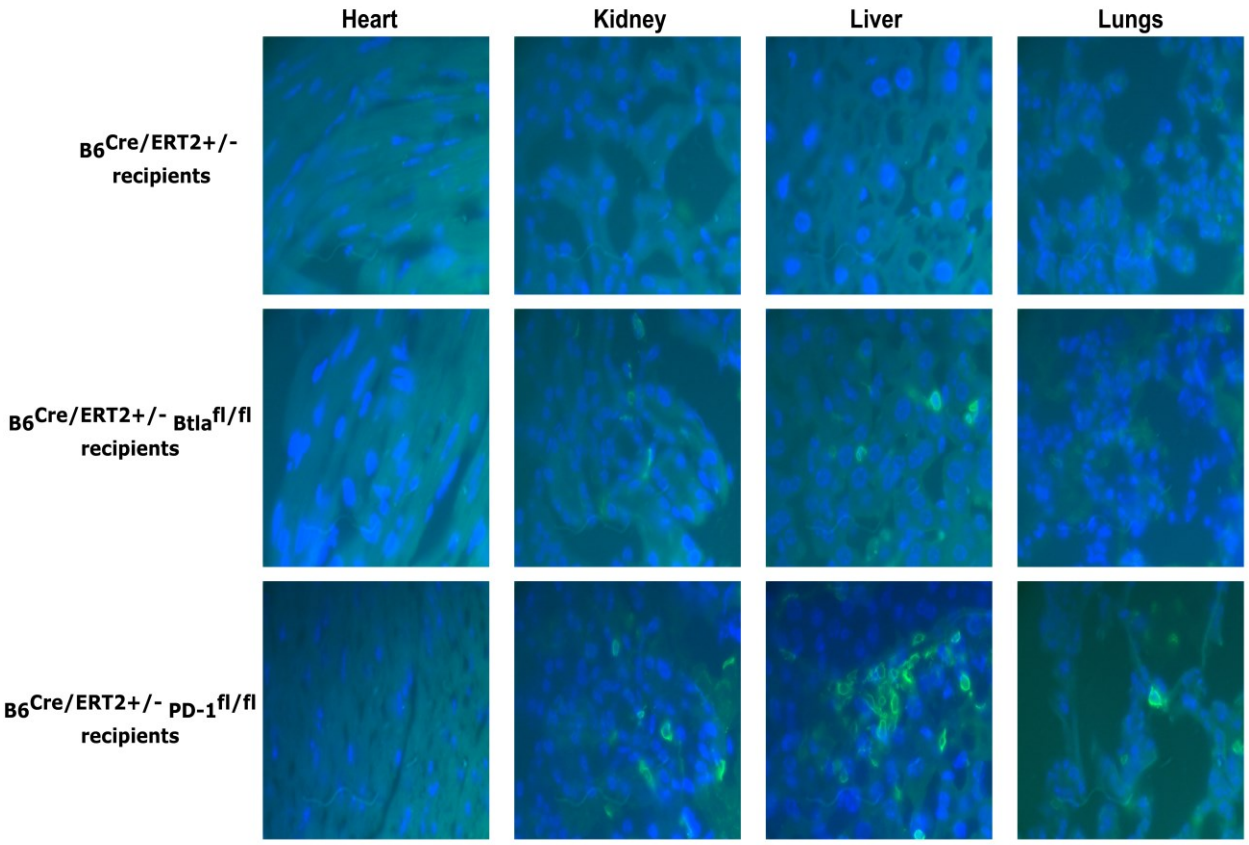


Figure 2.11. *Representative immunofluorescence staining (original magnification x1000) of heart, kidney, liver, and lungs of individual recipients of* $B6^{Cre/ERT2+/-}$, $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$, *and* $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ *thymocytes.* Blue: staining with the nuclear marker 4',6'-diamidino-2-phenylindole (DAPI); green: CD4 staining.

2.3.4 PD-1 signaling is crucial in neonates where all T cells are RTE

The importance of Btla and PD-1 in RTE suggests that these coinhibitors may be most critical during the neonatal period when all the T cells are RTE (315). Therefore, we hypothesized that, unlike PD-1 deficient T cells from adult mice, neonatal PD-1 deficient splenic T cells would induce autoimmunity in adult lymphopenic mice due to a much higher proportion of RTE in the neonatal spleen. To examine this, we first quantified the proportion of RTE in the neonatal spleen versus the adult spleen using the B6.Rag2p^{GFP} mice. Flow cytometry examination showed that splenic T cells in the neonatal mice are almost all RTE while the adult splenic T cells are mostly mature established peripheral T cells (Fig. 2.12). Further analysis showed that neonatal splenic T cells express higher levels of Btla (Fig. 2.13) and PD-1 (Fig. 2.14) relative to the adult mice. In addition, there is a higher percentage of Btla^{+} and PD-1^{+} T cells in the neonatal spleen relative to their adult counterparts (Fig. 2.13 – 2.14). To examine whether PD-1$^{-/-}$ signaling is needed during the neonatal period when all T cells are RTE or in adulthood where only a fraction of peripheral T cells are RTE, we adoptively transferred neonatal or adult PD-1$^{-/-}$ splenocytes into Rag$^{-/-}$ mice and monitored recipient mice for signs of autoimmunity. Indeed, the recipients of neonatal but not adult PD-1$^{-/-}$ splenocytes developed severe autoimmunity, evidenced by continuous weight loss, diarrhea, piloerection, dermatitis, and hunched posture (Fig. 2.15 A, B). This evidence further supports our hypothesis that coinhibitors are critical in RTE for self tolerance and important for establishing tolerance in neonatal T cells.

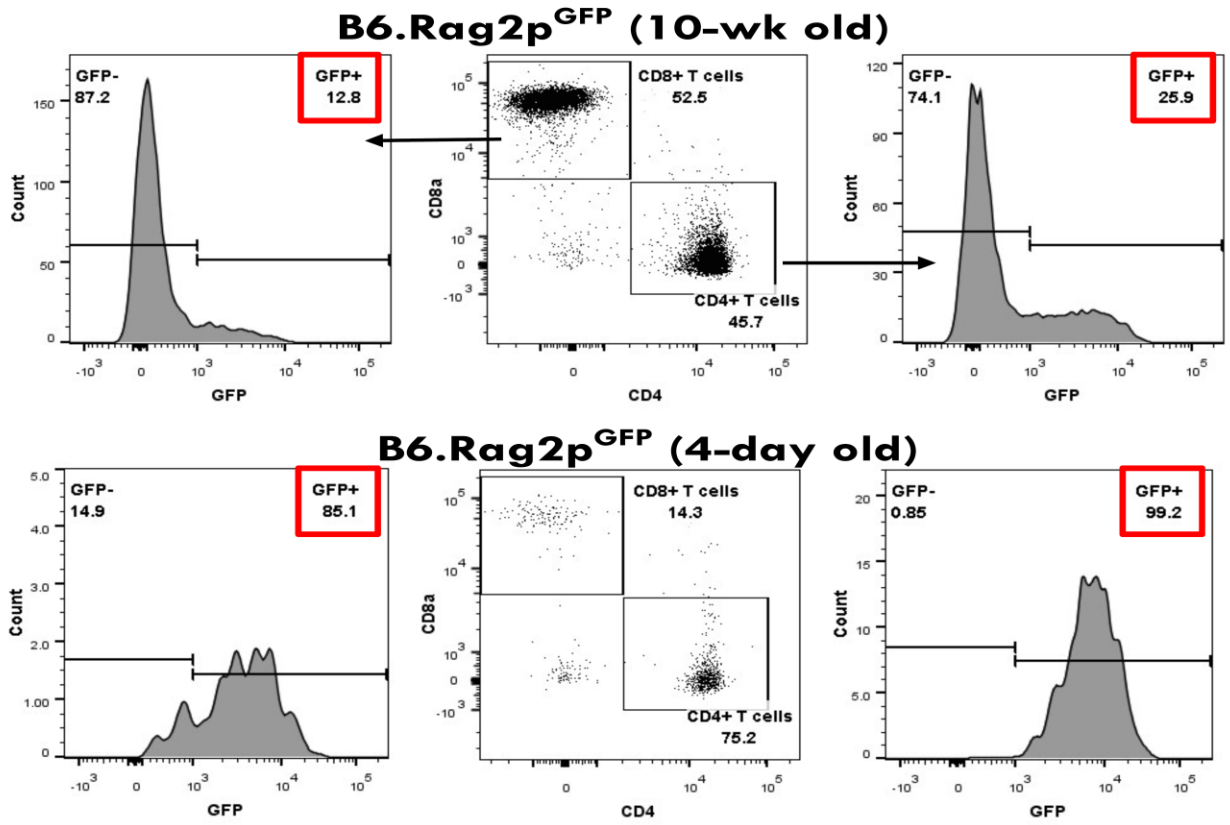


Figure 2.12. *A higher proportion of peripheral T cells in the neonatal mice are newly generated T cells.* Figure shows a representative flow cytometry data (n=3 mice/group) of the percentage of newly generated T cells in the spleen of adult (upper panel) or neonatal (lower panel) B6.Rag2p^{GFP} mice.

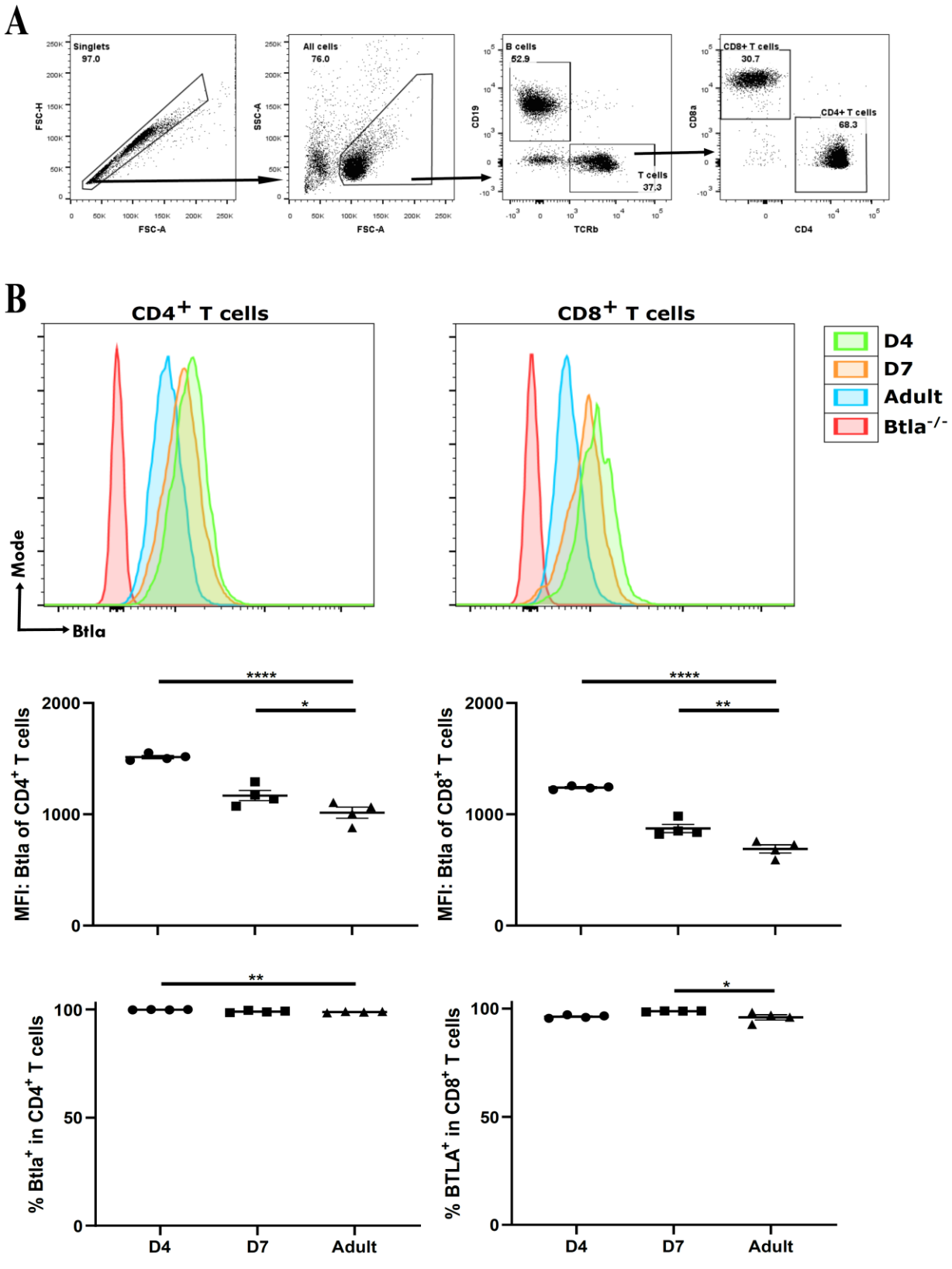

A
Singlets
97.0
FSC-H
FSC-A
All cells
76.0
SSC-A
FSC-A
B cells
52.9
T cells
37.3
CD19
TCRb
CD8+ T cells
30.7
CD4+ T cells
68.3
CD8a
CD4
B
CD4+ T cells
CD8+ T cells
D4
D7
Adult
Btla-/-
Mode
Btla
MFI: Btla of CD4+ T cells
MFI: Btla of CD8+ T cells
****
*
**
2000
1000
0
% Btla+ in CD4+ T cells
% BTLA+ in CD8+ T cells
100
50
D4
D7
Adult

Figure 2.13. *T cells in the neonatal mice express higher levels of Btla.* [**A**] Gating strategy show gating on singlets and then all cells and then T cells. [B] Upper panel show a representative histogram of Btla expression [i.e., mean fluorescence intensity (MFI)] in the splenic T cells of 4-day old, 7-day old, and 8-week old (adult) B6 mice, graph of the Btla MFI (middle panel) and proportion (lower panel) of Btla+ cells in the $CD4^+$ (left) or $CD8^+$ (right) T cells is shown. Data is from one experiment. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

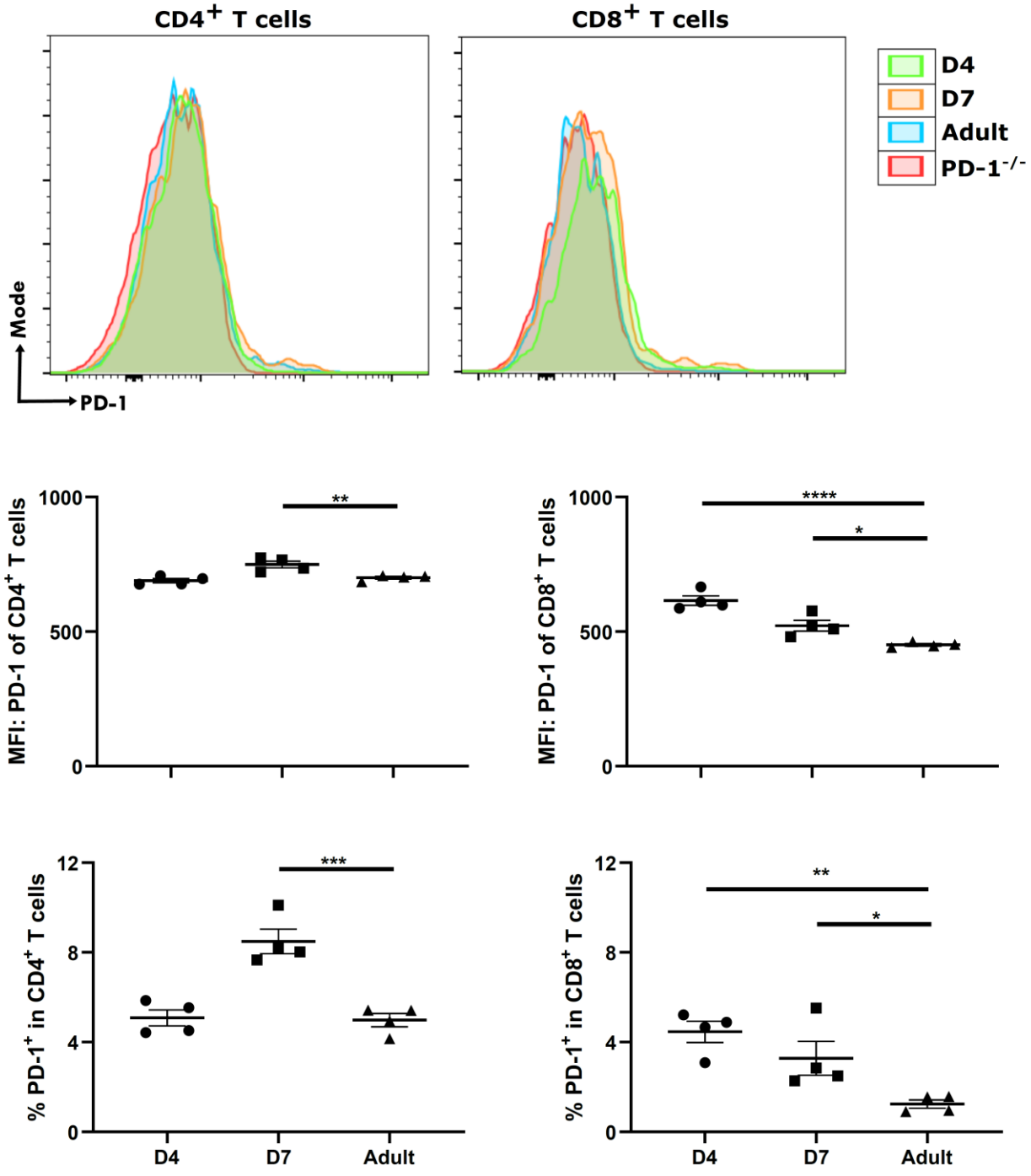

CD4+ T cells
CD8+ T cells
D4
D7
Adult
PD-1-/-
Mode
PD-1
MFI: PD-1 of CD4+ T cells
MFI: PD-1 of CD8+ T cells
% PD-1+ in CD4+ T cells
% PD-1+ in CD8+ T cells
1000
500
0
12
8
4
**
****
*
***
D4
D7
Adult

Figure 2.14. *T cells in the neonatal mice express higher levels of PD-1.* Upper panel show a representative histogram of PD-1 expression in the splenic T cells of 4-day old, 7-day old, and 8-week old (adult) B6 mice, graph of the PD-1 MFI (middle panel) and proportion (lower panel) of PD-1+ cells in the $CD4^+$ (left) or $CD8^+$ (right) T cells is shown. Data is from one experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

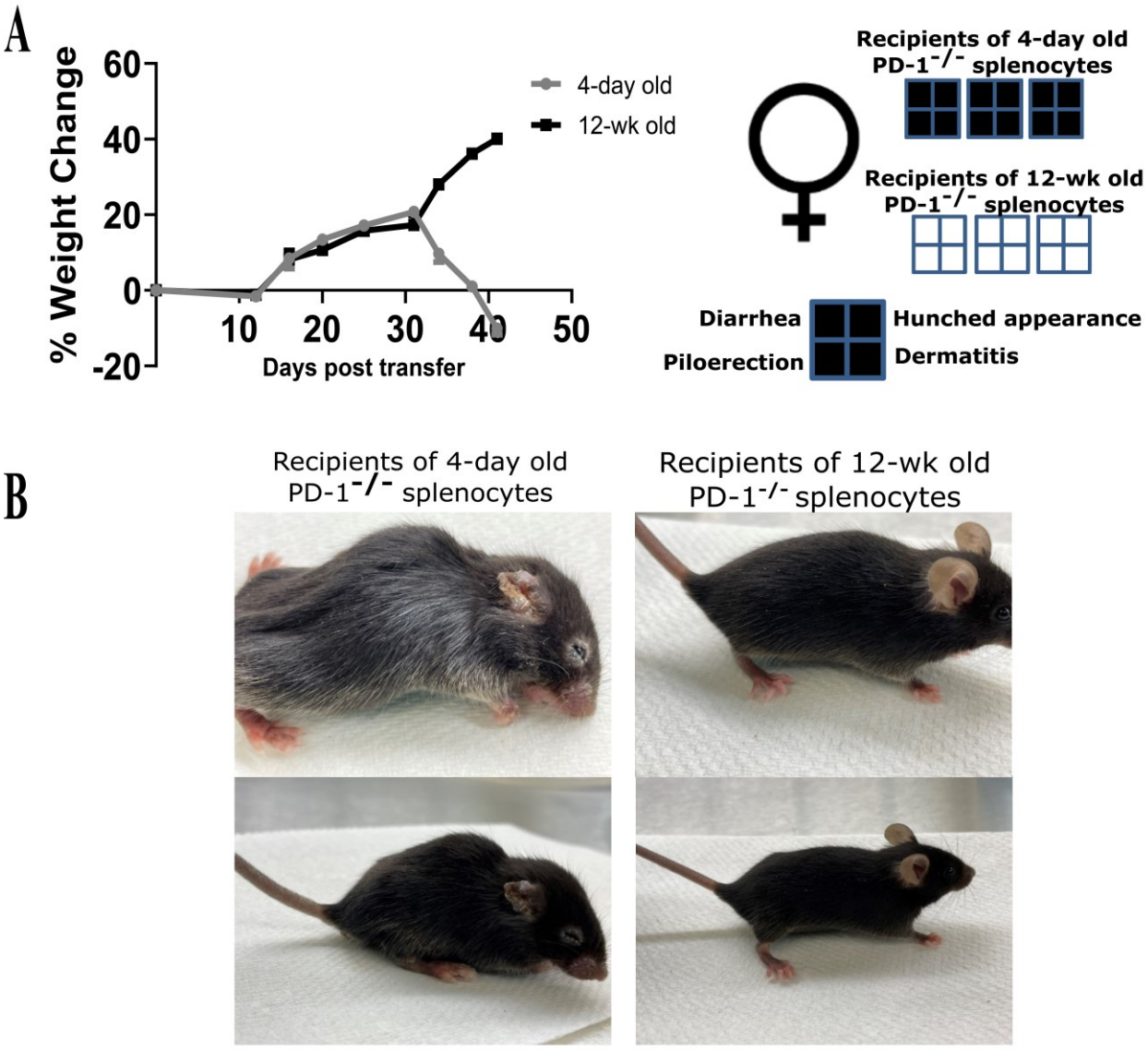


Figure 2.15. *PD-1 signaling is crucial in neonates where all T cells are RTE.* [**A**] shows body weight change (left panel) and disease signs (right panel) in the recipients of 1 x 10^6 SP splenic T cells from neonatal or adult B6.Rag2p^{GFP}-PD-1$^{-/-}$ mice (n=3 recipients per group). [B] Representative photos of diseased mice (left panel) and disease-free mice (right panel) that received the indicated splenocytes.

2.4 Discussion

Previous studies on T cell coinhibitory receptors have focused on what developmental stage of the organism these coinhibitory molecules are important. Studies on CTLA-4 and TGF-β1 show these coinhibitory molecules are needed early in life (290,289,296,316). In contrast, mice deficient in PD-1 or Btla appear relatively healthy from birth through adulthood but then develop late-life autoimmunity (86,105), suggesting these coinhibitors may be needed later in life, or perhaps early events take time for their consequences to become apparent. In addition, PD-1 and Btla have primarily been characterized as being highly expressed in T cells late in the response when they have been exposed chronically to antigen (the so called exhausted T cells) (317). However, a previous study from our group showed that PD-1 is needed early in establishing tolerance in polyclonal newly generated T cells in the context of lymphopenia (113). Further supporting this conclusion, using a TCR transgenic system, May *et al*. recently showed that establishment of tolerance to a tissue-restricted antigen was less successful when PD-1/PD-L1 signaling was absent both in the thymus and periphery when compared to being absent only in the periphery. Briefly, OT-I $Bim^{-/-} \rightarrow$ RIP-mOVA chimera T cells that typically do not generate autoimmunity were unable to establish tolerance to tissue-restricted ovalbumin, resulting in diabetes development, when PD-1/PD-L1 signaling was compromised in the thymus and periphery (114). While this study and studies from our group showed that PD-1 is required for establishing tolerance in newly generated T cells, it is hard to decipher whether PD-1 signaling is required during, or post thymic selection.

There have been many studies on PD-1 and CTLA-4, and antibodies blocking these coinhibitory molecules are currently used in the clinic for cancer treatment. In contrast, Btla, a lymphocyte coinhibitory receptor similar to CTLA-4 and PD-1, has not received much attention.

In the clinic, antibodies to PD-1 and its ligands, and CTLA-4 only benefit a fraction of cancer patients. Many patients receiving this immunotherapy develop a spectrum of AD termed immune-related adverse events (iRAEs) (318,319). The efficacy of anti-PD-1 antibody and anti-CTLA-4 antibody in some cancer patients suggests that other coinhibitory receptors might be an important target. In addition, it is also important to understand the role other T cell coinhibitory receptors play in regulating immune tolerance in T cells. As such, our group examined the role of Btla in newly generated T cells and found that Btla, like PD-1, is equally important in establishing tolerance in polyclonal newly generated T cells during lymphopenia (268). However, Btla signaling appears to be dispensable for establishing tolerance in newly generated TCR transgenic CD8 T cells that recognize a tissue specific antigen (114).

Previous studies showed that CD4 T cells and/or MHCII are required for disease development in the PD-1$^{-/-}$ RTE autoimmunity model (113,297). Likewise, only the recipients of Btla$^{-/-}$ CD4 T cells developed disease in this study. However, because the Btla$^{-/-}$ CD4 T cell recipient mice are MHCI and MHCII sufficient, it is possible that the few contaminating CD8 T cell contributes to disease pathology via MHCI. To exclude this possibility and to understand what MHC molecule is permissive for disease, we transferred whole (non-sorted) thymocytes to lymphopenic mice that lack MHCI or do not express MHCII. As expected, only the MHCI deficient recipients developed disease. Hence, we conclude that CD4 T cells and MHCII are required for disease generated by Btla$^{-/-}$ RTE. However, because naïve CD8 T cells, unlike their CD4 T cell counterparts, can use either MHCI or MHCII expressed on antigen-presenting cells to undergo LIP *in vivo* (320), it is still possible that CD8 contributes to disease pathology in our model. Supporting our findings, it has been previously reported that Btla regulates CD4^{+} T cell alloreactivity and proliferative responses to MHCII antigens (321).

In this study, we asked at what stage of T cell development Btla or PD-1 are needed to establish tolerance. We employed the inducible Cre-lox system (304,305) to delete Btla or PD-1 in T cells that otherwise expressed these coinhibitors. When Btla was deleted in T cell progenitor cells prior to thymic selection, host mice developed disease, which was consistent with a previous finding from our group in recipients of germline Btla knockout FLC (313). In the recipient mice of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ FLC, SP T cells populating the periphery became biased towards the effector memory T cell phenotype. Although T cells undergoing LIP can acquire effector memory function and thus contribute to the induction of autoimmunity (265), Btla has been shown to negatively regulate the LIP of $CD8^+$ T cells (294). The finding that loss of Btla in T cell progenitors generates autoimmunity by SP T cells populating the periphery suggests that Btla is needed at some point after deletion. To understand at what point during T cell development Btla may be needed to establish tolerance, we deleted Btla in RTE that had the capacity to express Btla during thymic development. We found that loss of Btla signaling post thymic selection in RTE resulted in autoimmunity in lymphopenic host mice, evidenced by loss of weight and T cell infiltration of the host liver and kidneys. Similarly, deletion of PD-1 in RTE that had the capacity to express PD-1 during thymic development resulted in autoimmunity in host mice. Unlike the recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ thymocytes, CD4 T cell infiltration was observed in the lungs of $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ thymocyte recipients, in addition to kidney and liver infiltration. However, the heart appeared to be spared of CD4 T cell infiltration in the recipients of $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ or $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ thymocytes. In contrast, although uncommon, myocarditis secondary to T cell infiltration of the heart has been reported in cancer patients undergoing anti-PD-1 immunotherapy, and it is often fatal (318,319,322). While it is unclear why lymphocyte infiltration of the heart was not observed in the current study, a

previous study from our lab showed lymphocyte infiltration of the heart in mice adoptively transferred with PD-1$^{-/-}$ HSC (113). We speculate that using thymocytes, as in the current study, rather than HSC tends to reduce disease overall and infiltration of the heart might be lost first.

All the T cells in neonatal mice are considered to be RTE (31,315). The finding that Btla and PD-1 are required in the RTE suggests that neonatal splenocytes, which are mostly RTE, could be potentially highly autoreactive when PD-1 or Btla signaling is absent. Jiang *et al.* found a positive correlation between PD-1 expression and the affinity of myelin oligodendrocyte glycoprotein (MOG)-specific CD4^{+} T cells for their cognate self-antigen, thus suggesting that PD-1 might be needed to prevent autoimmunity by these MOG-specific T cells (115). Indeed, the onset of EAE disease was faster when these MOG-specific T cells lacked PD-1 (115). Interestingly, our group had previously shown that adult mouse splenic T cells lacking PD-1 or Btla did not cause autoimmunity in lymphopenic mice (113,313). In contrast, our group also showed that the RTE subpopulation of splenocytes from adult PD-1$^{-/-}$ mice generated autoimmunity in adult lymphopenic mice (297). Consistent with our data, a substantial proportion of neonatal CD4 T cells, those undergoing natural LIP, were found to express PD-1 (297). Based on these findings from our group, we examined whether the absence of PD-1 signaling in neonatal splenocytes, in which most T cells are RTE, results in autoimmunity in adult lymphopenic recipients. As expected, all the recipients of neonatal PD-1$^{-/-}$ splenocytes developed autoimmunity in contrast to the recipients of adult PD-1$^{-/-}$ splenocytes. Lymphocytes generated in neonatal mice express receptors with significantly less diversity than the adult lymphocyte repertoires due to the delayed expression of TdT in neonates (198). Although TdT deficiency did not reduce autoantibody production and hypergammaglobulinemia in (NZB × NZW)F_1 TdT$^{-/-}$ mice compared to TdT$^{+/+}$ WT control mice, the absence of TdT abrogated the

occurrence of autoimmune nephritis in the (NZB × NZW)F_1 $TdT^{-/-}$ mice and also improved their survival (199). Also, a recent study showed that the neonatal TCR repertoire is biased toward TCR with high affinity and high cross-reactivity toward self-antigen (323). Furthermore, a previous study showed that neonatal thymocytes or splenocytes from normal BALB/c mice generated autoimmune oophoritis and autoimmune gastritis when adoptively transferred to syngeneic athymic nu/nu (or SCID) mice (324). These studies demonstrated the autoimmune potential of neonatal T cells, and our study showed the importance of Btla and PD-1 signaling in establishing tolerance in T cells at the RTE stage in lymphopenic recipients. Although this study established the importance of Btla signaling and PD-1 signaling in blocking autoimmunity in RTE during lymphopenia, the germline Btla or PD-1 knockout mice seem to be protected from autoimmunity during the neonatal period when there is physiological lymphopenia. This is probably because [1] their T cell repertoire is first generated early in life, a period naturally deficient in lymph node stroma, or [2] early events take time for their consequences to become apparent. Consistent with the first idea, our group previously showed that neonatal $Rag^{-/-}$ recipients of PD-$1^{-/-}$ FLC were resistant to disease as were adult recipients that lacked or had reduced lymph nodes (113). Supporting the second idea, T cells generated early in life in the NOD mice play an important role in the initiation of insulitis long before the onset of overt diabetes that manifests much later in life (325) , suggesting that the consequences of early events may take time to manifest.

Taken together, our data suggest that Btla and PD-1 signaling are most critical when T cells initially seed the periphery and during neonatal life when all T cells are RTE. Hence, on the one hand, to reduce the incidence of iRAEs in cancer patients undergoing immunotherapy blocking PD-1 signaling, patients should be closely monitored for LIP of RTE following

radiotherapy and/or chemotherapy. The same goes for patients currently enrolled in the anti-Btla antibody clinical trials for the treatment of cancer (326,327). It remains unclear what is being recognized by the T cells to cause the disease in this study. Previous studies from our lab showed that autoimmunity in the lymphopenic recipients of PD-$1^{-/-}$ HSC or thymocytes was independent of gut microbiota (288) but could be due to an exaggerated response to self-pMHC or other resources present during lymphopenia (293).

Chapter 3

Loss of Btla early in T cell ontogeny leads to a long-term Increase in CD5 expression by T cells

3.1 Introduction

T cell activation and effector function is dictated in part by signaling via the TCR, and in part by signaling via costimulatory or coinhibitory receptors. While costimulation tends to favour T cell activation, coinhibition limits T cell activation and effector function. Coinhibitory receptors like programmed cell death protein-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) are absent from naïve T cells and are upregulated upon activation (328,329). In contrast, there are a small number of coinhibitory receptors expressed constitutively by naïve T cells, including CD5, B and T lymphocyte attenuator (Btla), and V-domain immunoglobulin suppressor of T cell activation (VISTA) (75,141,330,331).

CD5 (T1 or Leu-1 in humans, or Lyt-1 in mouse), is a coinhibitory receptor that is constitutively expressed in thymocytes, mature T cells, and a subset of B cells (B1a cells) (141,330,332–334). CD5 is expressed as a 67-kDa type I transmembrane glycoprotein (gp), which belongs to the highly conserved superfamily of protein receptors known as SRCR superfamily (118). The proposed ligands for CD5 includes: CD72 (127), IL-6 (128,129), gp40–80 (130–132), gp150 (133), gp200 (134), IgV_H framework region (135), and the CD5 itself (136); however, their physiological relevance in interacting with CD5 remains an active area of investigation. CD5 also appears to function without its extracellular domain, putting in question whether a ligand for CD5 is relevant. CD5 expression is set during T cell development in the thymus and finely adjusted throughout the life of the T cell (119,145). As such, CD5 surface expression on mature SP thymocytes and T cells correlates directly with the avidity or signaling intensity of the positively selecting TCR–peptide MHC (pMHC)-ligand interaction (119,335). During T cell activation, CD5 is rapidly recruited and co-localizes with the TCR/CD3 complex at the immune synapse resulting in the dampening of downstream TCR signals (138,336).

Btla (CD272) is a negative regulator of antigen receptors on B and T cells, dendritic cells, macrophages, and NK cells (72,75,79,337). Btla contains both a ITIM and a ITSM as well as a Grb2 recognition motif in its cytoplasmic domain (75). Following T cell activation and the interaction of Btla with its ligand, herpesvirus entry mediator (HVEM), the tyrosine residues in ITIM and ITSM of Btla are phosphorylated and then recruit SHP-1 and SHP-2 phosphatases to dampen TCR signaling (76,79). Consistent with its coinhibitory function, mice lacking Btla develop systemic autoimmunity and multiorgan lymphocytic infiltration (86). In addition, our group recently showed that Btla signaling is indispensable for the establishment of tolerance in RTE during lymphopenia (Adegoke *et al*., Chapter 2).

Unlike the $Btla^{-/-}$ mice that develops late-life autoimmunity (86), the $CD5^{-/-}$ mice remain relatively healthy in late-life (149). However, the constitutive expression of Btla and CD5 on T cells indicates they play a unique non-redundant role, but the relationship between these constitutively expressed coinhibitors is not well defined. Matson *et al*. recently reported an increased expression levels of CD5 in SH2-containing tyrosine phosphatase 1 (SHP-1) knockout T cells relative to the WT T cells (338). Since Btla and CD5 exert their inhibitory function through lymphocyte activation-induced recruitment of SHP-1 to their cytoplasmic tail tyrosine residues, we explored the relationship between these coinhibitors and found an inverse relationship between CD5 and Btla expression levels in T cell ontogeny.

3.2 Materials and Methods

3.2.1 Mice

Male and female mice included B6.129S7-*Rag1*^{tm1Mom}/J (Rag1$^{-/-}$), B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J (Foxp3^{EGFP}) (306,307), B6.Nur77^{GFP} (339), and B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$/J (*B6.UBC*$^{Cre/ERT2+}$) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). B6.Rag2p^{GFP} mice (35,36) were kindly provided by Pamela Fink (University of Washington, Seattle, WA), and our lab previously generated B6.Rag2p^{GFP}-PD-1$^{-/-}$ mice (297). C57BL/6-Btla$^{-/-}$ (abbreviated as Btla$^{-/-}$), and C57BL/6-Pdcd1$^{-/-}$ [abbreviated as PD-1$^{-/-}$; backcrossed 11 generations to C57BL/6; originally generated by Prof. T. Honjo and colleagues (105)] mice were bred at the University of Alberta. The Foxp3^{EGFP} x Btla$^{-/-}$ mice were generated by crossing Foxp3^{EGFP} and Btla$^{-/-}$ strains. Similarly, Foxp3^{EGFP} x PD-1$^{-/-}$ mice were generated by crossing Foxp3^{EGFP} and PD-1$^{-/-}$ strains (288). We generated the B6.Nur77^{GFP} x Btla$^{-/-}$ mice by crossing the B6.Nur77^{GFP} mice to the B6.Btla$^{-/-}$ mice. The B6.PD-1$^{fl/fl}$ mice were purchased from Taconic Biosciences (Rensselaer, NY, USA), and B6.Btla$^{fl/fl}$ mice were kindly provided by John R Šedý (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, United States). All mice were between 7 – 12 weeks old at the time they were used in experiments unless otherwise indicated. Animal care was in accordance with the Canadian Council on Animal Care guidelines. The studies were performed under Animal Use Protocol 00000215 approved by the Animal Care and Use Committee Health Sciences of the University of Alberta. Mice were housed under clean conventional housing conditions at the University of Alberta Health Sciences Laboratory Animal Services (HSLAS) facilities.

3.2.2 Tamoxifen-induced conditional deletion

To induce Btla or PD-1 deletion, $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ or $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ mice or adult $Rag^{-/-}$ recipients were intraperitoneally injected with 1.4 mg tamoxifen (Sigma-Aldrich) in corn oil [+5% (vol/vol) ethanol] on days 0, 1, 3, 5, and 6. Complete deletion was achieved by day 14 post the last dose of tamoxifen as assessed by flow cytometry. Control $B6^{CreERT2+/-}$ mice also received tamoxifen injection.

3.2.3 Hematopoietic stem cell transfer

FLC, which was a source of HSC, were harvested from embryonic day 14 – 16 $B6^{Cre/ERT2+/-}$ or $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ fetuses. A single-cell suspension was made on ice by gently pipetting the fetal livers and filtration through a 70 μm cell strainer (Fisherbrand™). Viability was assessed by trypan blue exclusion and > 90% viable cells were used for experiments. Seven-week-old male and female $Rag^{-/-}$ mice were used as recipients and each recipient received 20 x 10^6 FLC, followed by tamoxifen injection as described above.

3.2.4 Flow cytometry

Fluorochrome conjugated antibodies for flow cytometry staining used in this study were purchased from ThermoFisher Scientific: murine anti-TCRβ (H57-597), CD4 (RM4-5), CD5 (53-7.3), CD8α (53-6.7), FoxP3 (FJK-16s), Btla (6F7), PD-1 (J43); or BioLegend: CD19 (6D5). GFP expression was also analyzed in mice expressing the GFP transgene. In the $Rag2p^{GFP}$ mice, GFP expression, under the control of recombination-activating gene 2 (Rag2) gene promoter, is turned on when the Rag proteins are transiently expressed during the late DN and DP stages of T cell development (35). GFP expression persists through T cell egress into the peripheral

circulation for up to 3 weeks even though Rag2 expression has been terminated intrathymically (36). This enables a reliable assessment of RTE. Peripheral blood samples, thymocytes, and splenocytes were stained after incubation with FcR block, which was a cocktail of anti-CD16/32 antibody (2.4G2; Bio Express, West Lebanon, NH) and mouse, rat, and hamster sera, to reduce background staining and in turn, increase the specificity of antibody binding. The antibody staining was done at 4°C for 20 minutes, followed by washing and resuspension in HBSS supplemented with 2% FBS. A BD LSR II (BD Biosciences) with FlowJo software was used for data acquisition and analyses.

3.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data of biological replicates are depicted as mean ± standard error. Data were statistically analyzed using Student's t-test with Welch's correction or Wilcoxon matched-pairs signed rank test (Figure 3.1). Statistical analysis in experiments with one variable and three groups was performed using one-way ANOVA with Dunn's multiple comparisons test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

3.3 Results

3.3.1 Btla expression in the thymus and spleen is inversely related to CD5 expression

To examine the relationship between CD5 and Btla, we assessed CD5 and Btla expression levels in steady state in both TCRβ^{hi} SP T cells in the spleen and thymus from 7 – 10-week-old B6 mice (Fig. 3.1 A). Consistent with earlier studies (119,340), thymic SP T cells expressed higher levels of CD5 relative to splenic SP T cells (Fig. 3.1 B, E). In contrast, expression of Btla is lower in the thymic SP T cells relative to the splenic SP T cells (Fig. 3.1 B, E). Analysis of the proportion of Btla$^+$ cells among thymic and splenic SP T cells also revealed a reduced frequency of Btla$^+$ SP T cells in the thymus relative to the spleen (Fig. 3.1 C). Since the RTE make up a small fraction of the splenic T cells, we hypothesized that these newly generated T cells also express lower levels of Btla relative to their established (mature) T cell counterparts. To examine this, we used the Rag2p-GFP mice, where GFP expression is restricted to RTE and newly generated B cells (35,36). Analysis of Btla expression between the GFP+ (RTE) and GFP– (mature) T cells in the spleen revealed that Btla expression is significantly lower in splenic RTE relative to their mature T cell counterparts (Fig. 3.1 D). Collectively, these data suggest an inverse relationship between CD5 and Btla expression levels, with low Btla expression in the thymus (including RTE) and higher Btla expression in the periphery, corresponding with high and low CD5 expression, respectively (Fig. 3.1 E).

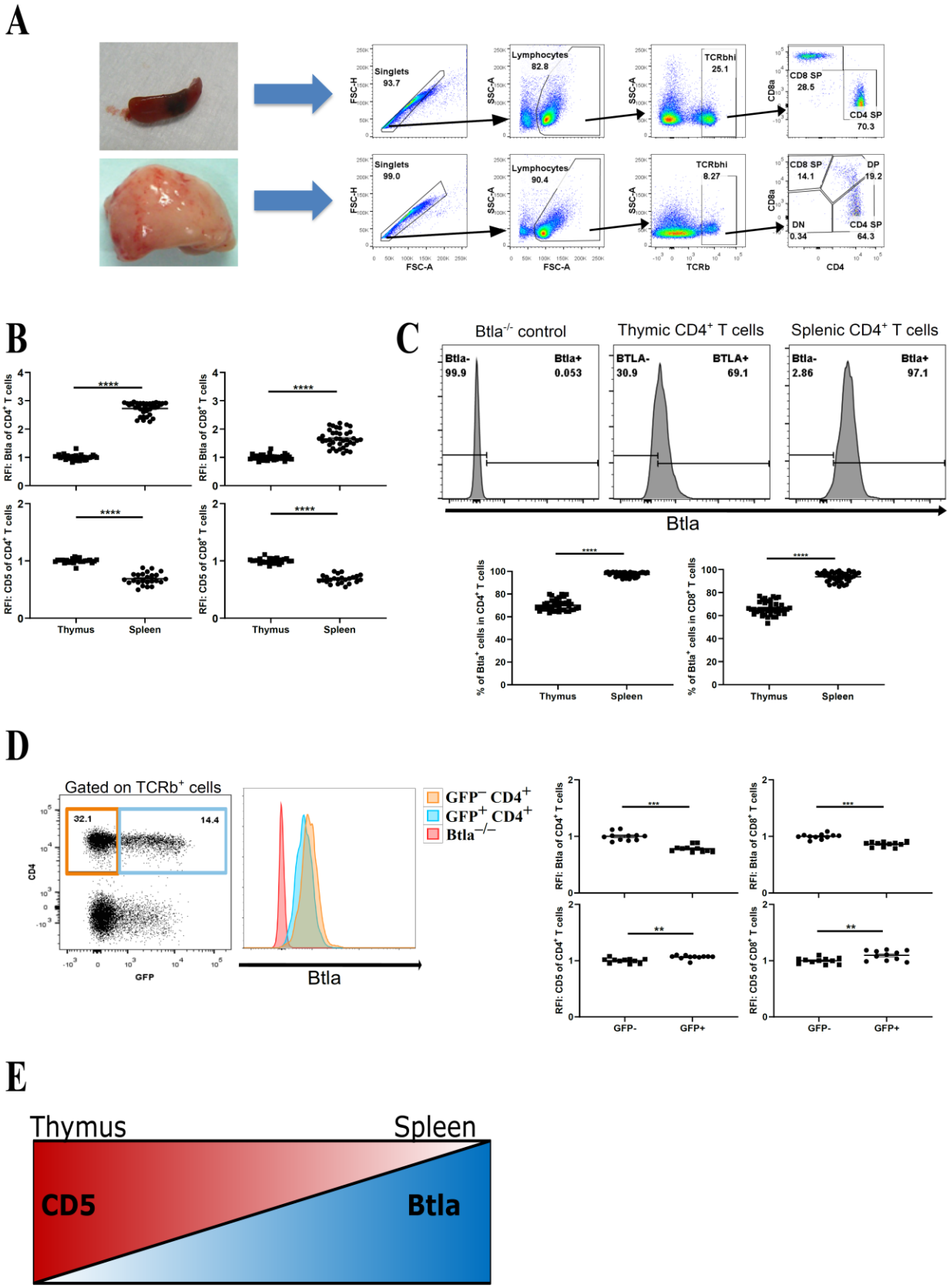

A
Singlets
93.7
Lymphocytes
82.8
TCRbhi
25.1
CD8 SP
28.5
CD4 SP
70.3
Singlets
99.0
Lymphocytes
90.4
TCRbhi
8.27
CD8 SP
14.1
DP
19.2
DN
0.34
CD4 SP
64.3
FSC-A
SSC-A
TCRb
CD4
CD8a
B
RFI: Btla of CD4+ T cells
RFI: Btla of CD8+ T cells
RFI: CD5 of CD4+ T cells
RFI: CD5 of CD8+ T cells
****
Thymus
Spleen
C
Btla-/- control
Thymic CD4+ T cells
Splenic CD4+ T cells
Btla-
99.9
Btla+
0.053
BTLA-
30.9
BTLA+
69.1
Btla-
2.86
Btla+
97.1
Btla
% of Btla+ cells in CD4+ T cells
% of Btla+ cells in CD8+ T cells
D
Gated on TCRb+ cells
32.1
14.4
CD4
GFP
Btla
GFP− CD4+
GFP+ CD4+
Btla−/−
RFI: Btla of CD4+ T cells
RFI: Btla of CD8+ T cells
RFI: CD5 of CD4+ T cells
RFI: CD5 of CD8+ T cells
***
**
GFP-
GFP+
E
Thymus
Spleen
CD5
Btla

Figure 3.1. *Btla expression in the thymus and spleen is inversely related to CD5 expression.* [**A**] Gating strategy for CD5 and Btla in the spleen and thymus. We gated on single cells, followed by lymphocytes, and then the TCRβhi SP T cells in the spleen (upper row) and thymus (lower row). [**B**] Relative fluorescent intensities (RFIs) of Btla (upper row) or CD5 (lower row) in CD4 SP T cells (left column) and CD8 SP T cells (right column) in the thymus and spleen (Btla, n = 39; CD5, n = 24). To calculate the RFI of Btla or CD5, MFI data were normalized to the average MFI of Btla or CD5 of the thymic SP T cells in each individual experiment. [**C**] Representative histograms showing the proportion of $Btla^+$ CD4 SP T cells (upper row) in the thymus and spleen. Lower row shows the proportion of $Btla^+$ CD4 SP T cells (lower left) and $Btla^+$ CD8 SP T cells (lower right) in the thymus and spleen (n = 39). [**D**] Representative flow cytometry dot plots (left) and histogram (middle) of the indicated markers in splenic $TCR\beta^+$ cells from 7 -10-wk old $B6.Rag2p^{GFP}$ mice. RFIs of Btla (upper right) and CD5 (lower right) on mature (GFP–) or newly generated (GFP+) SP T cells in the spleen (n = 11). [**E**] shows the inverse relationship between CD5 and Btla in the thymus and spleen. Data are normalized to the average Btla MFI or CD5 MFI of the GFP negative splenic SP T cells in each individual experiment. Dots indicate individual mice from six – nine independent experiments (B – C) or two independent experiments (D). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

3.3.2 T cell repertoire in Btla$^{-/-}$ mice express higher levels of CD5

The inverse relationship between CD5 and Btla expression levels in the thymus and spleen suggests the possibility there is a causal relationship between these coinhibitors affecting their expression. To determine if there is a specific causal relationship between Btla expression and CD5 expression, we compared CD5 expression levels between polyclonal WT and coinhibitor (Btla or PD-1) deficient T cells in the periphery. The expression levels of CD5 were significantly higher in the Btla$^{-/-}$ splenic CD4^{+} and CD8^{+} T cells relative to their WT and PD-1$^{-/-}$ counterparts (Fig. 3.2 A). Interestingly, CD5 expression levels in the PD-1$^{-/-}$ splenic CD8^{+} T cells were significantly higher than that in the WT splenic CD8^{+} T cells (Fig. 3.2 A). To assess whether the elevated CD5 expression in the splenic Btla$^{-/-}$ splenic T cells originated from the thymus or preferentially increased in the periphery, we compared CD5 expression levels between the WT, PD-1$^{-/-}$, and Btla$^{-/-}$ thymic TCRβ^{hi} SP cells. Examination of thymic TCRβ^{hi} cells revealed that these cells in the Btla$^{-/-}$ mice expressed higher levels of surface CD5 than do their WT or PD-1$^{-/-}$ counterparts (Fig. 3.2 B). In addition to being a coinhibitory receptor, the level of surface CD5 expression is directly proportional to the signaling strength of the TCR:self-antigens (self-pMHC) interaction (119,335). Similar to CD5, Nur77 serves as a specific reporter of antigen receptor signaling in murine and human T and B cells (341–345). Nur77 is an immediate early gene whose expression is rapidly upregulated by TCR signaling in murine T cells and human thymocytes (341,346). In Nur77^{GFP} reporter mice, GFP expression level is proportional to the TCR signaling strength (339). To complement our findings on CD5, we hypothesized that Nur77^{GFP} expression is enhanced in the Btla$^{-/-}$ T cells. To examine this, we generated Btla$^{-/-}$ Nur77^{GFP} mice by crossing the B6.Nur77^{GFP} mice to B6.Btla$^{-/-}$ mice and compared their GFP expression levels in the splenic and thymic T cells to that in WT Nur77^{GFP} mice. The splenic

Btla$^{-/-}$ CD8^{+} T cells expressed slightly higher levels of GFP relative to the WT CD8^{+} T cells and a trend towards an increased GFP expression was also observed in the thymic Btla$^{-/-}$ CD8^{+} T cells relative to the WT CD8^{+} T cells (Fig. 3.2 C). In contrast, GFP expression in the thymic and splenic Btla$^{-/-}$ CD4^{+} T cells was not significantly different from their WT CD4^{+} T cell counterparts (Fig. 3.2 C). Since Nur77^{GFP} expression in B cells also correlates to the B cell receptor affinity for antigen (345), we compared GFP expression levels in B cells from Btla$^{-/-}$ Nur77^{GFP} mice and the WT Nur77^{GFP} mice and found no significant difference between the two groups (Fig. 3.2 D). Although CD5 expression levels was enhanced in the PD-1$^{-/-}$ splenic CD8^{+} T cells (Fig. 3.2 A), whether Btla expression levels are regulated by PD-1 is unknown. Thus, we examined Btla expression levels between the thymic and splenic WT and PD-1$^{-/-}$ T cells and found no significant difference (Fig. 3.2 E). Together, these data suggests that Btla expression directly or indirectly determines the level of CD5 expression broadly across T cells.

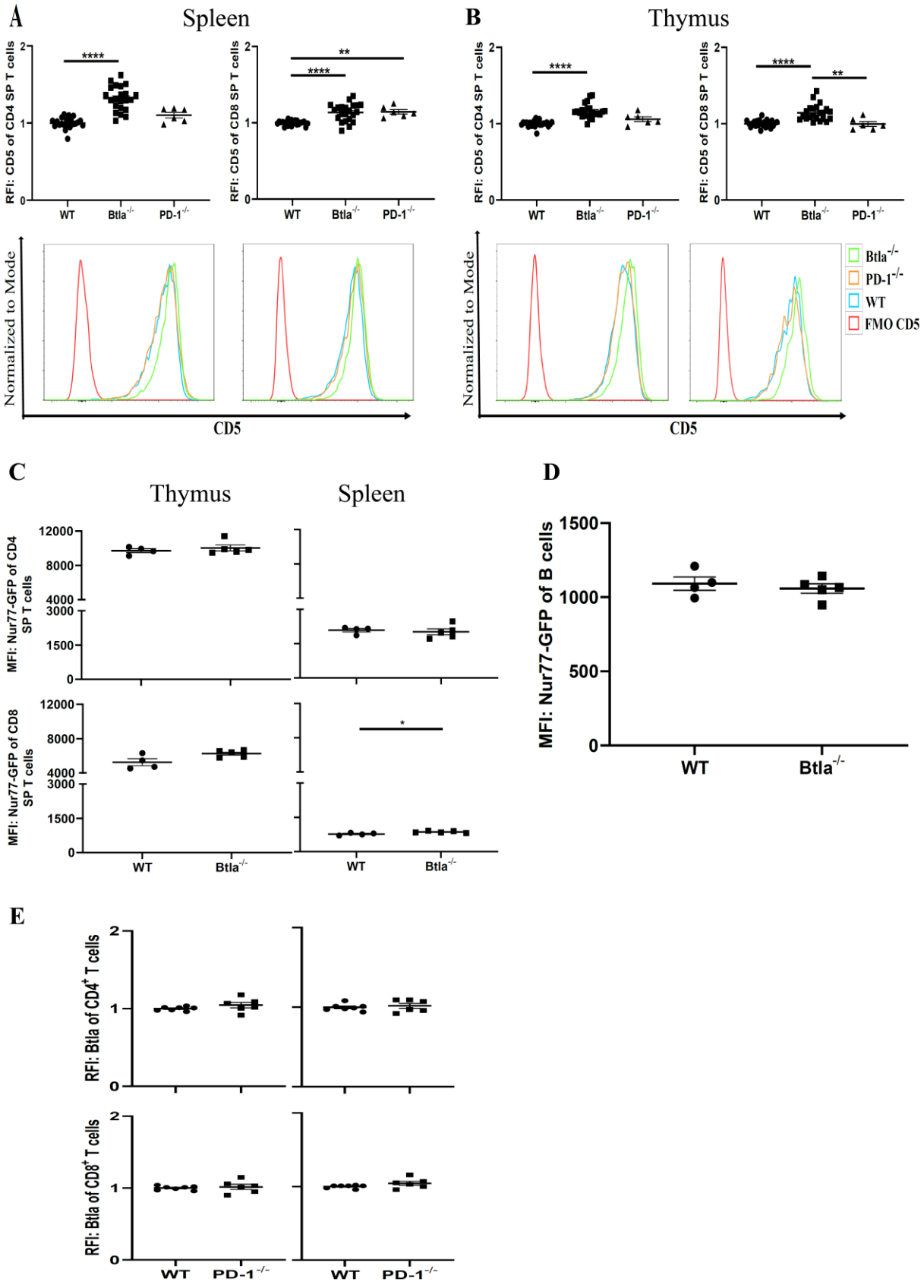

A
Spleen
RFI: CD5 of CD4 SP T cells
RFI: CD5 of CD8 SP T cells
WT
Btla-/-
PD-1-/-
****
**
B
Thymus
Normalized to Mode
CD5
Btla-/-
PD-1-/-
WT
FMO CD5
C
Thymus
Spleen
MFI: Nur77-GFP of CD4 SP T cells
MFI: Nur77-GFP of CD8 SP T cells
*
D
MFI: Nur77-GFP of B cells
E
RFI: Btla of CD4+ T cells
RFI: Btla of CD8+ T cells

Figure 3.2. *T cells in the Btla$^{-/-}$ mice express higher levels of CD5. RFIs of CD5 in splenic* [**A**] CD4 SP T cells (left column) and CD8 SP T cells (right column) or thymic [**B**] T cell subsets and their corresponding representative histograms (lower rows) from WT [n = 24; (B6.Foxp3^{GFP}, B6.Nur77^{GFP})], Btla$^{-/-}$ [n = 22; (B6.Foxp3^{GFP} x Btla$^{-/-}$, B6.Nur77^{GFP} x Btla$^{-/-}$)], and PD-1$^{-/-}$ [n = 6; (B6.Foxp3^{EGFP} x PD-1$^{-/-}$)] mice [**C**] MFI of Nur77^{GFP} in thymic (left column) and splenic T cells (right column) of WT and Btla$^{-/-}$ mice. [**D**] MFI of Nur77^{GFP} in splenic B cells of WT (n = 4) and Btla$^{-/-}$ (n = 5) mice. [**E**] RFIs of Btla in thymic (left column) or splenic T cells (right column) of WT [n = 7; (B6.Foxp3^{GFP})] and PD-1$^{-/-}$ [n = 6; (B6.Foxp3^{EGFP} x PD-1$^{-/-}$)] mice. To calculate RFIs, data are normalized to the average MFI of CD5 [B] or Btla [D] of the WT splenic or thymic SP T cells in each individual experiment. Dots indicate individual mice from five independent experiments [A and B], one and two independent experiments in [C and D], respectively. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

3.3.3 CD5 expression in Btla$^{-/-}$ mice is increased post thymic selection and independent of Tregs

Having shown that expression levels of CD5 in T cells of the Btla$^{-/-}$ mice is increased prior to thymic export (Fig. 3.2 B), we next sought to determine at what point during T cell ontogeny the surface CD5 expression levels is skewed in the Btla$^{-/-}$ mice. In the WT B6 mice, the increase in CD5 expression levels in developing thymocytes follows a stepwise progression from the immature DN T cells to the SP T cells (119). Thus, similar to the WT B6 mice, we hypothesized that the CD5 expression levels in the Btla$^{-/-}$ T cells increased post thymic selection. To examine this, we compared CD5 expression levels in the CD4^{-}CD8^{-} DN and CD4^{+}CD8^{+} DP T cells of the WT and Btla$^{-/-}$ B6 mice (Fig. 3.3 A). Supporting our hypothesis, WT, Btla$^{-/-}$, and PD-1$^{-/-}$ T cells expressed a similar level of CD5 prior to thymic selection (Fig. 3.3 B). Developing thymocytes expressing TCR with moderate to high affinity for self-pMHC are preferentially recruited to the Treg lineage (63,64). We examined whether the Btla$^{-/-}$ CD4 SP T cells expressing higher levels of CD5 are enriched for Treg cells both in the thymus and spleen relative to the WT CD4 SP T cells. The B6 WT, Btla$^{-/-}$ and PD-1$^{-/-}$ mice have a similar proportion of Foxp3^{+} (Treg marker) CD4^{+} T cells in the thymus and, consistent with our previous findings (288), the splenic PD-1$^{-/-}$ CD4^{+} T cells have a higher proportion of Foxp3^{+} cells relative to the splenic WT CD4^{+} T cells (Fig. 3.3 C). The absolute count of Foxp3^{+} T cells in the splenic CD4^{+} T cells is higher in the Btla$^{-/-}$ mice and their PD-1$^{-/-}$ counterparts relative to their WT counterparts (Fig. 3.3 D). Although the Treg cells make up a minute proportion of the bulk CD4^{+} T cell population, the preferential expression of higher levels of CD5 in the Treg cells (121), may skew the CD5 expression levels in the Btla$^{-/-}$ CD4^{+} T cells. To examine this, we analyzed CD5 expression levels in the splenic and thymic TCRβ^{+} CD4^{+} Foxp3^{+} (Treg) or TCRβ^{+}

$CD4^+$ $Foxp3^-$ (non-Treg) cells of WT and $Btla^{-/-}$ mice. The CD5 expression levels was significantly higher in both Treg and non-Treg cells of the B6.$Btla^{-/-}$ mice in the spleen and thymus (Fig. 3.3 E, F). Together, these data indicate that CD5 expression levels on developing $Btla^{-/-}$ T cells is preferentially increased post thymic selection, and the increased CD5 expression in the $Btla^{-/-}$ T cells occurs is both conventional T cells and Treg cells.

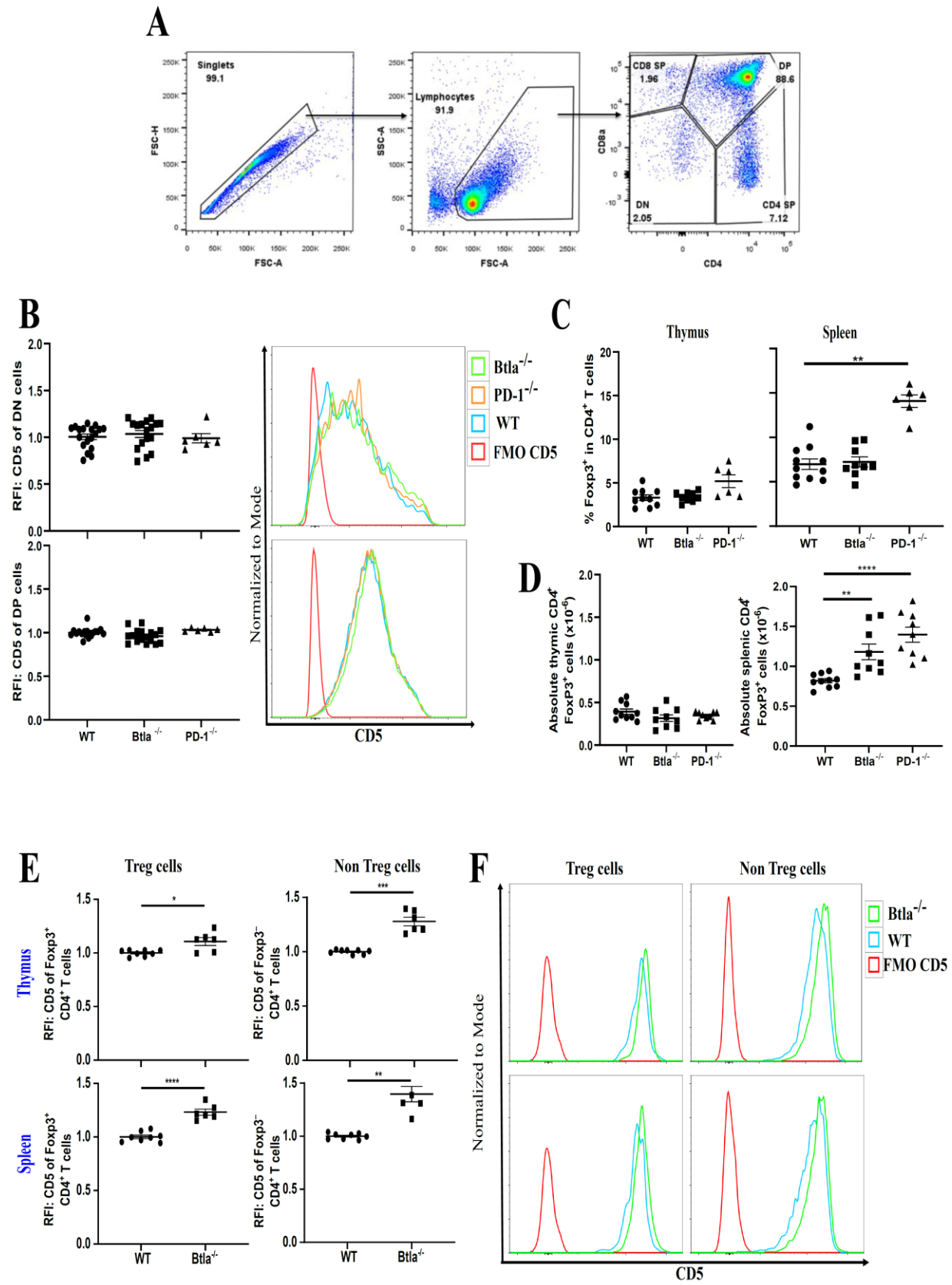

A
Singlets
99.1
Lymphocytes
91.9
CD8 SP
1.96
DP
88.6
DN
2.05
CD4 SP
7.12
FSC-H
FSC-A
SSC-A
CD8a
CD4
B
RFI: CD5 of DN cells
RFI: CD5 of DP cells
WT
Btla-/-
PD-1-/-
Normalized to Mode
CD5
Btla-/-
PD-1-/-
WT
FMO CD5
C
Thymus
Spleen
% Foxp3+ in CD4+ T cells
D
Absolute thymic CD4+ FoxP3+ cells (x10-6)
Absolute splenic CD4+ FoxP3+ cells (x10-6)
E
Treg cells
Non Treg cells
Thymus
Spleen
RFI: CD5 of Foxp3+ CD4+ T cells
RFI: CD5 of Foxp3- CD4+ T cells
F
Treg cells
Non Treg cells
Normalized to Mode
CD5
Btla-/-
WT
FMO CD5

Figure 3.3. *CD5 expression in developing T cells is increased in the Btla$^{-/-}$ mice post thymic selection.* [**A**] Gating strategy for CD5 expression in the immature T cells show gating on single cells, followed by lymphocytes, and then the DP and DN cells in the thymus. [**B**] RFIs of CD5 in DN and DP cells in the WT, Btla$^{-/-}$, PD-1$^{-/-}$ mice (left column) and representative histograms (right column). Comparison of the percent CD4^{+} Foxp3^{+} T cells [**C**] and absolute count of CD4^{+} Foxp3^{+} T cells [**D**] in the thymus and spleen of adult B6 WT, Btla$^{-/-}$, and PD-1$^{-/-}$ mice. [**E**] RFIs of CD5 in Treg and non-Treg cells of WT and Btla$^{-/-}$ mice, and representative histograms [**F**]. To calculate RFIs, data are normalized to the average MFI of CD5 of the WT thymic or splenic T cells in each individual experiment. Dots indicate individual mice from a minimum of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.3.4 Loss of Btla early in T cell ontogeny leads to a long-term increase in CD5 expression by T cells

The loss of Btla expression early in T cell ontogeny might alter the TCR repertoire indirectly affecting CD5 levels, or instead its loss might affect CD5 expression rapidly within mature T cells (Fig. 3.4 A). To examine the latter, we crossed the B6.Btla$^{fl/fl}$ strain to a tamoxifen-inducible Cre recombinase expressing strain, B6$^{Cre/ERT2}$, to generate B6$^{Cre/ERT2+/-}$ Btla$^{fl/fl}$ (Fig. 2.1). Adult 7-week-old B6$^{Cre/ERT2+/-}$ Btla$^{fl/fl}$ and WT control B6$^{Cre/ERT2+/-}$ mice were injected intraperitoneally with five doses of tamoxifen, and their thymus and spleen were examined for Btla expression and CD5 expression levels in T cells (Fig. 3.4 B). Splenic CD4 and CD8 SP T cells, which were 97 ± 2% and 94 ± 4% positive for Btla, respectively, in the B6$^{Cre/ERT2+/-}$ mice were reduced to 2 ± 1% and 3 ± 2% positive for Btla in the B6$^{Cre/ERT2+}$Btla$^{fl/fl}$ mice two weeks after the last dose of tamoxifen (Fig. 3.4 C). Initial assessment of blood samples at one week post tamoxifen showed CD5 expression levels in CD4 SP T cells that no longer express Btla was significantly higher in comparison to their Btla-positive counterparts (Fig. 3.4 D). In contrast, CD8 T cells that had lost Btla did not have increased CD5 (Fig. 3.4 D). Similarly, assessment of thymus and spleen at one week post tamoxifen showed significantly higher CD5 expression levels in the splenic CD4 SP T cells that no longer express Btla relative to their Btla-positive counterparts, but the reverse was seen among splenic CD8 SP T cells (Fig. 3.4 E). Interestingly, when Btla was completely deleted at two weeks post tamoxifen, the CD5 expression levels in the thymic and splenic CD4 or CD8 T cells of B6$^{Cre/ERT2+/-}$ Btla$^{fl/fl}$ mice were not significantly different compared to the B6$^{Cre/ERT2+/-}$ mice (Fig. 3.4 F). In contrast, CD5 expression levels were significantly lower in the splenic T cells from the B6$^{Cre/ERT2+/-}$ PD-1$^{fl/fl}$ mice post tamoxifen injection (Fig. 3.4 F). Thus, in contrast to germline Btla$^{-/-}$ mice, which

expressed higher levels of CD5, inducible deletion of Btla in adult mice resulted in a transient increase in CD5 expression levels at one week, and only in CD4 T cells. The increased CD5 expression levels in the germline $Btla^{-/-}$ mice suggests that it is the loss of Btla early in T cell ontogeny that leads to a long-term increase in CD5 expression by T cells. To examine this, FLC from embryonic day 14 – 16 $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ were adoptively transferred to adult $Rag^{-/-}$ mice, followed by 5 doses of tamoxifen injection intraperitoneally to induce Btla gene deletion in the transferred cells. The WT control group received FLC from $B6^{Cre/ERT2+/-}$ mice. In this experiment, FLC was used a source of HSC, allowing for T cell progenitors to undergo thymic development, and then seeding of the periphery. We detected SP T cells in the blood of recipient mice around 4 weeks post FLC transfer. Similar to the germline $Btla^{-/-}$ T cells, peripheral T cells in the recipients of FLC from $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ mice maintained an increased CD5 expression levels through the 8-week experimental period (Fig. 3.4 G). Together, our data indicates that loss of Btla early, but not later in T cell ontogeny, leads to a long-term increase in CD5 expression by T cells. Such calibration of CD5 levels early in T cell ontogeny might serve to reduce autoimmunity.

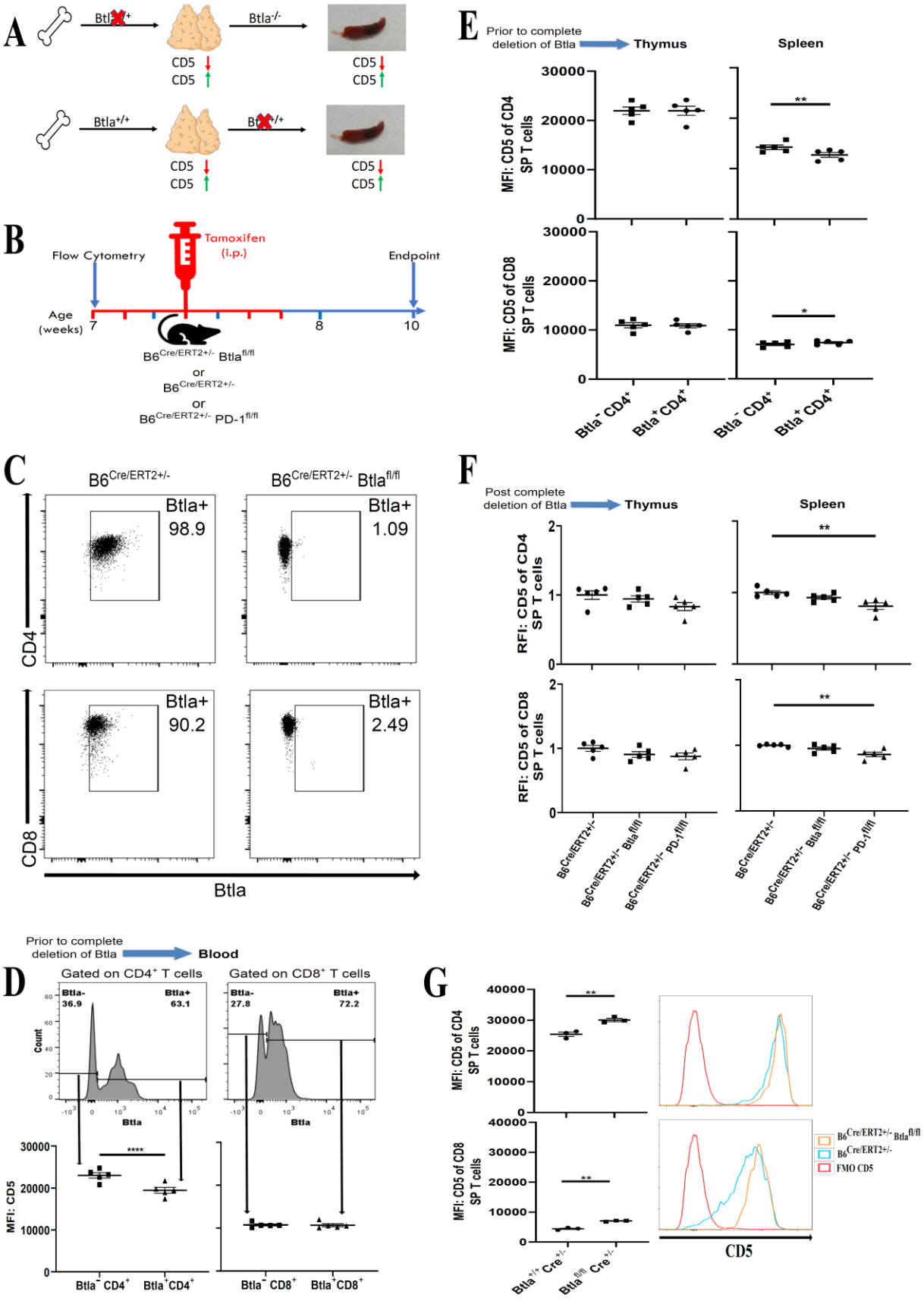

A
Btla+/+
Btla-/-
CD5
CD5
B
Flow Cytometry
Tamoxifen
(i.p.)
Endpoint
Age
(weeks)
7
8
10
B6Cre/ERT2+/- Btlafl/fl
or
B6Cre/ERT2+/-
or
B6Cre/ERT2+/- PD-1fl/fl
C
B6Cre/ERT2+/-
B6Cre/ERT2+/- Btlafl/fl
Btla+
98.9
Btla+
1.09
Btla+
90.2
Btla+
2.49
CD4
CD8
Btla
D
Prior to complete
deletion of Btla
Blood
Gated on CD4+ T cells
Gated on CD8+ T cells
Btla-
36.9
Btla+
63.1
Btla-
27.8
Btla+
72.2
Count
Btla
MFI: CD5
****
Btla- CD4+
Btla+CD4+
Btla- CD8+
Btla+CD8+
E
Prior to complete
deletion of Btla
Thymus
Spleen
MFI: CD5 of CD4
SP T cells
MFI: CD5 of CD8
SP T cells
**
*
Btla- CD4+
Btla+ CD4+
Btla- CD4+
Btla+ CD4+
F
Post complete
deletion of Btla
Thymus
Spleen
RFI: CD5 of CD4
SP T cells
RFI: CD5 of CD8
SP T cells
**
**
B6Cre/ERT2+/-
B6Cre/ERT2+/- Btla fl/fl
B6Cre/ERT2+/- PD-1fl/fl
B6Cre/ERT2+/-
B6Cre/ERT2+/- Btla fl/fl
B6Cre/ERT2+/- PD-1fl/fl
G
MFI: CD5 of CD4
SP T cells
MFI: CD5 of CD8
SP T cells
**
**
Btla+/+ Cre+/-
Btlafl/fl Cre+/-
CD5
B6Cre/ERT2+/- Btlafl/fl
B6Cre/ERT2+/-
FMO CD5

Figure 3.4. *Loss of Btla early in ontogeny leads to a long-term increase in CD5 expression by T cells.* [**A**] The loss of Btla expression early in T cell precursors might alter the TCR repertoire indirectly affecting CD5 levels (upper figure), or instead its loss might affect CD5 expression rapidly within mature T cells (lower figure). [**B**] Adult $B6^{Cre/ERT2+/-}$, $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$, and $B6^{Cre/ERT2+/-}$ $PD\text{-}1^{fl/fl}$ mice received five doses of tamoxifen on days 0, 1, 3, 5, and 6 (highlighted in red). [**C**] Representative dot plot of Btla expression in the splenic CD4 SP T cells (upper row) and CD8 SP T cells (lower row) in the $B6^{Cre/ERT2+/-}$ mice (left column) and $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ mice (right column) at two weeks post tamoxifen. [**D**] Representative histograms (upper row) and the MFI (lower row) of $Btla^{+}$ and $Btla^{-}$ cells in the CD4 gate (left column) and CD8 gate (right column) in the peripheral blood at one week post tamoxifen. [**E**] MFI (lower row) of $Btla^{+}$ and $Btla^{-}$ cells in the CD4 gate (upper row) and CD8 gate (lower row) in the thymus (left column) and spleen (right column) at one week post tamoxifen. [**F**] shows the RFI of CD5 in thymic T cells (left column) and splenic T cells (right column) of the indicated mice two weeks after the last dose of tamoxifen. [**G**] MFI of CD5 in CD4 SP T cells (upper) and CD8 SP T cells (lower) with respective representative histograms of peripheral T cells in the recipients of FLC from $B6^{Cre/ERT2+/-}$ and $B6^{Cre/ERT2+/-}$ $Btla^{fl/fl}$ at eight weeks post tamoxifen. Dots indicate individual mice from two independent experiments (Fig. D, F) and one experiment (Fig. E, G). $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

3.4 Discussion

In this study, we aimed to understand the relationship between the constitutively expressed T cell coinhibitory molecules Btla and CD5 in the steady state. Our assessment of CD5 and Btla expression in the WT B6 mice showed that Btla expression levels are lower in thymic SP T cells compared to their splenic SP T cell counterparts, corresponding to higher CD5 expression in the thymus and lower expression levels in the spleen. As anticipated, our data on CD5 expression levels in the thymus and spleen is consistent with findings in the literature (119,340). Although Btla is known to be expressed in the thymus and spleen (72,73), our data show that splenic SP T cells in the WT B6 mice expresses higher levels of Btla relative to the thymic SP T cells. Similarly, we found an increased proportion of $Btla^{+}$ SP T cells in the spleen relative to the thymus. Based on that, we speculate that the small proportion of newly generated T cells (i.e., RTE) in the spleen did not skew the overall Btla expression levels among splenic SP T cells. As such, we hypothesized that RTE in the spleen would express lower levels of Btla relative to the mature splenic SP T cells. Indeed, using the B6 $Rag2p^{GFP}$ mice that allowed us to differentiate RTE from the mature T cells based on GFP expression (35,36), our data supports our hypothesis that splenic RTE express lower levels of Btla relative to the mature splenic T cells. Interestingly, splenic RTE expressed higher levels of CD5 relative to their mature T cell counterparts). Overall, these findings suggested an inverse relationship in Btla expression and CD5 expression levels in the thymus and spleen. Furthermore, the data suggested that the mature T cell level of CD5 expression is more rapidly achieved than that of Btla expression once T cells are exported from the thymus into the periphery.

Analysis of CD5 expression in the $Btla^{-/-}$ mice showed that CD5 expression levels is enhanced in the $Btla^{-/-}$ T cells. Mice deficient in Btla develop late-life autoimmunity (86),

suggesting that either other coinhibitors compensate for the loss of Btla in early life or early events take time for their consequences to become apparent. Our findings that CD5 expression levels are elevated in the Btla$^{-/-}$ mice suggests that CD5 could serve as a compensatory coinhibitory receptor due to the loss of Btla signaling. While upregulation of a compensatory coinhibitor may serve to limit T cell autoreactivity, in cancer therapy, compensatory upregulation of other coinhibitors when one coinhibitory signaling pathway is blocked often limit the efficacy of treatment (347). Btla blockade has be shown to limit tumour growth and improve survival in a murine model (348); as a result, Btla blockade is currently being tested in clinical trials (349). However, to improve Btla blockade effectiveness and efficacy, our data on the increased CD5 expression levels when Btla signaling is lost indicate that more studies need to be done to understand how Btla regulates other immune signaling molecules. Supporting our finding in the PD-1$^{-/-}$ mice, where CD5 expression levels was only elevated in the splenic CD8^{+} T cells (Fig. 3.2 A), a recent report showed that blocking CD5 together with PD-1 substantially enhanced survival and the capacity of cytotoxic T cells to eradicate 4T1 breast tumour cells *ex vivo* as opposed to blocking CD5 or PD-1 alone (350). Although our data showed a disparity between CD5 and Nur77^{GFP} expression levels, Zinzow-Kramer *et al*., had previously reported that a broad range exists in the expression levels of CD5, Ly6C, and Nur77^{GFP} *in vivo* for naïve T cells even within the same TCR niche (351). This suggests that CD5 and Nur77 are distinctly expressed on T cells and can be regulated independently of each other.

In the WT mice, developing T cells in the thymus express levels of CD5 as follows: DN T cells < DP T cells < SP T cells (119). Having found that CD5 expression levels are altered in the Btla$^{-/-}$ SP T cells, we wondered at what point in T cell development this alteration took place and assessed CD5 expression levels in the DN and DP T cells between the Btla$^{-/-}$ and WT mice.

Interestingly, there was no significant difference in CD5 expressions in the immature T cells of the WT and Btla$^{-/-}$ mice, suggesting that CD5 expression levels preferentially increased post thymic selection in the Btla$^{-/-}$ SP T cells. In the WT mice, CD4^{+} T cells expressing high levels of CD5 are enriched for Foxp3^{+} regulatory T cells both in the thymus and spleen (63,64,352). Although there was no significant difference in the proportion of splenic and thymic CD4^{+} Foxp3^{+} Tregs in the WT and Btla mice, we found an increased amount of Tregs in the splenic Btla$^{-/-}$ T cells.

In addition to Btla signaling enforcing tolerance in RTE (Adegoke *et al*., Chapter 2), it also appears to regulate CD5 expression levels in T cells. Supporting the finding that germline Btla deletion enhanced CD5 expression, we employed the inducible Cre-lox system (304,305) to delete Btla in mice that otherwise expressed Btla and observed a transient upregulation of CD5 expression in the proportion of CD4 T cells that no longer expressed Btla. Of note, these B6$^{Cre/ERT2+/-}$ Btla$^{fl/fl}$ mice were only kept for two weeks post tamoxifen injection. Thus, whether or not the CD5 expression level is increased in these mice long after Btla deletion is unknown. In addition, if Btla deletion in the B6$^{Cre/ERT2+/-}$ Btla$^{fl/fl}$ mice preferentially recruited CD5^{hi} T cells during thymic selection, it may take up to two weeks for these CD5^{hi} T cells to exit the thymus and, at least, another three weeks to replace the CD5^{lo} or CD5^{int} T cells in the B6$^{Cre/ERT2+/-}$ Btla$^{fl/fl}$ mice post tamoxifen injection. Indeed, when Btla was deleted in HSC, SP T cells populating the periphery showed increased CD5 expression, which was maintained long-term. Our study is the first to show that loss of Btla early on in T cell ontogeny results in a long-term increase in their CD5 expression levels. However, how Btla regulates CD5 expression levels is unknown. We speculate that Btla either controls the level of CD5 gene expression, i.e., independent of the TCR repertoire, or instead Btla levels affect which TCRs are selected into the repertoire and thus

changes the repertoire's affinity for self and hence changes CD5 expression levels. In a preliminary study, we performed scRNASeq on WT and Btla$^{-/-}$ CD4 SP thymocytes and found distinct gene clusters and TCR clonotypes between WT and Btla$^{-/-}$ CD4 SP T thymocytes (Appendix 1). Our data showed an inverse relationship between CD5 and Btla expression levels in the steady state, however, whether the loss of CD5 leads to a long-term increase in Btla expression levels is also unknown. In this study, we did not examine CD6 expression, which is a receptor protein expressed in T cells and is highly homologous to the CD5 receptor in terms of structure and function (353–357). In addition, it is currently not known whether HVEM, the ligand for Btla (76), also regulates CD5 expression levels in T cells. In conclusion, we showed that Btla regulates CD5 expression levels in the T cell repertoire and this may have important implications for the prevention of autoimmunity by acting as a compensatory coinhibitor and by modulating T cell survival.

Chapter 4

Anti-CD52 treatment blocks EAE independent of PD-1 signals, increases proportion of DN T cells, newly generated T and B cells, and prevents EAE relapse when the thymus is functional

Some of the studies in this chapter have been published as two separate manuscripts:

***Adegoke A**, *Haile Y, Laribi B, Lin J, Anderson CC. Anti-CD52 blocks EAE independent of PD-1 signals and promotes repopulation dominated by double negative T cells and newly generated T and B cells. *(*co-first authors)* Eur J Immunol. 2020 Sep;50(9):1362-1373. doi: 10.1002/eji.201948288.

Adegoke AO., Lin J., Anderson CC. Loss of thymic function promotes EAE relapse in anti-CD52-treated mice. Curr Res Immunol. 2022 Mar 8;3:37-41. doi: 10.1016/j.crimmu.2022.03.001

Yohannes Haile contributed data to Figures 4.1, 4.2, 4.3, 4.4, and 4.6; Bahareh Laribi contributed data to Figures 4.1; Jiaxin Lin performed thymectomy and edited the manuscript.

4.1 Introduction

Lymphocyte depletion is an approach for treating AD. Targeting B cells has been a successful approach in several AD, including MS (358). More recently, polyclonal T cell depleting antibodies have been employed to reset the MS patient's immune system, with impressive efficacy (359). Anti-CD52 (alemtuzumab, lemtrada®) is instead a humanized IgG1 monoclonal antibody (mAb) that eliminates CD52-expressing lymphocytes and generates a marked lymphopenia. Administration of this depleting mAb has been employed clinically for the treatment of lymphoma (360), the prevention of transplant rejection (361–363), and more recently it has been shown to efficiently inhibit relapses in MS patients (282,364). CD52 is highly expressed on the surface of T and B lymphocytes and to a lesser extent on NK cells, monocytes, macrophages, DCs, eosinophils, and neutrophils (365–369). Although the function of CD52 on immune cells is still unclear, some evidence suggests that it may modulate T cell activation and induce regulatory CD4 T cells (363). Activated CD4 T cells with higher CD52 expression were able to suppress other effector T cells (370). Cross-linking of CD52 on human B and T lymphoma cell lines mediates growth inhibition followed by apoptosis (371,372).

The mechanisms by which alemtuzumab eliminates CD52 expressing cells involves induction of antibody dependent cell mediated cytotoxicity via neutrophils and NK cells (373), complement-dependent cytotoxicity, and triggering of apoptotic pathways on CD52 positive cells (374–378). Alemtuzumab for the treatment of RRMS is typically administered for 5 consecutive days initially and then for 3 days one year later. Following alemtuzumab induced lymphopenia, immune cells slowly repopulate. Lymphopenia poses a considerable risk for the development of autoimmune disease (379), and anti-CD52 treated MS patients develop other AD at a high rate, with approximately 40% developing autoimmunity targeting the thyroid and more

rarely immune thrombocytopenic purpura (ITP) or renal disease (anti-glomerular basement membrane disease or membranous nephropathy) (285,380). The characteristics of immune cell recovery post alemtuzumab may determine the effectiveness of inhibiting autoimmunity. In humans, monocytes typically recover first, approaching baseline levels at 3 months. T cells recover more slowly than B cells, reaching approximately 30 and 45% of baseline at 12 months for CD4 and CD8 T cells, respectively (275–277). Similarly in mice, B cells recover faster than T cells. However, T cell repopulation post anti-CD52 treatment is quite rapid in mice, taking several weeks in contrast to more than a year in humans (278,279). The mechanism of repopulation could involve (1) homeostatic expansion of mature lymphocytes that escaped depletion, and (2) production of RTE and generation of new B cells from precursors (280,281). However, the relative contribution of homeostatic expansion of established cells vs. generation of new lymphocytes in repopulation is not fully established, nor is their role in recurrence of disease. Furthermore, the mechanisms that prevent relapses post immune repopulation in alemtuzumab-treated MS patients are unknown.

Coinhibitory receptors, the target of new cancer immunotherapies, are key candidate molecules in the prevention of relapses and alternate autoimmunity, as they are known to control T cells undergoing lymphopenia-induced activation (298). In addition, Foxp3 expressing regulatory T cells (Treg) (381,382), as well as CD4 and CD8 DN T cells (383–385), are subsets that suppress autoimmunity and could play a role during immune repopulation. While Treg are known to increase in frequency post anti-CD52 in both mouse and humans (280,374), effects on peripheral DN T cells have not been reported.

Herein, we examined the contribution of newly generated lymphocytes to repopulation post anti-CD52 treatment in the EAE mouse model of MS and tested whether reversal of disease

depends on the PD-1 coinhibitory receptor pathway of immune tolerance. We found that anti-CD52 is effective at reversing EAE even in the absence of PD-1 and that DN T cells and newly generated T and B cells predominate early in the process of repopulation. Thus, DN T cells and the rapid repopulation by newly generated lymphocytes, with a consequent reduction in the period of lymphopenia, may contribute to the resistance of mice to relapses of EAE post anti-CD52 treatment. Subsequently, we examined the hypothesis that eliminating the rapid repopulation by newly generated T cells in mice would reduce the efficacy of anti-CD52 treatment of EAE and thus model more closely the human condition. The data suggest that the thymus and the ability to generate RTE reduces EAE relapse following anti-CD52 treatment.

4.2 Materials and Methods

4.2.1 Mice and induction of EAE and blood glucose monitoring

B6.129S7-*Rag1*^{tm1Mom}/J (termed Rag1$^{-/-}$), B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J (Foxp3^{EGFP}) (306,307), and NOD/ShiLtJ (termed NOD), mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-*Pdcd1*$^{-/-}$ (termed PD-1$^{-/-}$; backcrossed 11 generations to C57BL/6) were originally generated by Prof. T. Honjo and colleagues (105). B6.Rag2p^{GFP} mice (35,36) were kindly provided by Pamela Fink (University of Washington, Seattle, WA) and we previously generated B6.Rag2p^{GFP}-PD-1$^{-/-}$ mice (297). NOD.Rag2p^{GFP} mice were generated by crossing B6.Rag2p^{GFP} mice to NOD mice. F1 mice were backcrossed to NOD mice for over 14 generations and selected for the expression of GFP transgene and NOD MHC. The diabetes incidence of NOD.Rag2p^{GFP} mice was similar to NOD mice (Unpublished observation). EAE was induced in 6–14-week-old B6 (Rag2p^{GFP}, Rag2p^{GFP}-PD-1$^{-/-}$, Foxp3^{EGFP}) and NOD (WT, Rag2p^{GFP}) mice by subcutaneous injection of each hind flank with 50 μg myelin oligodendrocyte

glycoprotein (MOG_{35-55} peptide) emulsified into 100 µL with 50 µL CFA (Sigma-Aldrich) supplemented with an additional 5 mg/mL heat-killed *Mycobacterium tuberculosis* H37Ra (Difco). Mice were also injected intraperitoneally with 300 ng PT (List Biological Laboratories) in 200 µL PBS (final concentration 1.5 µg/mL) on the day of immunization and 2 days later. Both male and female mice were used and were distributed in equal numbers to the experimental groups. EAE induced mice were treated once they showed a symptom of EAE, with either 10 mg/Kg of mouse IgG2a anti-mouse CD52 monoclonal antibody [provided by Genzyme (278)] subcutaneously or the same volume of PBS for 5 consecutive days. Other non-EAE (healthy control) groups were also treated with 10 mg/Kg anti-mouse CD52 or the same volume of PBS. The clinical score of the mice was assessed daily using the following criteria. 0: no clinical signs; 0.5: partially limp tail; 1.0: paralyzed tail; 1.5: hind limb paresis, uncoordinated movement; 2.0: one hind limb paralyzed; 2.5: both hind limbs paralyzed; 3.0: hind limbs paralyzed, weakness in forelimbs; 3.5: hind limbs paralyzed, one forelimb paralyzed; 4.0: hind limbs paralyzed, both forelimbs paralyzed. Mice were euthanized at a score of ≤ 4 to prevent suffering. EAE relapse was defined as an increase in the clinical disease score occurring more than ten days post the termination of anti-CD52 treatment. Glycemia in NOD mice was quantified by a glucometer (OneTouch, LifeScan, Canada) and two consecutive blood glucose readings of ≥ 14 mmol/L were considered diabetic. Animal care was in accordance with the guidelines of the Canadian Council on Animal Care and the studies were performed under Animal Use Protocol 00000369 approved by the Animal Care and Use Committee Health Sciences of the University of Alberta. Mice were housed under clean conventional housing conditions at the University of Alberta Health Sciences Laboratory Animal Services (HSLAS) facilities.

4.2.2 Thymectomy and adoptive transfer experiments

To assess the resistance of RTE or newly generated cells to anti-CD52 mediated lymphocyte killing, splenocytes were harvested from Rag2p^{GFP}-PD-1$^{-/-}$ mice, and 200 x 10^6 cells in 300 µl/mouse were intravenously injected into Rag1$^{-/-}$ mice, followed by anti-CD52 injection. After 17 hours, the mice were bled, and cells were analyzed by flow cytometry. To assess whether the population of RTE was due to newly emigrated cells from the thymus or not, Rag2p^{GFP}-PD-1$^{-/-}$ mice were thymectomized. Mice were anesthetized and restrained on a surgical board. For the exposure of thymus, a 1-2 mm incision was made on the base of the manubrium. A suction cannula was inserted with the tip placed over the lower pole of one thymic lobe in a manner such that the thymic lobe totally occluded the cannula opening. Negative pressure was applied to remove both lobes separately. The success of surgery was confirmed by having both thymic lobes removed and inspected *in vitro*. Skin was secured with suture after surgery. After two days recovery, mice received either anti-CD52 or vehicle control for 5 consecutive days. Similarly, a group of mice that were not thymectomized were treated with anti-CD52. One day after the end of the 5-day treatment, mice were bled, and cells were analyzed using flow cytometry. Thymectomized mice were examined at the end of the experiment to confirm there were no thymic remnants.

Thymectomy was also performed in mice as described above to block the repopulation by RTE post anti-CD52 mAb treatment. Similarly, sham surgery was performed on another group of mice, or no surgical procedure was performed (euthymic group). After seven days of recovery, mice were immunized to induce EAE. Thymectomized mice were examined at the end of the experiment to confirm there were no thymic remnants.

4.2.3 Antibodies and flow cytometry

The baseline data, depletion, and repopulation of lymphocytes were monitored using flow cytometry. Flow cytometry assessed murine TCRβ [APC-eFluor 780 (H57-597)], CD4 [Alexa Fluor 700 (RM4-5)], CD19 [APC (1D3)], CD25 [PE-Cyanine7 (PC61.5)], CD44 [PE-Cyanine7 (IM7)], CD62L [PerCP-Cyanine5.5 (MEL-14)], NK1.1 [Super Bright 645 (PK136)], IgG2a [(m2a-15F8)], Foxp3 [APC (FJK-16s)], Granzyme-B [PE (NGZB)], IgG2a κ [PE (eBR2a)], antibodies and Fixable Viability Dye [eFluor 780] were purchased from ThermoFisher; CD8a [V500 (53-6.7)] was purchased from BD Biosciences; TCRβ [brilliant violet 570 (H57-597)] was purchased from BioLegend. Antibodies were titrated for optimal staining or used at manufacturer's recommended concentrations. Flow cytometry was done in accordance with guidelines (386). Single-cell suspensions of murine blood and lymphoid organs were first incubated with a cocktail of Fc block (3 mL each of normal mouse, rat, and hamster serum, with addition of 0.3 mg of anti-CD16/32 antibody, clone 2.4g2, Bio X Cell) for 10 min at 4°C to block nonspecific background staining, followed by fluorescently labeled cell surface antibodies for 20 min at 4°C. For intranuclear staining, cells were fixed and permeabilized using a Foxp3 Staining Kit (eBioscience) according to the manufacturer's protocol. To determine the expression of CD52 receptor on newly generated cells, cells from Rag2p^{GFP}-PD-1$^{-/-}$ mice were stained with a primary anti-CD52 antibody (1 μg/μL) followed by PE-conjugated rat anti-mouse IgG2a secondary antibody (ThermoFisher, 0.2 μg/μl). Cells with secondary antibody but not primary antibody were used to determine background staining. For cell subsets within all cells, gating in FSC/SSC was on all cells but excluding low FSC/SSC (i.e., debris/red blood cells). For cell subsets with lymphocyte populations, gating was on lymphocytes defined by FSC/SSC. T cell gating for all tissues (including thymus) and all subpopulations (including DP and DN T

cells), was on cells expressing high levels of TCRβ characteristic of mature T cells and did not include cells with the low level of TCR characteristic of immature DN and DP thymocytes. GFP assessment in mice expressing the transgene was performed at different time intervals. In the Rag2p^{GFP} mice, GFP expression, under the control of recombination-activating gene 2 (Rag2) gene promoter, is turned on when the Rag proteins are transiently expressed during the late DN and DP stages of T cell development (35). GFP expression persists through T cell egress into the peripheral circulation for up to 3 weeks even though Rag2 expression has been terminated intrathymically (36). This enables a reliable assessment of RTE. Cells were analyzed by flow cytometry using a BD LSR II instrument for acquisition and analysis was performed using FlowJo (Treestar software, Portland, OR, USA).

4.2.4 Histological analysis

Sections of spinal cord, liver, spleen, kidney, heart, skin, stomach and thyroid glands were collected at termination of experiments from selected mice and fixed in 10% neutral buffered formalin. Following fixation for a minimum of 24 hours, tissues were trimmed with a scalpel to a thickness of 2 – 3 mm and a section of each was placed in a tissue cassette. Tissues in cassettes were processed into paraffin, embedded in a paraffin block, sectioned on a microtome to a thickness of 5 microns, placed on a microscope slide, stained with hematoxylin and eosin (H&E) stain and cover-slipped following standard histology techniques. Tissue sections were processed in the Biological Sciences Microscopic Unit at the University of Alberta. Slides were examined by a board-certified veterinary pathologist (Dr. Nick Nation) and each section of tissue was scored as detailed in Figure 4.3.

4.2.5 Statistical analyses

Statistical analysis was performed using GraphPad Prism software. Data are depicted as mean ± standard error of the mean (SEM) or as Kaplan–Meier survival curves. Data were statistically analyzed using grouped analysis and T-tests with Holm-Sidak correction for multiple comparisons. Statistical analysis in experiments with two variables and three groups was by 2-way ANOVA. EAE clinical scores up to 52 days post EAE induction were compared by generating an area under the curve (AUC) for each mouse followed by a Mann-Whitney test. Asterisks denote *$p < 0.05$, ** $p < 0.001$, ***$p < 0.0001$.

4.3 Results

4.3.1 Murine anti-CD52 mAb therapy blocks a severe form of EAE independent of PD-1

Our laboratory has recently shown that PD-1 is critical in preventing lymphopenia-potentiated autoimmunity (113,288). Given the severe lymphopenia post anti-CD52, we tested whether the ability of anti-CD52 to treat EAE depends on PD-1. We compared treatment of EAE in PD-1 deficient vs. WT Rag2p^{GFP} mice; Rag2p^{GFP} mice were used to allow us to track the frequency of RTE (described in detail in the materials and methods section). Deficiency or lack of PD-1 has been associated with an early onset and more aggressive disease outcome in EAE (108) and also restores disease susceptibility in otherwise EAE-resistant mice (387). WT controls immunized with MOG_{35-55} peptide started losing weight at two weeks, which coincided with the EAE disease onset (Fig. 4.1 A). Weight loss was substantial with a peak disease score of approximately 3. To mimic the clinical dosing regimen of alemtuzumab in the MS patients, we treated EAE-induced mice with 10 mg/kg anti-CD52 mAb for 5 consecutive days once each

mouse started to show early symptoms (clinical score of 0.5 or greater). As expected based on previous studies (278), symptoms of EAE were substantially reversed post anti-CD52 mAb treatment compared to controls and anti-CD52 partially blocked the loss in body weight (Fig. 4.1 A). PD-1 deficient mice (Rag2p^{GFP}-PD-1$^{-/-}$) had significantly more severe disease with EAE maintained at a persistently high disease score of grade 3 (Fig. 4.1 A). Despite the more severe score, the anti-CD52 mAb was just as effective in reversing disease of the PD-1$^{-/-}$ mice (Fig. 4.1 A); symptoms of disease were almost fully eradicated in the PD-1$^{-/-}$ mice and they gained weight, exceeding their baseline weight. Anti-CD52 caused a marked reduction in the proportion of circulating T and B cells, with minimal effects on the frequency of NK cells. Apparently the low level of CD52 expression on NK cells (278) makes these cells less susceptible to anti-CD52 mAb-mediated cell death. The rapid repopulation pattern in B and T cells was consistent with an earlier study in alemtuzumab-treated human CD52 transgenic mice (374) and a study using the same mAb to murine CD52 employed herein (278). Near complete recovery of T and B cells in blood post anti-CD52 took 5-6 weeks in WT mice, however, despite B cell recovery, T cell frequency did not recover to pre-treatment values in PD-1$^{-/-}$ mice (Fig. 4.1 B). The proportion of T cells with a naïve (CD44^{lo} CD62L^{hi}) phenotype decreased while T cells with an effector memory (CD44^{hi} CD62L^{lo}) phenotype increased following anti-CD52 treatment in both WT and PD-1$^{-/-}$ mice, with a reversal of this trend by 6 weeks post EAE induction (Appendix 2 and Fig. 4.2). Despite the prolonged T cell lymphopenia, in a preliminary analysis we did not observe a development of alternate forms of autoimmune disease post anti-CD52 treatment in PD-1$^{-/-}$ mice. Histologic evaluation of most organs eight months post EAE induction, including thyroid, showed normal tissue morphology; the only organ showing consistent infiltration in PD-1$^{-/-}$ mice was the liver, and this occurred whether or not these mice were treated with anti-CD52

(Fig. 4.3). Altogether, these results showed the efficacy of anti-CD52 mAb treatment in reversing an aggressive form of EAE and that treatment efficacy occurred independent of tolerogenic signals from PD-1.

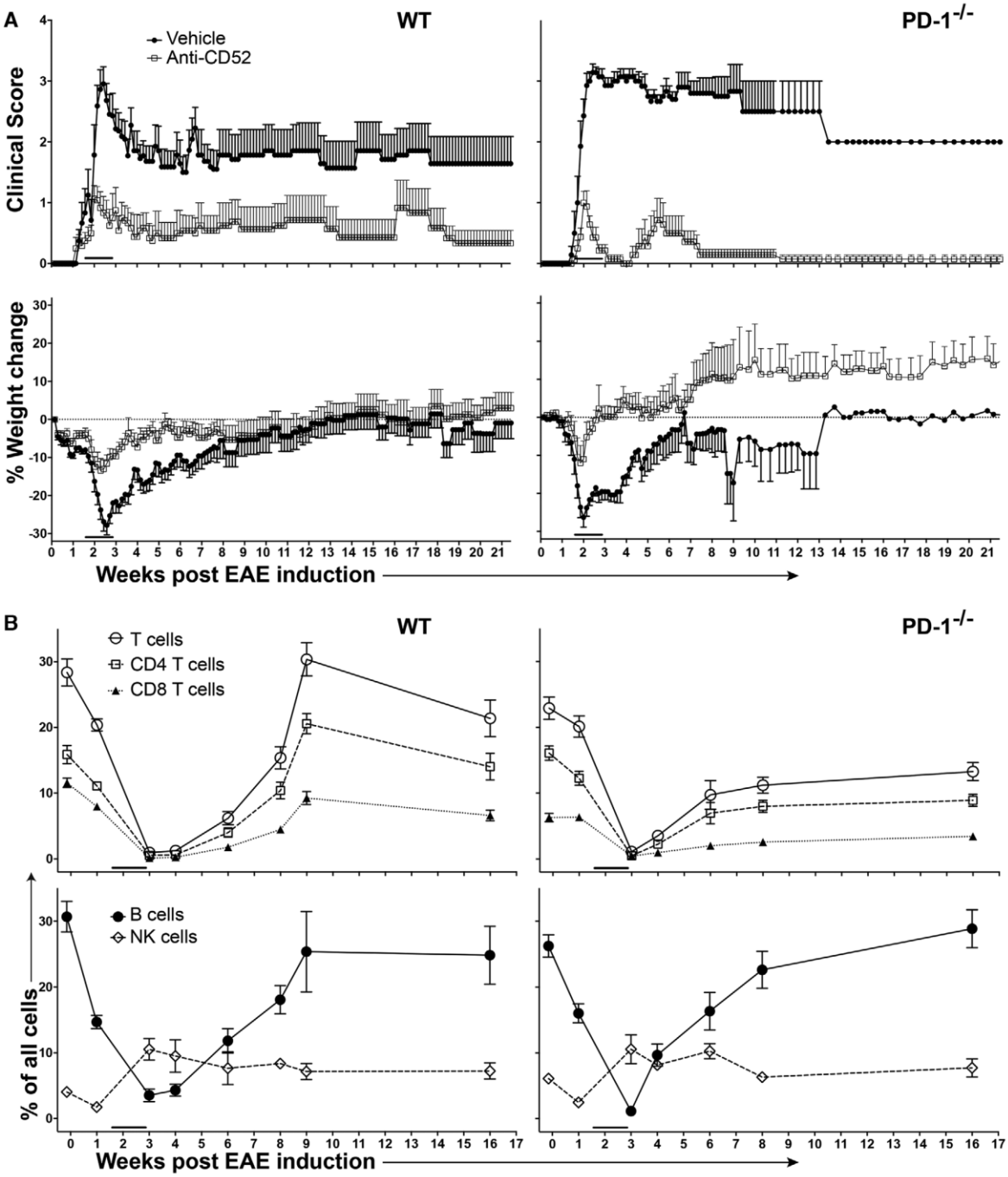

A
WT
PD-1-/-
Vehicle
Anti-CD52
Clinical Score
% Weight change
Weeks post EAE induction
B
T cells
CD4 T cells
CD8 T cells
B cells
NK cells
% of all cells
Weeks post EAE induction

Figure 4.1. *Anti-CD52 reversal of EAE in the absence of PD-1 mediated signals.* EAE was induced at time zero and mice were treated with anti-CD52 or vehicle for 5 days after the first sign of EAE; Rag2p^{GFP} mice (n=12 anti-CD52, n=12 vehicle) or Rag2p^{GFP}-PD-1$^{-/-}$ mice (n=8 anti-CD52, n=7 vehicle). **[A]** Clinical score and body weight were assessed and **[B]** lymphocytes in peripheral blood were determined prior to EAE induction and various time points post EAE. Analysis included cells expressing CD4^{+} TCRβ^{+}, CD8^{+} TCRβ^{+}, NK1.1^{+} TCRβ^{-}, and CD19^{+}. Dark solid line above the x-axis denotes the anti-CD52 treatment range. Data was pooled from three independent experiments. Statistical significance was calculated by Mann-Whitney test; comparison of clinical scores for vehicle vs. anti-CD52, $p = 0.002$ for Rag2p^{GFP} and $p = 0.001$ for Rag2p^{GFP}-PD-1$^{-/-}$. Clinical scores for vehicle control of Rag2p^{GFP} vs. Rag2p^{GFP}-PD-1$^{-/-}$, $p = 0.007$.

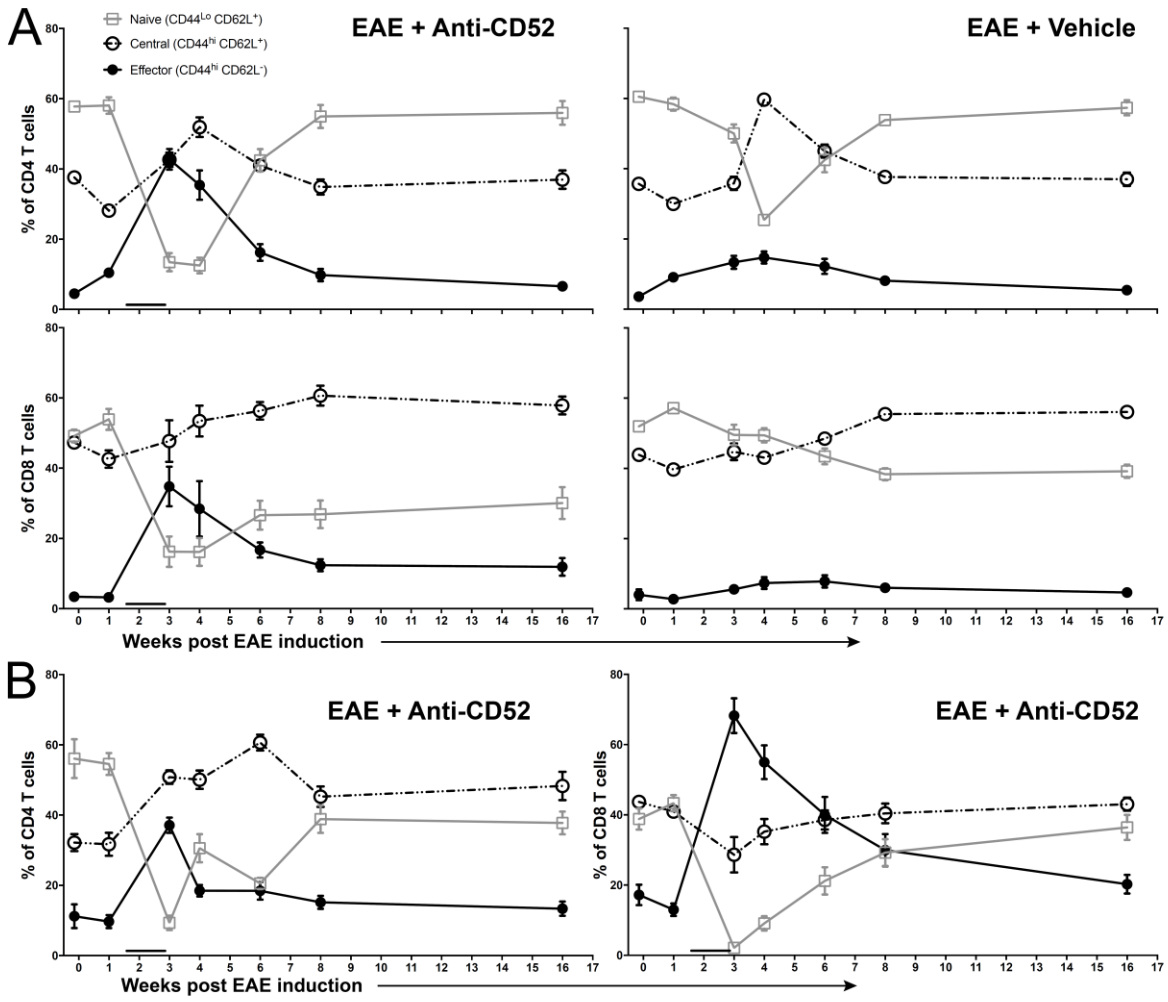


Figure 4.2. *Decreased naive T cells and increased effector memory T cells in mice with EAE and treated with anti-CD52.* Some of the mice shown in Fig. 4.1 were analyzed for the indicated markers of naïve vs. memory T cell subsets. **[A]** Analysis of CD4 T cells (top) and CD8 T cells (bottom) in blood of Rag2p^{GFP} mice (n=8) and **[B]** Rag2p^{GFP}-PD-1$^{-/-}$ mice (n=7). Dark solid line above the x-axis denotes the anti-CD52 treatment range. Data was pooled from two independent experiments.

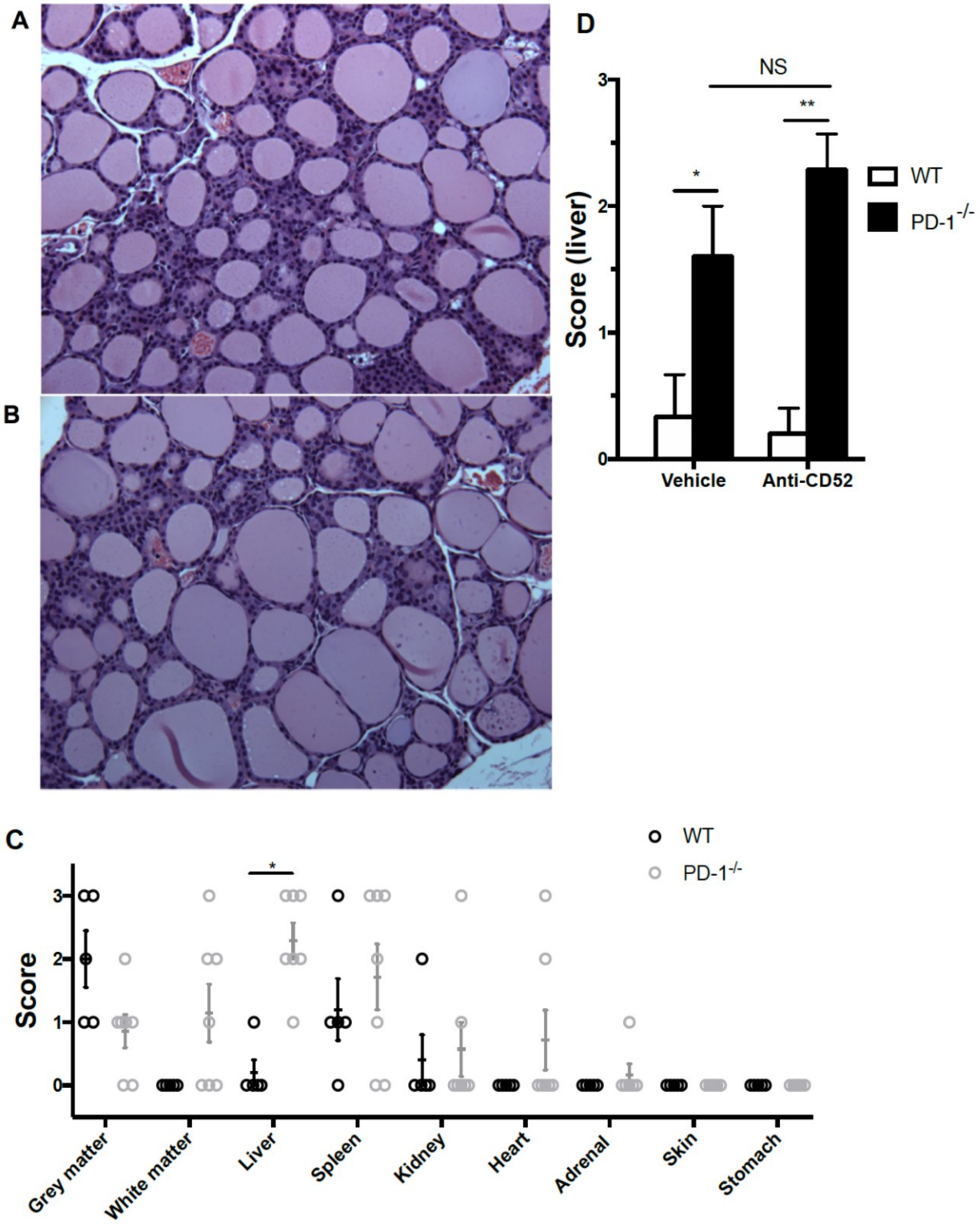

A
B
C
D
NS
**
*
WT
PD-1-/-
Score (liver)
Vehicle
Anti-CD52
Score
Grey matter
White matter
Liver
Spleen
Kidney
Heart
Adrenal
Skin
Stomach

Figure 4.3. *Histologic assessment of organs in mice with EAE and treated with anti-CD52.* Some of the anti-CD52 treated Rag2p^{GFP} and Rag2p^{GFP}-PD-1$^{-/-}$ mice shown in Fig. 4.1 were euthanized and tissues were stained with H and E and tissue state was scored by a pathologist (N. Nation) blinded to the treatment groups (see scoring method below). **[A, B]** Representative histology of the thyroid from Rag2p^{GFP}-PD-1$^{-/-}$ mice with EAE treated with **[A]** vehicle or **[B]** anti-CD52 (n=7). Tissues, including thyroid tissue, were taken 8 months after EAE induction for the anti-CD52 group, while in the vehicle group tissues were taken at 1-4 months due to severe EAE disease. One of 7 of the Rag2p^{GFP}-PD-1$^{-/-}$ thyroids (EAE + anti-CD52 group) had cells with a foamy cytoplasm and another 1/7 had a cyst with local inflammation. **[C]** Histologic score of tissues from Rag2p^{GFP} (WT) and Rag2p^{GFP}-PD-1$^{-/-}$ (PD-1$^{-/-}$) mice with EAE and treated with anti-CD52 at 8 months post EAE induction. **[D]** Liver histology score comparison between EAE induced Rag2p^{GFP} mice with vehicle (n=6) vs. anti-CD52 (n=5) and Rag2p^{GFP}-PD-1$^{-/-}$ mice with vehicle (n=5) vs. anti-CD52 (n=7). Statistical significance was calculated by grouped analysis T-tests with Holm-Sidak correction for multiple comparisons; *$p < 0.05$, ** $p < 0.001$, ***$p < 0.0001$.

KEY TO HISTOLOGY SCORES IN FIGURE 4.3

SPINAL CORD:

GREY MATTER GLIAL CELL REACTION

0 = NORMAL
1 = MILD
2 = MODERATE
3 = SEVERE

WHITE MATTER/AXONS

0 = NORMAL
1 = FOCI OF LYMPHOCYTES
2 = FOCI OF LYMPHOCYTES AND AXONAL SWELLING/VACUOLATION
3 = FOCI OF LYMPHOCYTES AND AXONAL DIGESTION CHAMBERS

LIVER

0 = NORMAL
1 = OCCASIONAL FOCI OF LYMPHOCYTES IN PORTAL TRIAD AREAS OR AROUND PORTAL VEINS
2 = FOCI OF NECROSIS AND/OR MULTIPLE SEPARATE FOCI OF LYMPHOCYTES IN PARENCHYMA
3 = MULTIPLE FOCI OF LYMPHOCYTES AND MICROGRANULOMAS THROUGHOUT THE PARENCHYMA OR MULTIPLE VERY LARGE LYMPHOID ACCUMULATIONS AROUND VEINS IN PORTAL TRIADS

SPLEEN

0 = NORMAL
1 = MILD TO MODERATE HYPERPLASIA OF LYMPHOID GERMINAL CENTRES
2 = SCORE #1 AND/OR MILD TO MODERATE INCREASE IN SINUS LYMPHOCYTE POPULATION
3 = LYMPHOID GERMINAL CENTRE HYPERPLASIA AND/OR MODERATE OR SEVERE INCREASE IN SINUS LYMPHOID POPULATION

KIDNEY

0 = NORMAL
1 = OCCASIONAL INFILTRATE OF LYMPHOCYTES AROUND RENAL VEINS1
2 = SCORE #1 AND/OR THREE OR MORE CORTICAL LYMPHOID NODULES
3 = LARGE ACCUMULATIONS OF LYMPHOCYTES BESIDE RENAL VEINS OR AROUND ARCUATE ARTERIES

HEART

0 = NORMAL
1= LOCAL INFLAMMATION OF MYOCARDIUM
2 = LOCAL INFLAMMATION OF A MAJOR VESSEL
3 = SEVERE BUT LOCALIZED INFLAMMATION AROUND CORONARY ARTERY

ADRENAL

0 = NORMAL
1 = SUBCAPSULAR LYMPHOCYTES

4.3.2 Anti-CD52 reduces newly generated B cells while increasing the proportion of RTE and DN T cells

In contrast to anti-CD52 treatment of MS in humans, immune repopulation post anti-CD52 treatment in mice is quite rapid (several weeks in contrast to more than a year in humans) and does not appear to trigger alternate forms of autoimmune disease. To begin to investigate why immune repopulation and self tolerance is enhanced, we assessed the effect of anti-CD52 on various lymphocyte subsets. In addition to the homeostatic expansion of cells that escaped depletion, newly generated T and B cells might contribute to the more rapid repopulation in mice. To examine this possibility, we used the Rag2p^{GFP} model to track the frequency of lymphocytes newly exported from central lymphoid organs. We initially examined the proportion of RTE in mice treated with anti-CD52, without EAE induction. Consistent with the previous results in mice with EAE, anti-CD52 mAb significantly depletes circulating T and B cells in naïve Rag2p^{GFP} and Rag2p^{GFP}-PD-1$^{-/-}$ mice (Fig. 4.4 A, B), including CD4, CD8, and DN T cells. In addition to the peripheral blood, depletion was also evident in the spleen, but less evident in lymph nodes, and no depletion was seen in the thymus. Overall cell numbers and T and B cell numbers were reduced in the spleen, while thymus cellularity was not significantly affected (Fig. 4.4 C). However, the proportion of both CD4 and CD8 T cells that were RTEs was significantly increased in the blood post anti-CD52 mAb treatment (Fig. 4.4 A, B). In contrast, the proportion of newly generated B cells was significantly reduced (Fig. 4.4 A). Although reduced in frequency overall post depletion, the remaining newly generated B cells had very high GFP expression (Fig. 4.4 A, histograms), suggesting they were very recently generated B cells. Interestingly, the proportion of T cells that were DN T cells increased substantially post anti-CD52 treatment, from 1.7% to 11% of T cells in Rag2p^{GFP} mice and from 3.5% to 34% in

Rag2p^{GFP}-PD-1$^{-/-}$ mice and these cells did not express granzyme-B (Fig. 4.4 and Fig. 4.5). This increased proportion of DN T cells was apparent in other lymphoid tissues, with the exception of the thymus (Fig. 4.4 B). The majority of DN T cells did not express NK1.1 (i.e. were not NK T cells) and this was independent of anti-CD52 treatment (Fig. 4.4 B).

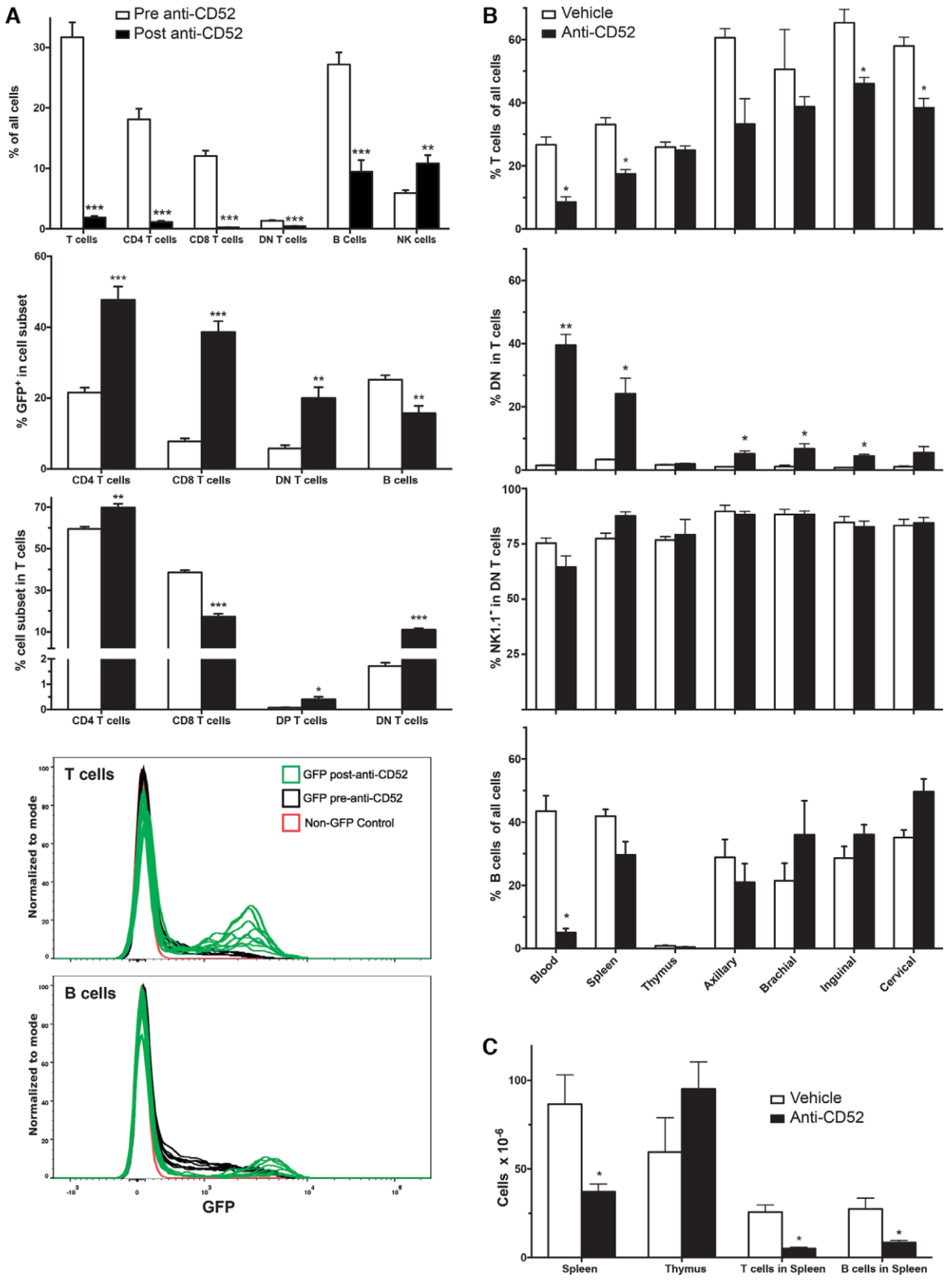

A
Pre anti-CD52
Post anti-CD52
% of all cells
T cells
CD4 T cells
CD8 T cells
DN T cells
B Cells
NK cells
% GFP+ in cell subset
CD4 T cells
CD8 T cells
DN T cells
B cells
% cell subset in T cells
CD4 T cells
CD8 T cells
DP T cells
DN T cells
T cells
GFP post-anti-CD52
GFP pre-anti-CD52
Non-GFP Control
Normalized to mode
B cells
Normalized to mode
GFP
B
Vehicle
Anti-CD52
% T cells of all cells
% DN in T cells
% NK1.1+ in DN T cells
% B cells of all cells
Blood
Spleen
Thymus
Axillary
Brachial
Inguinal
Cervical
C
Vehicle
Anti-CD52
Cells x 10-6
Spleen
Thymus
T cells in Spleen
B cells in Spleen

Figure 4.4. *Immediate effects of anti-CD52 include an increased frequency of newly generated T cells and DN T cells and decreased newly generated B cells.* **[A]** Rag2p^{GFP} mice were treated with anti-CD52 for 5 consecutive days and cells in peripheral blood were analyzed prior to treatment and one day after the last injection (pre-anti-CD52, n=11; post anti-CD52, n=9; data show results from one of two experiments with similar results. Newly generated T (TCRβ^{+}) and B cells (CD19^{+}) were defined by expression of GFP, and representative histograms of GFP within T and B cells are depicted (bottom). **[B, C]** Cell subsets were assessed in blood, spleen, thymus, and the indicated lymph nodes of Rag2p^{GFP}-PD-1$^{-/-}$ mice one day after 5 consecutive days of vehicle or anti-CD52 mAb injection (n=4; mean ± SEM). Data show results from one experiment with similar results in a second experiment in which only blood was analyzed. Statistical significance was calculated by grouped analysis T-tests with Holm-Sidak correction for multiple comparisons; *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

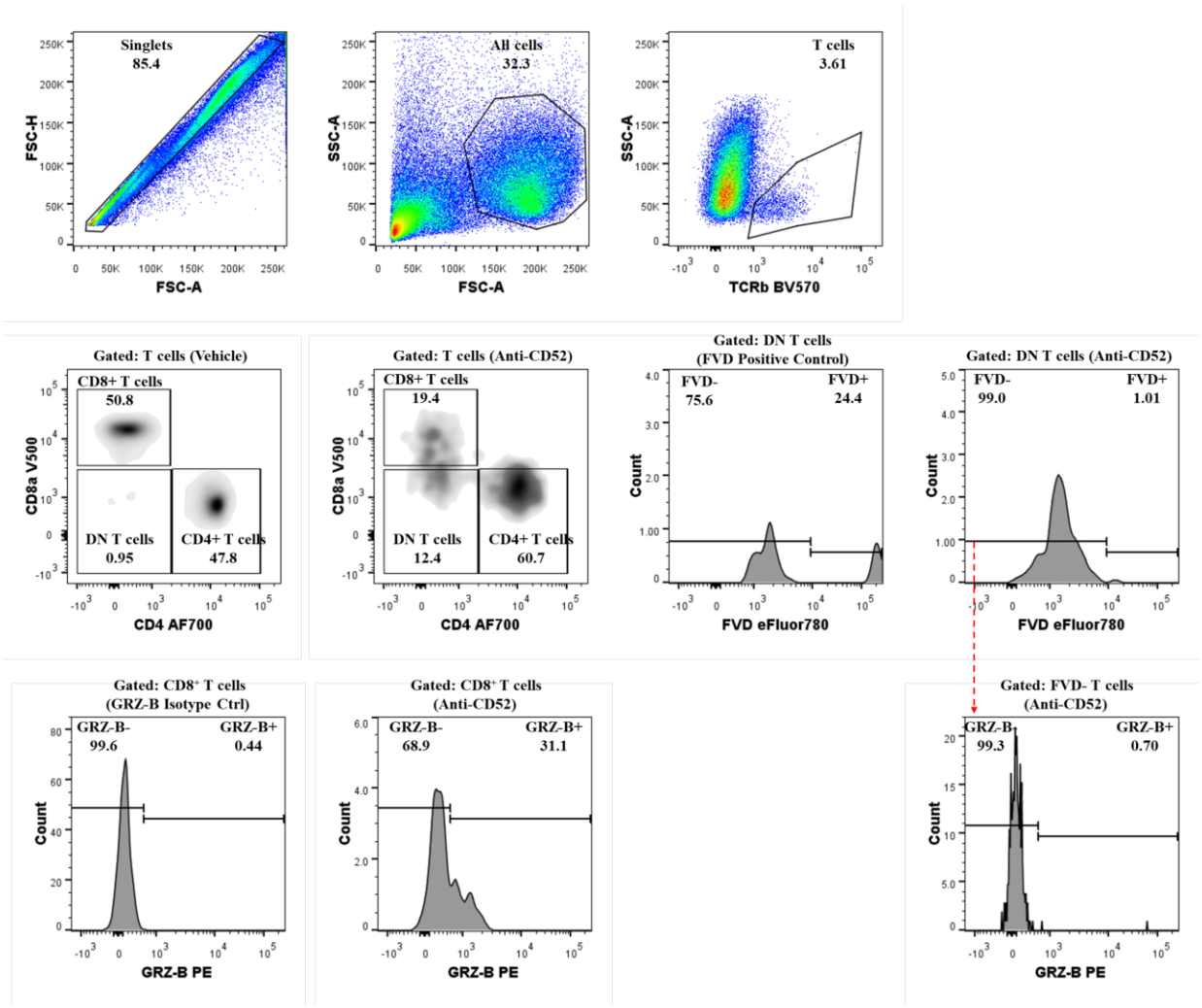


Figure 4.5. *DN T cells are found within the live cell gate post anti-CD52 and do not express Granzyme-B.* B6.Rag2p^{GFP} mice were treated with anti-CD52 mAb (n=6) or vehicle (n=6) for 5 consecutive days and both blood and spleen DN T cells were analyzed for live/dead cells one day after the last dose. We show here representative flow cytometry data from an anti-CD52 mAb-treated mouse showing gating strategy for live/dead DN T cells (top and bottom rows) and from a vehicle-treated mouse (middle row, left most plot). Splenocytes were gated on singlets and then all cells and then T cells. TCRβ^+ gated DN T cells were analyzed for viability with fixable viability dye (FVD). For the FVD positive control, 1 x 10^6 splenocytes from anti-CD52 mAb-

treated mouse was incubated for 15 mins at 56°C to heat kill the cells and then mixed with 1 x 10^6 live cells. Also depicted: Isotype control (Ctrl) of granzyme-b (GRZ-B) staining in $CD8^+$ T cells. Data is from one experiment on individual biological replicates.

4.3.3 Increased frequency of RTE post anti-CD52 is due to continued export from the thymus

Changes in the proportion of T and B cell subsets could be due to differential susceptibility to killing (e.g. due to differences in the level of CD52 expression) or it may result from differential repopulation kinetics. We therefore examined CD52 expression levels on T and B cell subsets and compared established lymphocytes (GFP−) to newly generated lymphocytes (GFP+). CD52 expression did not differ between established CD4 and CD8 T cells and RTE. Although, total DN RTE appeared to express significantly less CD52 (Fig. 4.6 A, lower panels), this was due to inclusion of the $NK1.1^{+}$ DN T cell subpopulation that expressed high levels of CD52. Both established (GFP−) and newly generated $NK1.1^{-}$ DN T cells expressed low levels of CD52 (Fig. 4.6 A, histograms). In contrast, newly generated B cells expressed significantly higher CD52 than established B cells, consistent with the preferential depletion of newly generated B cells by anti-CD52 (Fig. 4.4). T cell CD52 expression was similar between blood and lymphoid organs, with the exception of greater CD52 on splenic and thymic B cells and the small number of splenic TCR positive cells that were CD4/CD8 DP or DN (Fig. 4.6 A).

While RTE increased in frequency post anti-CD52, the similar expression levels of CD52 between established T cells and RTE suggested they should be equally susceptible to depletion. To directly examine whether RTEs are resistant to anti-CD52 mediated lymphocyte killing, we adoptively transferred splenocytes from $Rag2p^{GFP}$-$PD\text{-}1^{-/-}$ mice to $Rag1^{-/-}$ mice, followed by anti-CD52 mAb treatment. This method allows determination of susceptibility to killing without confounding output of new T cells from the thymus. Should RTE be less susceptible to killing than established T cells, RTE would be expected to be increased in proportion relative to established T cells post depletion. Post anti-CD52 mAb treatment, total T and B cells were

significantly reduced compared to the control group (Fig. 4.6 B). However, no statistically significant difference was observed in the proportion of RTEs between anti-CD52 mAb-treated and control groups (Fig. 4.6 B), whereas newly generated B cells were significantly reduced in the anti-CD52 mAb-treated mice, again indicating that newly generated B cells were more susceptible to anti-CD52 mediated depletion compared to established B cells (Fig. 4.6 B), consistent with their increased expression of CD52.

The above data suggested that the increased proportion of RTEs in circulation post anti-CD52 treatment was likely due to their rapid replacement by export of new T cells from the thymus. To directly test this hypothesis we thymectomized a cohort of Rag2p^{GFP}-PD-1$^{-/-}$ mice and two days later treated them either with anti-CD52 or vehicle. We left another anti-CD52 treated cohort euthymic. Anti-CD52 treatment, regardless of thymectomy, significantly reduced the overall T and B cells compared to vehicle-treated thymectomized mice (Fig. 4.6 C). Importantly, the percentage of RTE, including CD4, CD8, and DN RTE, was significantly reduced in the thymectomized vs. euthymic mice treated with anti-CD52. In contrast, the frequency of newly generated B cells in anti-CD52 treated mice was not affected by thymectomy (Fig. 4.6 C). These data indicate that the primary cause for the increased proportion of RTE post anti-CD52 treatment was due to export of new RTEs from the thymus.

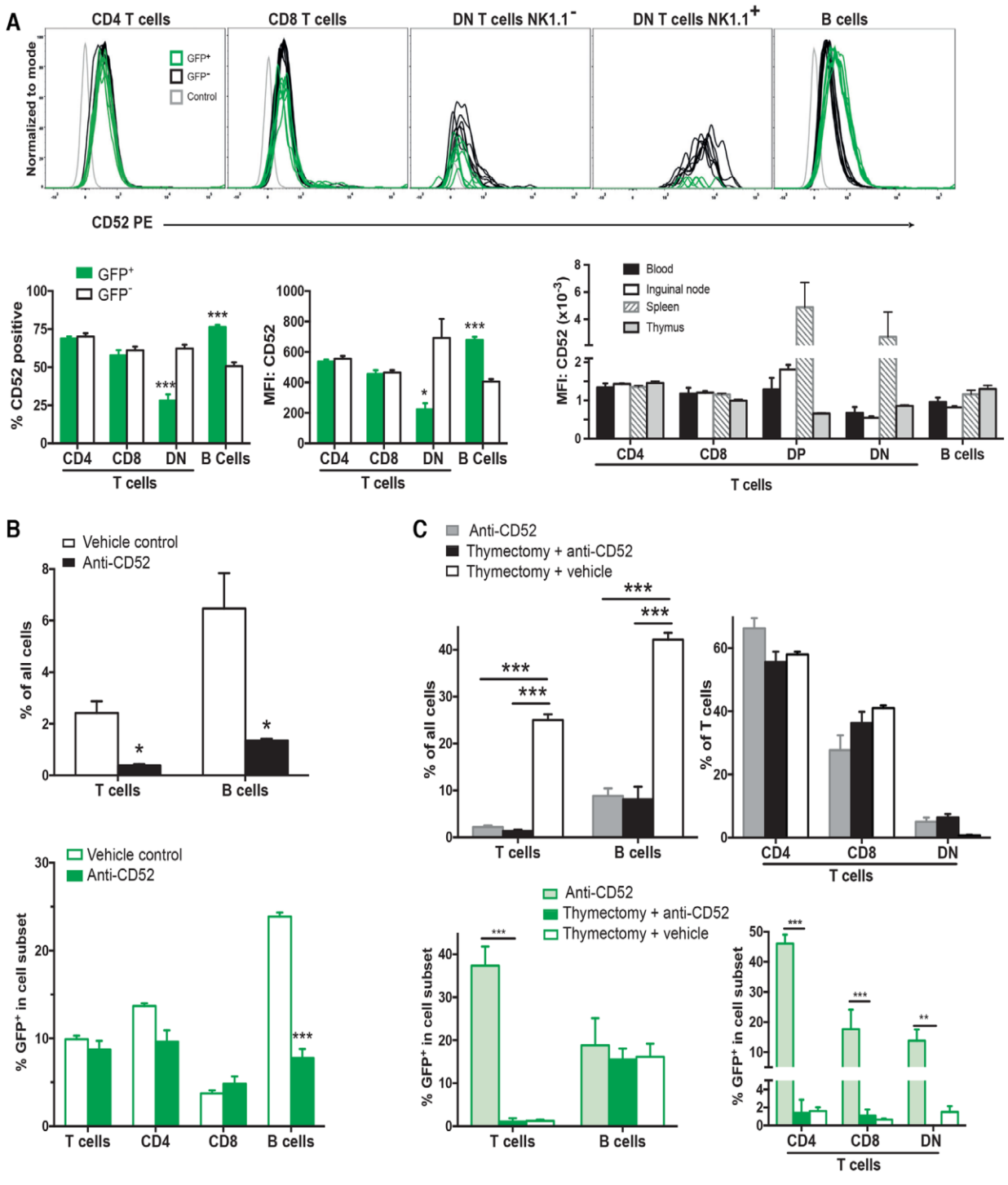

A
CD4 T cells
CD8 T cells
DN T cells NK1.1-
DN T cells NK1.1+
B cells
Normalized to mode
GFP+
GFP-
Control
CD52 PE
% CD52 positive
MFI: CD52
MFI: CD52 (x10-3)
Blood
Inguinal node
Spleen
Thymus
CD4
CD8
DN
B Cells
DP
B cells
T cells
B
Vehicle control
Anti-CD52
% of all cells
% GFP+ in cell subset
C
Anti-CD52
Thymectomy + anti-CD52
Thymectomy + vehicle
% of T cells

Figure 4.6. *Increased RTE and decreased newly generated B cells post anti-CD52 are associated with thymic output and the level of CD52 expression.* **[A]** CD52 expression assessed on cell subsets in the blood (Rag2p^{GFP}-PD-1$^{-/-}$ mice, n=7) or in the indicated tissues (bottom right, n=4). Data from two separate experiments is depicted. **[B-D]** Two hundred million splenocytes from Rag2p^{GFP}-PD-1$^{-/-}$ were injected into Rag1$^{-/-}$ mice after which the mice were treated either with anti-CD52 mAb or vehicle control (onetime treatment); GFP analysis was in lymphocyte gate. The population of GFP expressing RTEs and non-RTEs in the blood was assessed 17 hours after anti-CD52 treatment (n=4). **[C]** Rag2p^{GFP}-PD-1$^{-/-}$ mice were thymectomized or left euthymic and then treated for 5 consecutive days with anti-CD52 or vehicle. The indicated cell populations were assessed in the blood one day after the last injection of anti-CD52 mAb (n=5 for thymectomy + anti-CD52, n=4 for thymectomy + vehicle, n=3 for anti-CD52 euthymic). Data from one experiment is depicted. Statistical significance was calculated by 2-way ANOVA; *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

4.3.4 Repopulation post anti-CD52 treatment of EAE favours newly generated lymphocytes and DN T cells

The increased proportion of DN T cells and RTE along with a reduced proportion of newly generated B cells post anti-CD52 was unanticipated. These changes might play a role in the prevention of relapses. However, the effect of anti-CD52 on cell subsets in the setting of treating active EAE could be different from that seen when treating healthy naïve mice with anti-CD52. Furthermore, we had examined the changes in the proportion of these subsets only shortly after anti-CD52 treatment. For the changes to have substantial effects on disease they would likely need to be relatively long lasting. We therefore examined the aforementioned cell subsets over time in mice with active EAE. Induction of EAE without treatment was followed by a decline in the proportion of T and B cells that were newly generated cells (Fig. 4.7 A). The decrease of newly generated cells was not simply due to ageing related atrophy as the immunization to induce EAE itself caused a significant reduction in newly generated B and T cells that was maintained for at least 2 and 3 weeks, respectively (Fig. 4.7 B). In contrast, mice with EAE and treated with anti-CD52 showed an initial decline in newly generated B cells, followed by prolonged increases in the proportion of T and B cells that were newly generated; nearly 40% of T cells and 70% of B cells were newly generated cells 3 weeks after the last injection of anti-CD52. Consistent with previous findings in mice and humans (280,374), Foxp3 expressing Treg cells were increased in frequency by anti-CD52. Even more strikingly, the frequency of DN T cells increased over a prolonged time course following anti-CD52 treatment of mice with EAE; with 8.4-fold increase 10 days post anti-CD52 and a 2-fold increase being maintained over 3 weeks after termination of treatment (Fig. 4.7 C). The majority of DN T cells were not NK T cells, and the subset of DN T cells lacking NK1.1 transiently increased following

anti-CD52 treatment (Fig. 4.7 C). Together these findings show that anti-CD52 treatment of mice with EAE leads to a prolonged increase in the proportion of DN T cells and newly generated T and B cells.

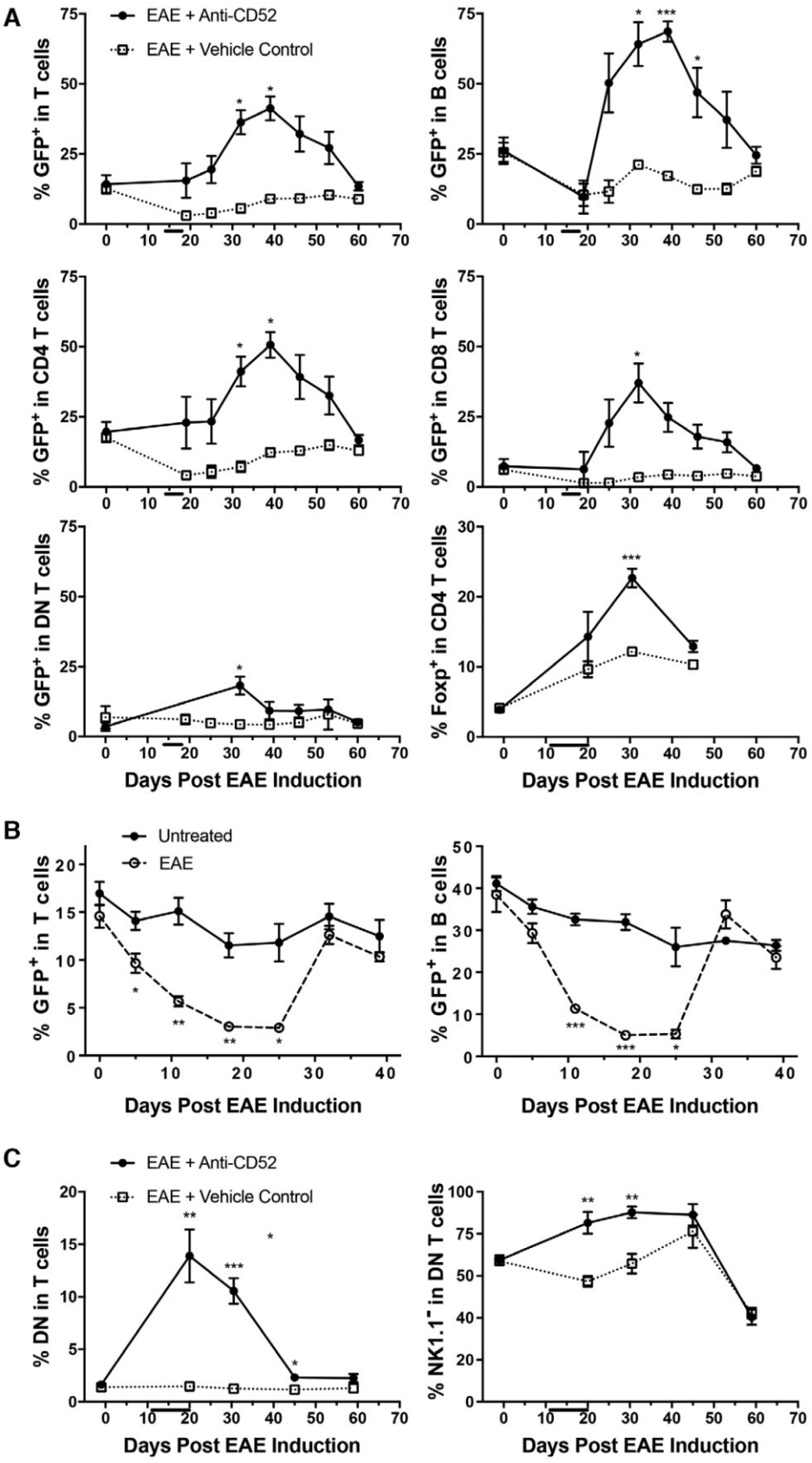

A
EAE + Anti-CD52
EAE + Vehicle Control
% GFP+ in T cells
% GFP+ in B cells
% GFP+ in CD4 T cells
% GFP+ in CD8 T cells
% GFP+ in DN T cells
% Foxp+ in CD4 T cells
Days Post EAE Induction
Days Post EAE Induction
B
Untreated
EAE
% GFP+ in T cells
% GFP+ in B cells
Days Post EAE Induction
Days Post EAE Induction
C
EAE + Anti-CD52
EAE + Vehicle Control
% DN in T cells
% NK1.1- in DN T cells
Days Post EAE Induction
Days Post EAE Induction

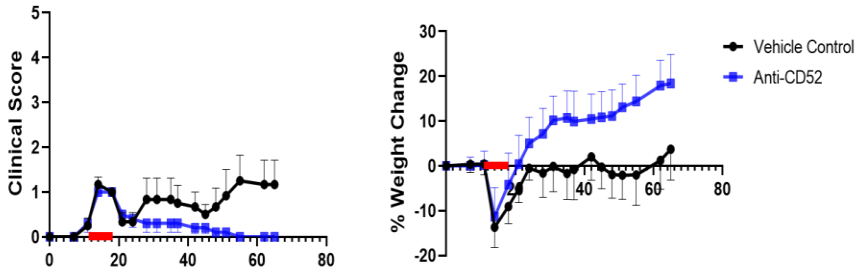
Figure 4.7. *Anti-CD52 treatment in mice with EAE causes a prolonged increase in the proportion of newly generated T and B cells as well as DN T cells.* EAE was induced in Rag2p^{GFP} or Rag2p^{GFP}-PD-1$^{-/-}$ mice at time zero and left untreated or were treated with anti-CD52 or vehicle for 5 days after the first sign of EAE. The frequency of the indicated lymphocyte gated cell subsets in peripheral blood was determined prior to EAE induction and at various time points post EAE induction. Dark solid line below the x-axis denotes the anti-CD52 treatment range. **[A]** Analysis of GFP or Foxp3 expression in cell subsets of Rag2p^{GFP} mice with EAE and treated with anti-CD52 or vehicle (GFP expression, n=4 per group, with a second separate experiment demonstrating similar effects of anti-CD52 over time; Foxp3 expression, n=11-12 per group pooled from two experiments). **[B]** Analysis of GFP expression in T and B cells of Rag2p^{GFP}-PD-1$^{-/-}$ mice left untreated or immunized (with MOG/CFA and PT) to induce EAE (Untreated, n=5; EAE, n=6, pooled from two experiments) **[C]** Analysis of DN T cell frequency and subsets (n=11-12 per group pooled from two experiments). Statistical significance was calculated by grouped analysis T-tests with Holm-Sidak correction for multiple comparisons; *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

4.3.5 Anti-CD52 mAb treatment reversed EAE in NOD mice

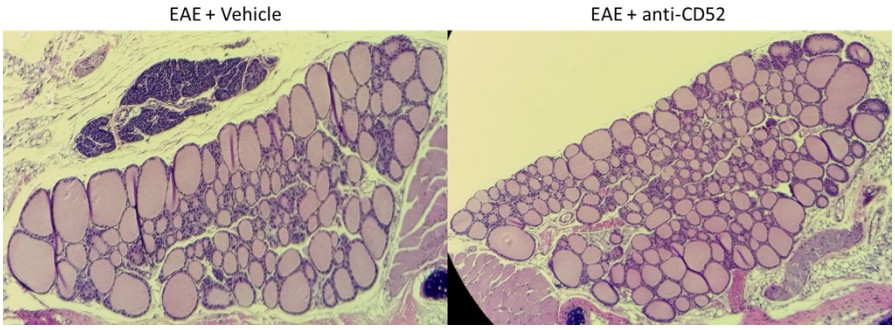
Having shown that anti-CD52 mAb treatment reversed the symptoms of EAE in both WT B6 mice and their PD-1$^{-/-}$ counterparts that had a more severe form of EAE (388), there was no evidence of immune cell infiltration in the thyroid glands of either WT or PD-1$^{-/-}$ B6 mice post anti-CD52 mAb treatment. We then hypothesized that inducing EAE in the autoimmune-prone NOD mouse genetic background and treatment with anti-CD52 mAb will generate alternate autoimmunity of the thyroid gland. To address this, EAE was induced in 8 – 10-week old female NOD mice and anti-CD52 mAb was administered for 5 consecutive days starting from the first sign of disease (clinical score 0.5 – 1.0; i.e., weak tail – paralyzed tail). Disease onset in these NOD mice occurred at 11 days post EAE induction, which was evident in the NOD mice having clinical scores of 0.5 and above, and a gradual decline in body weight (Fig. 4.8 A)**.** Anti-CD52 mAb-treated mice quickly recovered their baseline body weight and their clinical scores dropped to below 0.5 post treatment compared to vehicle-treated mice with clinical scores of above 1.0 (Fig. 4.8 A). To determine whether the NOD mice with EAE generates a model for studying alternate autoimmunity of the thyroid post anti-CD52 treatment, we performed H&E staining to assess the general tissue morphology and infiltration of the thyroid glands. Similar to our previous findings in the B6 mice, we saw no evidence of immune cell infiltration in the thyroid glands of EAE-induced NOD mice treated with anti-CD52 vs. vehicle-treated mice (Fig. 4.8 B). Type-1-diabetes has been reported in the clinic as a possible alternate autoimmunity following alemtuzumab treatment of MS patients (389,390). Since CFA, which is part of the EAE induction regimen prevents diabetes in the NOD mice (391), development of diabetes in the anti-CD52 mAb-treated mice would suggest a possible alternate autoimmunity. To examine this, we monitored the blood glucose of EAE-induced NOD mice pre- and post anti-CD52 mAb-

treatment and compared this to the vehicle-treated mice. There was no significant difference in the blood glucose level of anti-CD52 mAb-treated mice and their vehicle-treated counterparts (Fig. 4.8 C). Taken together, these data showed that similar to the B6 mice, anti-CD52 treatment effectively reversed signs of EAE in the autoimmune-prone NOD mice. Evidence from the thyroid histology and blood glucose measurements suggested that there was no development of alternate autoimmunity in the anti-CD52 mAb-treated mice within the time frame examined (note: that thyroid histology in Fig. 4.8 B has not yet been analyzed by a pathologist).

A



B



C

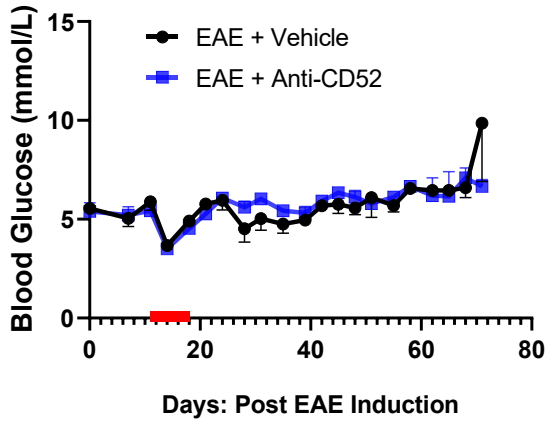


Figure 4.8. *Anti-CD52 mAb reversed EAE in the NOD mice.* EAE was induced on day zero and mice were treated with anti-CD52 or vehicle for 5 days after the first sign of EAE. NOD/ShiLtJ mice (n = 2 anti-CD52, n = 2 vehicle) or NOD.Rag2p^{GFP} mice (n = 3 anti-CD52, n = 4 vehicle). **[A]** Clinical score and body weight were assessed and **[B]** representative micrographs (H&E

staining; magnification x100) of the thyroid glands from anti-CD52 or vehicle-treated mice. **[C]** Blood glucose levels in the anti-CD52 or vehicle-treated mice was monitored pre-EAE induction and at various time points post EAE. Red solid line on the x-axis denotes the anti-CD52 treatment range. Data were pooled from two independent experiments and mean ± SEM. Statistical significance was calculated by Mann-Whitney test; comparison of clinical scores for vehicle versus anti-CD52, $p = 0.002$; comparison of percent weight change for vehicle versus anti-CD52, $p = 0.0003$.

4.3.6 Anti-CD52 treatment promotes repopulation dominated by RTE, DN T cells, and Treg cells in NOD mice

In B6 mice, anti-CD52 mAb treatment promoted lymphocyte repopulation dominated by DN T cells and newly generated T and B cells (388). Thus, we examined whether lymphocyte repopulation in the NOD mice follows the same pattern as in B6. EAE was induced in NOD and NOD.Rag2p^{GFP} mice and they were then treated with anti-CD52 mAb at disease onset for 5 consecutive days. As expected, anti-CD52 mAb treatment of EAE-diseased NOD and NOD.Rag2p^{GFP} mice, resulted in a significant decline in the frequency of T and B cells (Fig. 4.9 A). Our results showed a significant increase in the proportion of newly generated T cells, which was maintained for up to 8 weeks post anti-CD52 treatment (Fig. 4.9 B). Although the proportion of newly generated B cells trended towards an increase post anti-CD52 treatment in the treated group, the increase was not significantly different when compared to the vehicle-treated group (Fig. 4.9 B). This finding contrasts with the more substantial and significantly increased proportion of newly generated B cells that we observed previously for anti-CD52 treated mice on the B6 background (388). Similar to our previous findings in B6 mice, there was a significant increase in the frequency of DN T cells and Foxp3^{+} CD4^{+} regulatory T cells post anti-CD52 mAb treatment (Fig. 4.9 C). CD5 is a negative regulator of TCR signaling, and CD5^{hi} CD4^{+} T cells preferentially become committed to a regulatory T cell lineage (352). The increased MFI of CD5 in CD4^{+} T cells post anti-CD52 treatment is consistent with the increased frequency of Foxp3^{+} CD4^{+} T post anti-CD52 treatment (Fig. 4.9 C). There was a trend to an increased ratio of memory (central and effector memory) to naïve T cell populations, particularly for CD4 T cells, with anti-CD52 treatment (Fig. 4.9 D). Together, these results show that lymphocyte

repopulation post anti-CD52 mAb treatment in the NOD mice includes a high proportion of RTE, DN T cells and regulatory T cells.

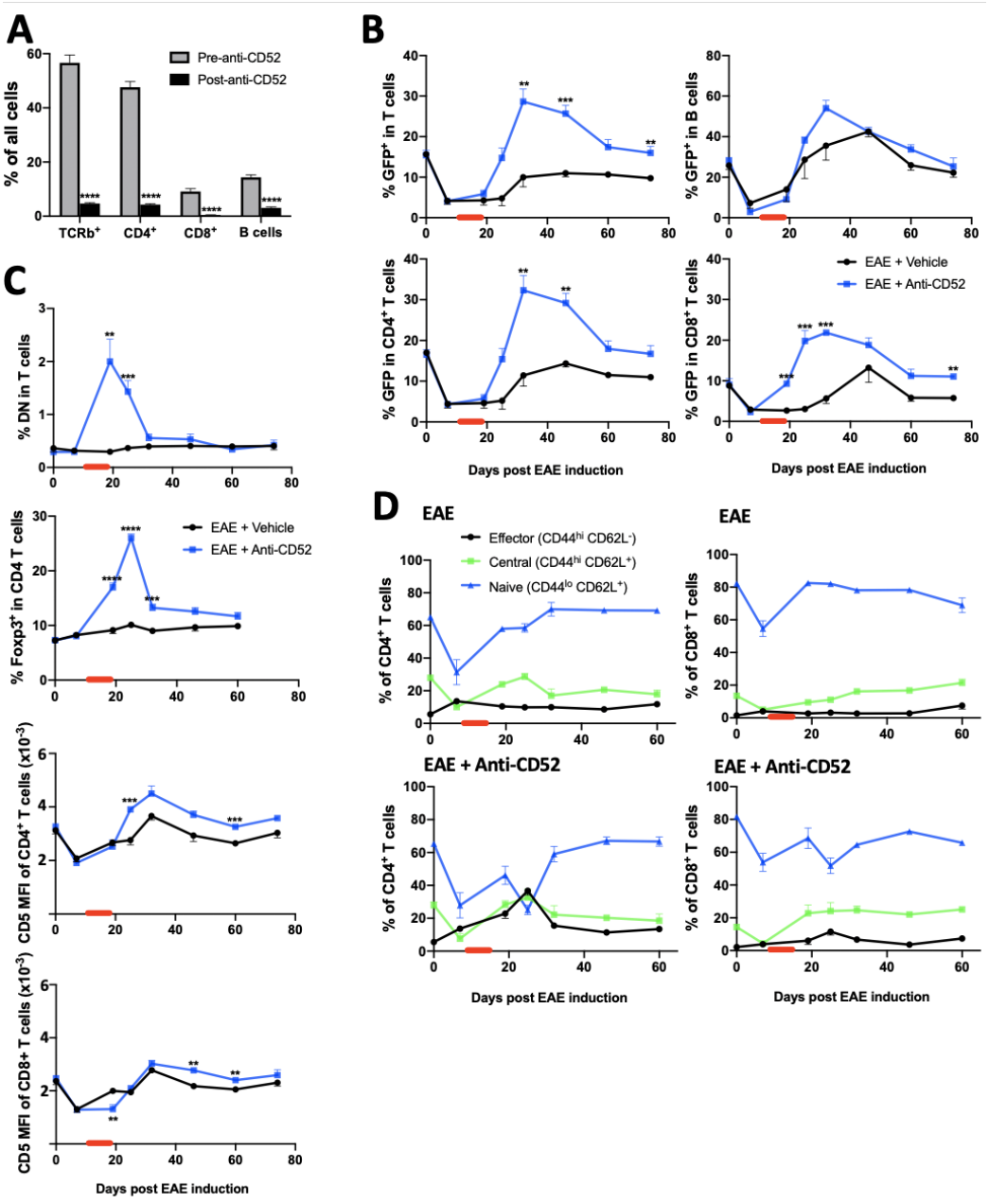
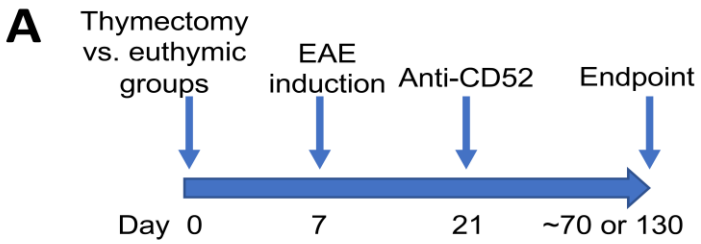


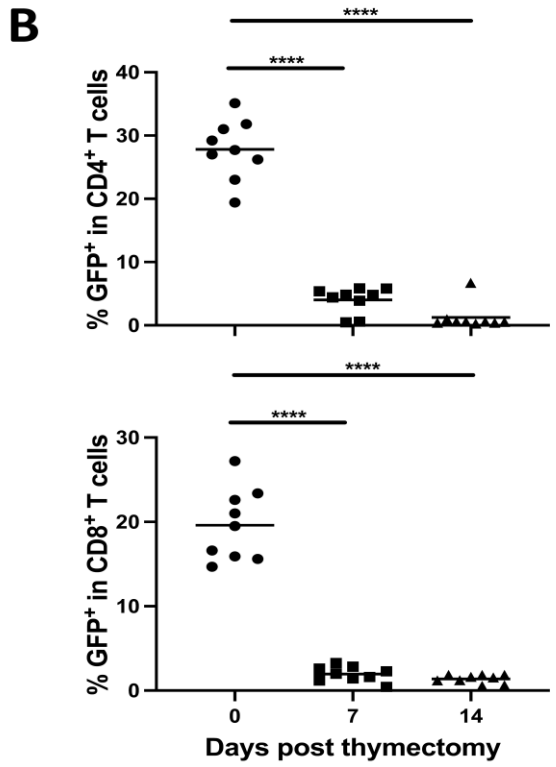
Figure 4.9. *Lymphocyte depletion and repopulation pattern in the anti-CD52-treated NOD mice described in Figure 4.8.* **[A]** The proportion of T and B cells before EAE and anti-CD52 vs. one day post the last injection of anti-CD52 in peripheral blood mononuclear cells (PBMC). **[B]** Proportion of GFP expressing T and B cells in NOD.Rag2p^{GFP} mice. **[C]** Proportion of DN T cells, Foxp3^{+} regulatory T cells, and MFI of CD5 in T cell subsets was assessed at various time points pre- and post anti-CD52 mAb treatment. **[D]** Changes in memory and naïve T cells over time and with anti-CD52 treatment. Red solid line on the x-axis denotes the anti-CD52 treatment range. Data show results from two independent experiments. All data were analyzed by flow cytometry. Statistical significance was calculated by grouped analysis T-tests with Holm-Sidak correction for multiple comparisons; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

4.3.7 Thymectomy promotes EAE relapse in anti-CD52-treated mice

Thymopoiesis is reduced in MS patients treated with anti-CD52 mAb (281). This results in prolonged T cell lymphopenia and skewing the T cell pool to an oligoclonal repertoire, which is associated with the development of alternate autoimmunity of the thyroid in humans (281). In contrast, T cell repopulation in mice treated with anti-CD52 is rapid and involves a high proportion of newly generated T cells (388). Whether or not the reduced thymic function in humans, and subsequently delayed repopulation of the T cell repertoire after anti-CD52 treatment, contributes to MS relapses is unknown. We wondered why anti-CD52 treatment is highly effective in preventing relapses in mice but not as effective in humans. Thus, we hypothesized that it is due to the rapid repopulation of T cells post anti-CD52 in mice and in contrast to the prolonged lymphopenia in humans. To block the repopulation by RTE post anti-CD52 in the mouse model (388), we performed thymectomy on mice of the B6 (B6.Rag2p^{GFP}; and B6.Foxp3^{EGFP}) and NOD (NOD.Rag2p^{GFP}) background, and left mice undisturbed for 7 days (Fig. 4.10 A). These mice would be expected to repopulate their T cells either exclusively from the homeostatic expansion of the remaining residual T cells or homeostatic expansion with a small contribution from RTE exported from cervical thymus tissue that is present in some mice (392,393). The proportion of RTE (GFP+ T cells) pre- and post thymectomy was examined to determine if thymectomy was successful. As described above, GFP expression in Rag2p-GFP mice allows us to assess RTE frequency reliably. The frequency of RTE was significantly reduced by day 7 post thymectomy and more fully by day 14 [7 days post EAE] (Fig. 4.10 B). Consistent with our previous data (388), euthymic mice induced with EAE without anti-CD52 treatment maintained an average clinical score of 2 while anti-CD52 treatment given after disease onset reduced the score to 0, and this reversal of disease was maintained long-term (Fig.

4.11). We then examined if this lack of relapse in mice given anti-CD52 after disease onset depended on thymic function. Surprisingly, while 11 of 11 mice with intact thymus function (no surgery and sham surgery groups) remained relapse-free, 8 of 12 (66.67%) thymectomized mice had a relapse of EAE from three weeks post anti-CD52 (Fig. 4.10 C and Fig. 4.11). EAE relapse post anti-CD52 treatment occurred in both male and female mice (Table. 2). Relative to the no surgery and sham surgery groups, thymectomy was associated with less weight gain and eventually a decline in weight in those mice experiencing an EAE relapse (Fig. 4.11). In the analysis of the effect of thymectomy on EAE relapse in mice on the NOD background, relapse was less frequent but again only occurred in the thymectomy group (1 of 5 thymectomized vs 0 of 4 sham surgery; Fig. 4.11). Assessment of the lymphocytes in the thymectomized B6 mice showed a long-term reduction in the frequency of CD4 T cells in PBMC together with a low proportion RTE in both CD4 and CD8 T cells relative to the euthymic (no surgery/sham surgery) mice pre- and post anti-CD52 treatment (Fig. 4.12 A). Recovery of T cells, particularly CD4 T cells, was associated with an increased proportion of RTE (Fig. 4.12 A). Thymectomy did not reduce the proportion of CD4 T cells expressing Foxp3 at day 7 post thymectomy (mean and SEM, thymectomized = 11 ± 1; euthymic 11 ± 1). Assessment of the thyroid histology in the mice that had EAE relapse vs. EAE relapse-free mice appeared to show no sign of immune cell infiltration (Fig. 4.12 B); [note: that thyroid histology has not yet been analyzed by a pathologist]. Overall, these results suggests that the thymus contributes to the prevention of EAE relapse post anti-CD52 mAb treatment through generation and export of T cells and the consequent amelioration of T cell lymphopenia. These findings suggest that monitoring thymic function of MS patients treated with anti-CD52 may help identify those patients who will require further interventions to prevent relapses.


A
Thymectomy vs. euthymic groups
EAE induction
Anti-CD52
Endpoint
Day 0
7
21
~70 or 130


B
****
****
% GFP+ in CD4+ T cells
40
30
20
10
0
****
****
% GFP+ in CD8+ T cells
30
20
10
0
0
7
14
Days post thymectomy

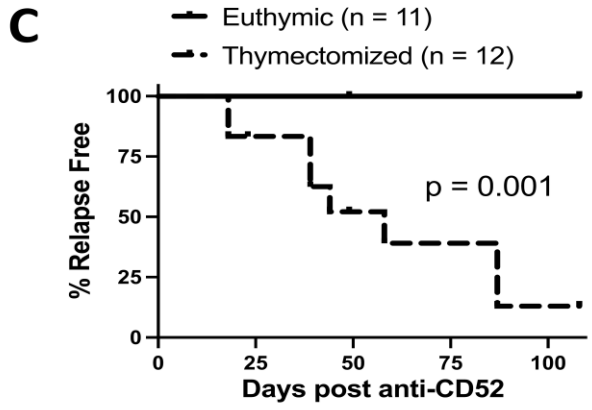
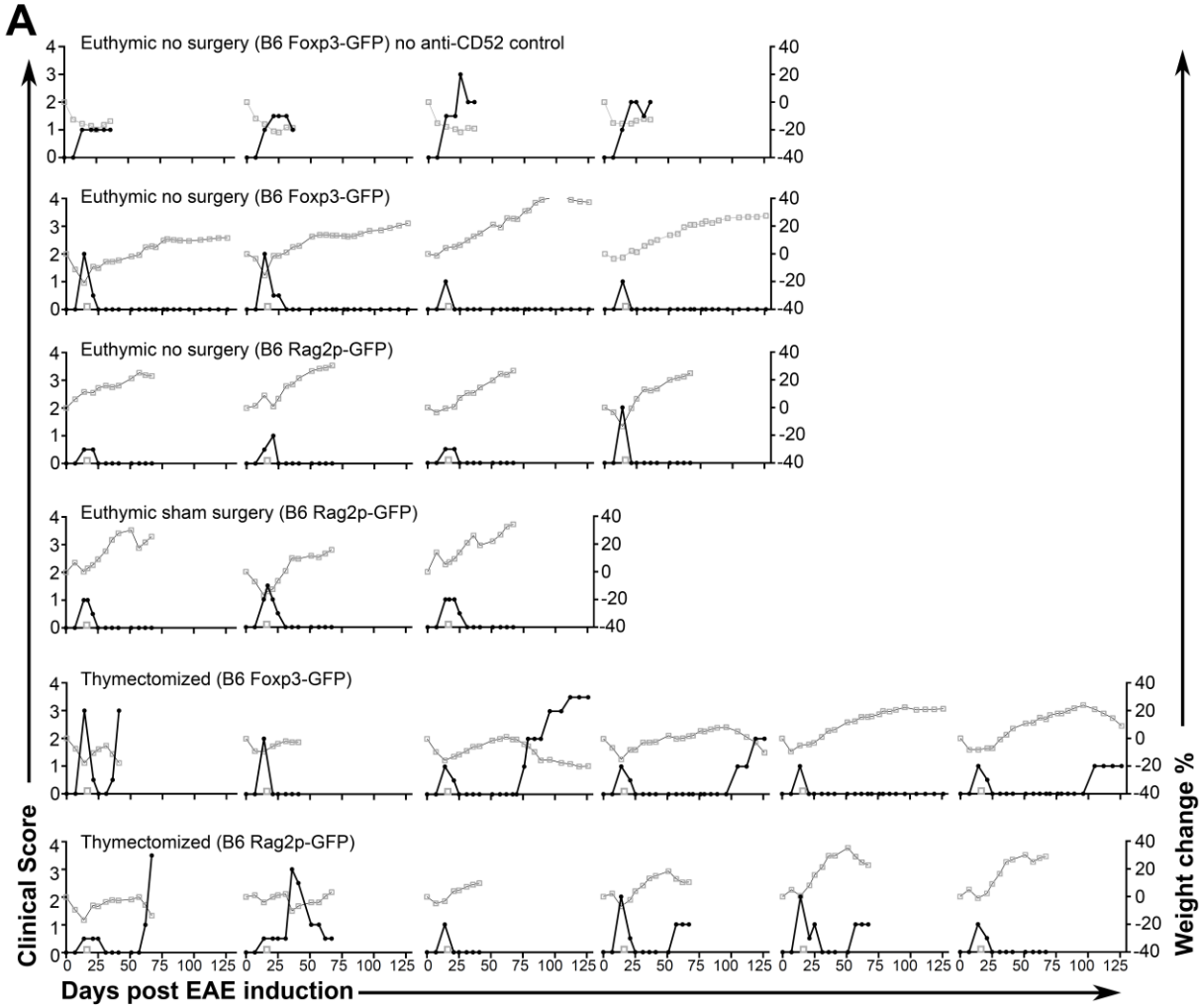

C
Euthymic (n = 11)
Thymectomized (n = 12)
% Relapse Free
100
75
50
25
0
p = 0.001
0
25
50
75
100
Days post anti-CD52

Figure 4.10. *Thymectomy reduces RTE and promotes EAE relapse in anti-CD52-treated mice.* **[A]** Mice of the B6 background were euthymic (no surgery or sham surgery) or were thymectomized, and subsequently underwent EAE induction and treatment with anti-CD52. **[B]** The proportion of GFP expressing CD4 and CD8 T cells in PBMC was assessed at days 7 and 14 post thymectomy of B6.Rag2p^{GFP} mice, n = 9; ****$p < 0.0001$, one-way ANOVA with Bonferroni's multiple comparisons test. **[C]** Kaplan–Meier survival curve analysis of EAE relapse in B6 mice among thymectomized (B6.Rag2p^{GFP}: 4 of 6; B6.Foxp3^{EGFP}: 4 of 6) and euthymic groups (no surgery: B6.Rag2p^{GFP}: 0 of 4; B6.Foxp3^{EGFP}: 0 of 4; and sham surgery: B6.Rag2p^{GFP}: 0 of 3); thymectomized vs. euthymic, p = 0.001 and thymectomized vs. sham surgery, $p = 0.055$ (Logrank, Mantel-Cox method). Data are combined from five independent experiments. For clinical scores and percent body weight change of individual mice see Fig. 4.11.


A
Euthymic no surgery (B6 Foxp3-GFP) no anti-CD52 control
Euthymic no surgery (B6 Foxp3-GFP)
Euthymic no surgery (B6 Rag2p-GFP)
Euthymic sham surgery (B6 Rag2p-GFP)
Thymectomized (B6 Foxp3-GFP)
Thymectomized (B6 Rag2p-GFP)
Clinical Score
Weight change %
Days post EAE induction

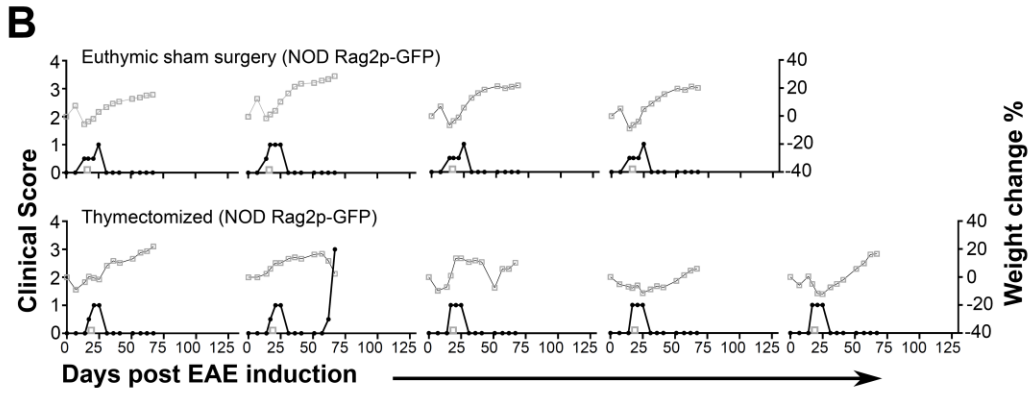

B
Euthymic sham surgery (NOD Rag2p-GFP)
Thymectomized (NOD Rag2p-GFP)
Clinical Score
Weight change %
Days post EAE induction

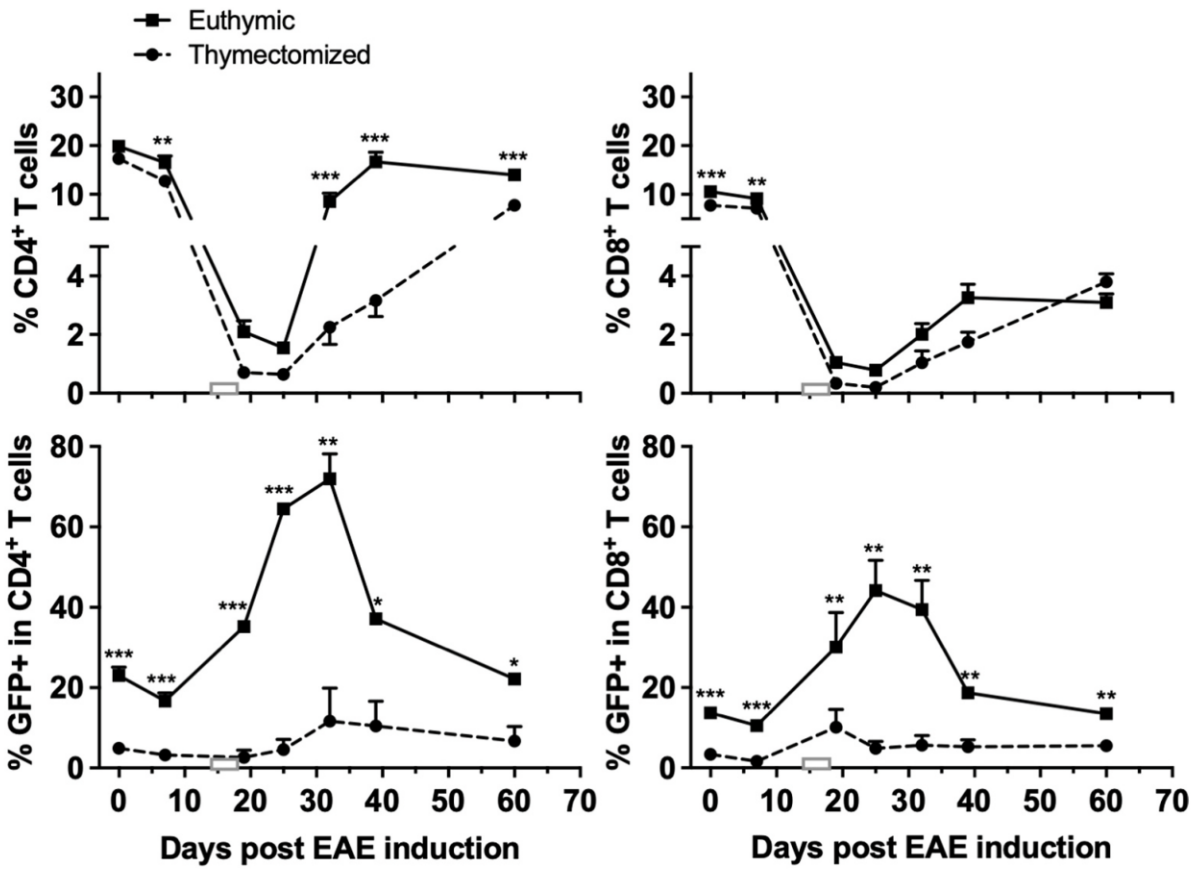
Figure 4.11. *Thymectomy promotes EAE relapse in anti-CD52-treated mice.* **[A]** Clinical scores and weight change of individual mice on the B6 background are depicted (grouped analysis is in Fig. 4.10 C) and for **[B]** female mice on the NOD background. Box on the x-axis denotes the anti-CD52 treatment range in individual mice. All mice depicted, except those in the top row of part A, were treated with anti-CD52. Anti-CD52 treatment began on day 14 when all mice had begun to have symptoms (a clinical score of 0.5 or greater), except for the thymectomized NOD group that had anti-CD52 treatment start on day 17 due to a slightly later onset of symptoms. On the graphs, grey squares are weight change, and the black circles are clinical score.

Table 2. Relapse post anti-CD52 in EAE mice occurs in both male and female mice.

	Male	**Female**
Thymectomized	4/5	4/7
Euthymic	0/3	0/8

The number of B6 background mice with an EAE relapse out of the total number analyzed is shown for each sex. The euthymic group includes mice with no surgery and those with a sham surgery.

A


Euthymic
Thymectomized
% CD4+ T cells
% CD8+ T cells
% GFP+ in CD4+ T cells
% GFP+ in CD8+ T cells
Days post EAE induction
Days post EAE induction

B

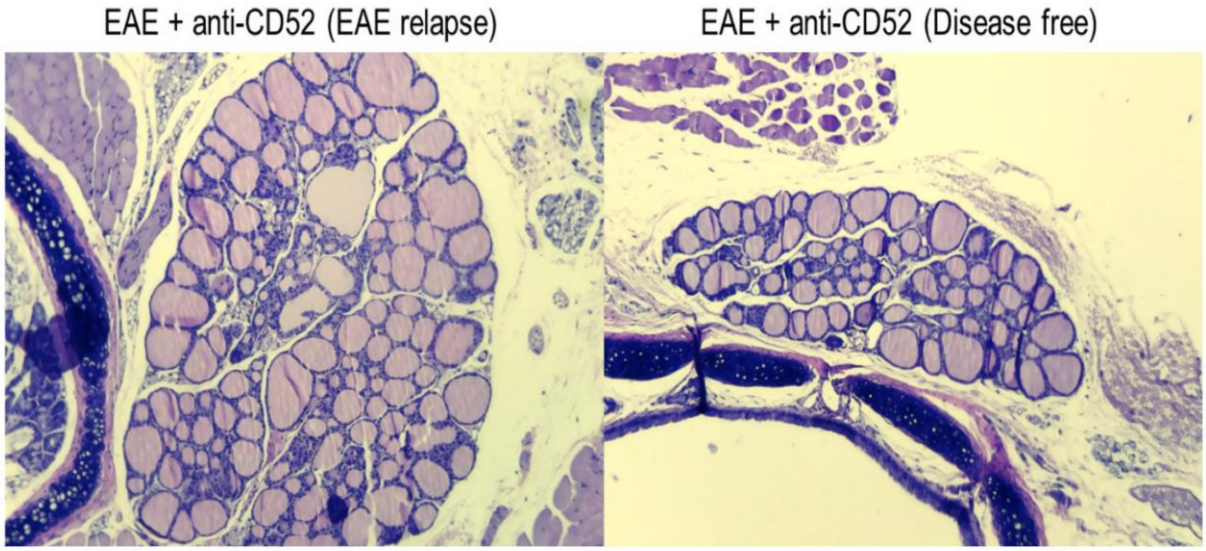

EAE + anti-CD52 (EAE relapse)
EAE + anti-CD52 (Disease free)

Figure 4.12. *Thymectomy-promoted EAE relapse in anti-CD52 treated mice is associated with prolonged T cell lymphopenia but not the development of thyroiditis.* **[A]** The mean and SEM percent of CD4 (top left) and CD8 (top right) T cells in PBMC pre- and post anti-CD52 in the thymectomized vs. euthymic (no surgery and sham surgery) mice is shown (Thymectomized, n = 12; Euthymic, n = 11). The proportion of newly generated (GFP+) CD4 (bottom left) and CD8 (bottom right) T cells is also shown. Box on the x-axis denotes the anti-CD52 treatment range. **[B]** Representative micrographs of H&E-stained thyroid glands harvested from B6.Rag2p^{GFP} mice that had a relapse of EAE post anti-CD52 treatment [left] vs. those that were EAE relapse-free [right]. Analysis: T-tests with Holm-Sidak correction for multiple comparisons; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.4 Discussion

While there are substantial differences between the EAE mouse model and human MS, the clinical situation is sufficiently similar that anti-CD52 also effectively reverses the signs of CNS autoimmunity in EAE. Surprisingly, we found that anti-CD52 was as effective at reducing the clinical disease score in mice lacking PD-1 as it was in WT mice. Treatment efficacy occurred despite the more severe EAE we observed in PD-1 deficient mice. Reduced EAE due to high dose antigen was also recently shown to occur in a PD-1 independent fashion (394). PD-1 deficiency has previously been shown to enhance disease in EAE (108,395–397) and allows disease induction without PT injection (398). As our laboratory has previously shown that PD-1 is critical to prevent autoimmunity in newly generated T cells during lymphopenia (113,288), the lack of recurrence of EAE after immune repopulation in PD-1 deficient mice might be considered unexpected. In addition, as in our data, anti-CD52 has been shown to primarily affect circulating lymphocytes, sparing many cells in lymphoid organs. These remaining lymphocytes together with the rapid repopulation from newly generated T and B cells may provide sufficient competitors (399) for homeostatic resources (self-peptide/MHC and cytokines) such that any new RTE are kept under control. Consistent with this possibility, we previously showed that established PD-1 deficient T cells were able to promote homeostasis, and prevent autoimmunity caused by PD-1 deficient RTE (288).

In the clinical setting, thymic function appears reduced in MS (400) and immune repopulation post alemtuzumab treatment of MS patients is extremely slow (276); in the first year repopulation of T cells appears to be primarily mediated by homeostatic expansion (281). This leads to an oligoclonal T cell repertoire, with decreased repertoire diversity and reduced thymic output being associated with the development of alternate autoimmunity targeting the

thyroid (281). The rapid repopulation by RTE in the mouse model, as seen in our study, may explain why the repertoire did not become oligoclonal when it was examined in human CD52 transgenic mice treated with alemtuzumab (279). Anti-CD52 had little impact on the cellularity in the thymus, possibly due to [1] low levels expression of CD52 in T cell precursors [2] reduced accessibility of the anti-CD52 mAb to the thymus [3] rapid cellular turnover from T cell precursors in the thymus, thus maintaining cellularity. Hence RTE rapidly (within 24 hours) increased in proportion post anti-CD52. However, the increased frequency of RTE was delayed in mice with EAE, and this may be as a result of the ability of PT to inhibit thymocyte emigration (401,402).

Peripheral B cell tolerance is defective in MS (403,404); however, whether anti-CD52 helps restore B cell tolerance is unclear. Although the contribution of homeostatic B cell proliferation is unclear, newly generated (transitional 1) B cells dominate early during repopulation post alemtuzumab in MS patients (405), elevating from approximately 7% to 54% of B cells, pre vs. one month post alemtuzumab, respectively (226,406). Whether this finding would be recapitulated in the EAE model post anti-CD52 was unknown. The Rag2p^{GFP} mice with trackable newly generated T and B cells allowed us to address this question. The immediate effect of anti-CD52 was a reduction of new B cells, a result that might be due to their higher expression of CD52. A correlation between the level of CD52 expression and the extent of depletion has been noted previously in tumour cell killing (373). However, we found that in the longer-term there is indeed an increase in the proportion of B cells that are newly generated (elevating from approximately 26% to 67% of B cells, pre vs. post anti-CD52, respectively), suggesting that homeostatic B cell expansion (407,408) has a more minor role, if any, in B cell repopulation post anti-CD52 treatment. Whether this rapid B cell repopulation via generation of

new B cells (405) is a pre-requisite for the development of alternate autoimmune disease is unknown (380). Answering such questions will require the development of an animal model of alternate autoimmunity post anti-CD52 therapy.

An unanticipated finding in our studies was the striking increase in the proportion of DN T cells post anti-CD52 treatment. A substantially increased frequency of DN T cells was recently identified in MS patients treated with fingolimod (409), suggesting both a clincal relevance of our findings and that multiple treatments of CNS disease preferentially spare DN T cells. DN T cells may arise from the thymus or may be generated in the periphery due to loss of either the CD4 or CD8 co-receptor on SP T cells (410–412). Loss of CD4 can be caused by chronic antigen stimulation (413). While the proportion of DN T cells increased post anti-CD52, there was only a small transient increase in the proportion of DN T cells that were newly generated. This suggests pre-existing DN T cells either had increased survival or increased proliferation compared to CD4 and CD8 T cells, or that co-receptor expressing cells had downregulated their co-receptor. While in some cases DN T cells can have proinflammatory activity and may even be effectors in disease (383,414,415), DN T cells have frequently been associated with suppression of responses, both in mice and in humans (383,384,416). For example, DN T cells are associated with suppression of graft vs. host disease (417,418), and they reduce diabetes in association with a reduction in autoantibodies (385,419). It is tempting to speculate that increased DN T cells post anti-CD52 therapy may help prevent EAE relapses. Further characterization and functional tests of the repopulating DN T cells will be required to determine if they are pro- or anti-inflammatory. To our knowledge, the contribution of DN T cells to repopulation post alemtuzumab in human MS has not been examined, and could be determined in ongoing clinical trials (420). Our findings suggest that a search for an association between DN T cell frequency

and disease relapses or autoimmune disease development post alemtuzumab could be informative.

Autoreactive T cells, such as the myelin-specific T cells, are part of the normal peripheral T cell repertoire because the thymic negative selection process eliminates most but not all potentially autoreactive T cells (421). Indeed, priming of the myelin-specific T cells already present *in vivo* with MOG_{35-55} peptide is the basis of EAE induction in mice. Anti-CD52 therapy depletes CD52-expressing lymphocytes from the periphery, which results in a transient but long-term lymphopenic state in humans (276,281). This strategy of resetting the immune system by purging lymphocytes from the periphery, including the myelin-specific T cells, and allowing repopulation of the peripheral lymphoid organs has been very effective in the treatment of MS but not in all patients (388,422). The failure of alemtuzumab to eliminate disease relapse in some MS patients reflects in part the complexity of MS pathology, and the complexity of processes leading to immune tolerance to self (282,423).

Activation in situ and homeostatic expansion of myelin-specific T cells is partly controlled in healthy individuals by competition with other T cells for survival resources, such as peptide:MHC and homeostatic cytokines (424–426). This T cell competition for survival resources is reduced during anti-CD52-mediated lymphopenia due to T cell lymphopenia. Given that anti-CD52-mediated T cell depletion is not complete in peripheral lymphoid tissues (388), it is conceivable that myelin-specific T cells that escaped depletion can outcompete other T cells during lymphopenia to promote disease relapse. Surprisingly, there were no substantial EAE relapses reported post anti-CD52 treatment in the B6, NOD, and SJL mouse strains, unlike the variable efficacy in human MS (278,282,388,423), which we attribute to the rapid repopulation of T cells in mice. This rapid repopulation of CD4 T cells post anti-CD52 treatment in mice

increases intra- and interclonal competition among T cells for resources required for their survival (399). Indeed, we have shown that the thymus is an important source of repopulating T cells in mice post anti-CD52-mediated lymphopenia. However, in severely lymphopenic mice, such as the anti-CD52 treated thymectomized used in our study, competition for survival resources is reduced, enabling the clonal expansion and survival advantage in the myelin-specific CD4 T cells causing disease (427–429). CD4 T cell lymphopenia in alemtuzumab-treated patients can persist through year 4, and the MS relapse rate increased through year 3 (277). In addition, epitope spreading of the repopulating myelin-specific CD4 T cells may have contributed to the EAE relapses in our study (430,431). Spontaneous EAE relapse post anti-CD52 treatment has been observed in the ABH mouse strain when treatment was given late, after onset of severe disease (432). This relapse might be due to the late initiation of treatment. However, it may instead reflect strain differences in T cell repopulation kinetics. No CD4 T cell recovery was seen in the ABH strain within the first four weeks post anti-CD52 treatment (432), while we observed substantial recovery of CD4 T cells in B6 mice within the first 2-3 weeks post treatment (Fig. 4.1 B and Fig. 4.12 A). Thus, prolonged T cell lymphopenia in the ABH strain may have predisposed it to relapses.

A role for the maintenance of thymus function in disease prevention is increasingly appreciated (433,434). Spontaneous remission of EAE in rodents is severely impaired in the absence of a functional thymus (435,436). Also, reduced thymic function post alemtuzumab has been reported to drive T cell repopulation by homeostatic expansion of the few residual T cells that escaped depletion, which results in an oligoclonal T cell repertoire, with decreased repertoire diversity (281). Although several studies show an increase in the frequency of regulatory T cells

post alemtuzumab (277,388,432), RTEs are the main precursor population of de novo generated regulatory T cells during lymphopenia (287,288).

In this study, we provide compelling new evidence indicating that the thymus contributes to the maintenance of EAE remission. The results suggest that the thymus contributes to preventing EAE relapse post anti-CD52 treatment through generation and export of T cells and the consequent amelioration of T cell lymphopenia. Continuous thymic output appears critical in ameliorating EAE relapse, as T cell repopulation by the homeostatic expansion of the few cells that escaped depletion is not sufficient for complete regulation of myelin-specific T cells. These findings suggest that monitoring the thymic function of MS patients treated with anti-CD52 may help identify those patients who will require further interventions to prevent relapses.

Chapter 5

Discussion and future directions

5.1 Discussion

In this thesis, I have attempted to clarify some fundamental issues about immune tolerance that are not well understood. Since each data chapter includes a detailed discussion section on the relevant subject, I shall keep this section brief. Some remarks here are speculative, and some further discusses the data chapters.

5.1.1 RTE: friend or foe?

RTE are newly generated T cells in the periphery that have most recently completed selection and thymic egress and constitute a population that is phenotypically and functionally distinct from its more mature counterpart (315). This T cell subset helps to maintain the naïve T cell pool and the T cell repertoire diversity (315). Our group has shown that RTE lacking coinhibitors, such as PD-1 and Btla, generated autoimmunity in lymphopenic settings (113,268). In contrast, PD-1 or Btla deficient mature T cells did not generate autoimmunity in $Rag^{-/-}$ mice (113,268). Reducing resources (e.g., peptide:MHC) or adding competitor cells reduced autoimmunity by these coinhibitor deficient RTE (113,268). Interestingly, we showed in this study that paucity of RTE also promoted autoimmunity, suggesting that RTE is a nexus between tolerance and autoimmunity in lymphopenic conditions (Fig. 5.1). However, in our study, RTE only generated autoimmunity in lymphopenic mice when the RTE were deficient of PD-1 or Btla, suggesting that in the presence of these coinhibitors, RTE can be considered as "friends." Similarly, other groups have shown that molecules with inhibitory functions such as protein tyrosine phosphatase N2 (PTPN2) and transforming growth factor-β limit LIP of newly generated T cells (296,437). Furthermore, lymphoproliferative disorders due to uncontrolled LIP, and the resultant mortality in neonatal $CTLA\text{-}4^{-/-}$ mice suggests that CTLA-4 also regulates LIP

in newly generated T cells (290). Further discussion in section 5.1.2. below details how PD-1$^{-/-}$ RTE do not result in severe autoimmunity when lymphopenia is incomplete.

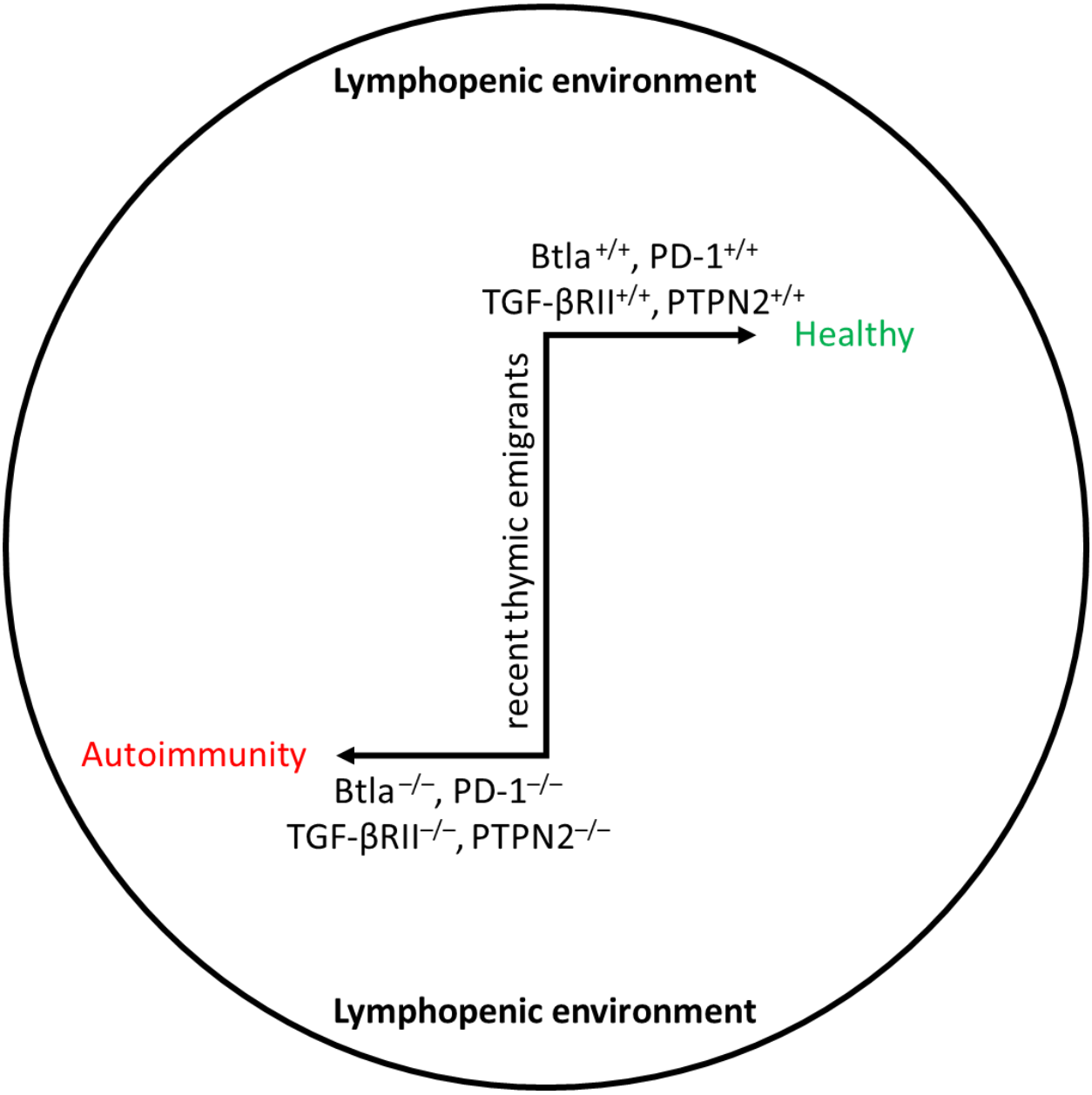


Figure 5.1. *Recent thymic emigrants are a nexus between tolerance and autoimmunity in lymphopenic settings.* RTE in the presence of coinhibitors like Btla and PD-1 do not generate autoimmunity in lymphopenic mice. In contrast, autoimmunity often occurs when Btla or PD-1 signaling is absent in RTE in lymphopenic mice.

5.1.2 Dynamics of RTE in lymphopenic setting when PD-1$^{-/-}$ signaling is absent

In scenario A as in Fig. 5.2. below, RTE are seeding an "absolute" lymphopenic environment where resources are abundant, and competition is minimal. In this scenario, T cells repopulate by thymic output and homeostatic proliferation of the first waves of T cells newly exported from the thymus that are still undergoing peripheral tolerance. As previously reported by our group, adoptive transfer of a PD-1$^{-/-}$ RTE population sorted from the spleen of lymphoreplete mice caused autoimmunity in Rag$^{-/-}$ mice (297), indicating that RTE are not immediately tolerized in the periphery. Similarly, we showed in this study that PD-1$^{-/-}$ neonatal splenocytes, naturally enriched for RTE, also generated autoimmunity in Rag$^{-/-}$ mice. Thus, in scenario A, intra- and interclonal competition for resources are between PD-1$^{-/-}$ RTE that are not yet tolerized, hence generating autoimmunity in lymphopenic mice. However, in scenario B, lymphopenia is not "absolute" because some residual T and B cells in the peripheral lymphoid organs appeared to have escaped anti-CD52-mediated depletion (Fig. 4.4 B). Hence, in this scenario, resources are not as abundant as in scenario A. Repopulation is partly via thymic output and partly by homeostatic expansion of RTE and the residual mature lymphocytes that escaped anti-CD52-mediated depletion. As a result, intra- and interclonal competition in scenario B are between RTE and established (i.e., tolerized matured) residual T cells that escaped anti-CD52-mediated depletion and undergo LIP. As previously shown by our group, adoptive co-transfer of

PD-1$^{-/-}$ RTE with their established matured T cell counterparts did not generate autoimmunity in the lymphopenic recipient mice (113). In addition, repopulation is substantially faster in scenario B because RTE from the thoracic thymus and the cervical thymus continues to seed the periphery as anti-CD52 depletes the peripheral lymphocytes. Unlike scenario A, where there is "absolute" lymphopenia and LIP potential is higher, LIP potential is lower in scenario B, reducing the development of autoimmunity. The LIP potential of T cells is partly influenced by the ratio of lymphocytes to the available resources and partly by the strength of TCR signaling (high-affinity signaling from cognate peptides versus low-affinity signaling from self-peptides). Similar to scenario A, the first set of RTE in the neonatal mice seeds an "absolute" lymphopenic environment but these PD-1$^{-/-}$ mice do not develop autoimmunity until late-life. This, we attribute to low LIP potential in neonatal mice because of reduced available resources. Indeed, the adoptive transfer of PD-1$^{-/-}$ FLC did not generate autoimmunity in neonatal mice, and the resulting PD-1$^{-/-}$ RTE did not acquire an effector memory phenotype typically associated with LIP (113).

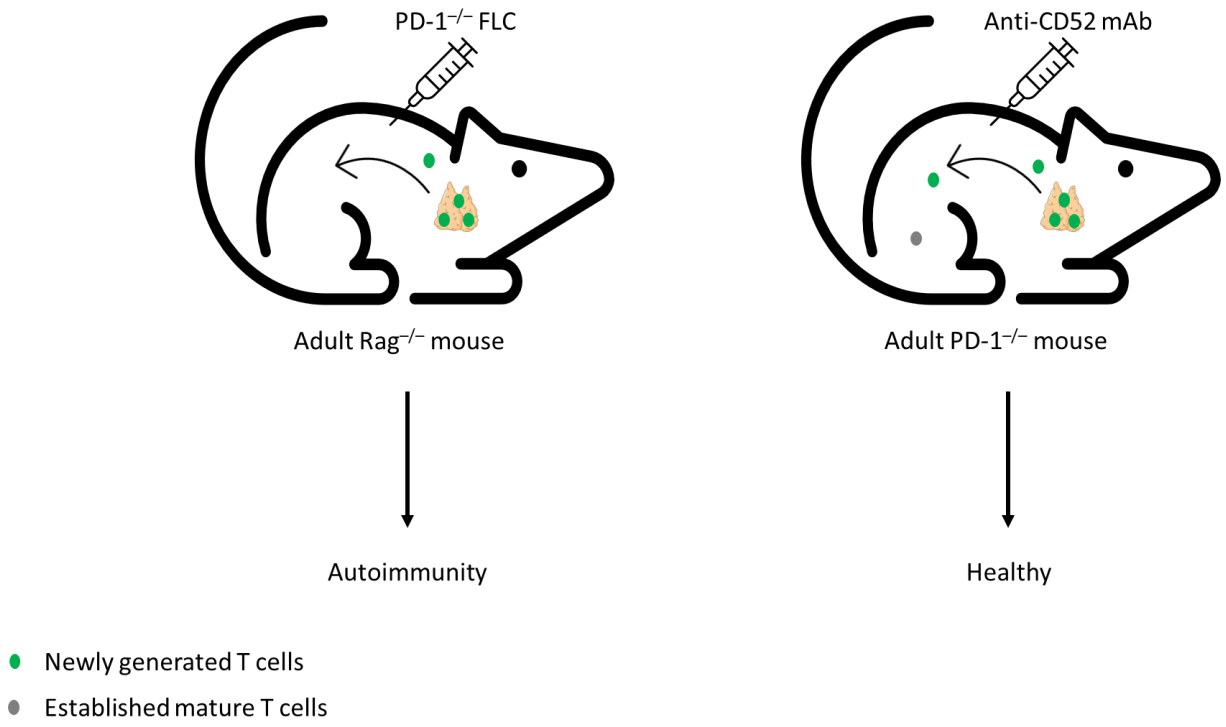


Figure 5.2. *Dynamics of RTE in lymphopenic setting when PD-1$^{-/-}$ signaling is absent.* Left panel (scenario A): adoptive transfer of PD-1$^{-/-}$ FLC or thymocytes often results in autoimmunity in the lymphopenic host shortly after newly generated T cells from the thymus are exported into the periphery. Right panel (Scenario B): immune system reset of the PD-1$^{-/-}$ mice with anti-CD52 mAb did not result in autoimmunity when RTE populated the periphery.

5.2 Conclusions and limitations

Our study in **Chapter 2** showed that Btla and PD-1 are needed to establish tolerance in RTE. The disease caused by neonatal PD-1$^{-/-}$ splenocytes in **Chapter 2** does not exclude the potential differences in the TdT enzyme and Treg cells between neonatal and adult T cells, which

might underlie the differences in disease incidence observed. In addition to enforcing tolerance in RTE, the coinhibitor Btla regulates CD5 expression levels in T cells. Our data in **Chapter 3** was the first to show that loss of Btla early in T cell ontogeny results in a long-term increase in CD5 expression levels. Whether or not Btla deficiency early in T cell ontogeny alters the repertoire of T cells by promoting thymic selection of $CD5^{hi}$ T cells is unknown. In **Chapter 4**, we showed that paucity of RTE promotes EAE relapse in diseased mice treated with anti-CD52 mAb, suggesting the contribution of a functional thymus and RTE in preventing disease relapse. In this study, two-thirds of thymectomized B6 mice had an EAE relapse post anti-CD52 treatment, while no surgery and sham surgery euthymic controls remained relapse-free. Given that lymphocyte depletion is not complete in secondary lymphoid organs, one speculates that the cells that resisted anti-CD52-mediated depletion and underwent LIP contributed to EAE relapse. Although mice with preserved thymic function remained EAE relapse-free in this study, our study did not answer whether restoration of thymic function could prevent disease.

5.3 Future directions

5.3.1 Examining if PD-1 or Btla influence the quorum required for T cell activation and disease generation in the lymphopenic recipients of $Btla^{-/-}$ or $PD\text{-}1^{-/-}$ RTE

The "Quorum Hypothesis" postulates that *"if a quorum (i.e., a minimum number) of T cells is needed to achieve T cell activation, and only a few T cells specific for a peripheral self-antigen exist, the self-antigen will rarely, if ever, activate its corresponding T cells"* (55). Having shown that Btla and PD-1 are required for establishing tolerance in RTE in a lymphopenic setting, these coinhibitory receptors' role in deciding T cell quorum (i.e., the minimum number of antigen-specific T cells required for their activation) is unknown. One could examine the

relationship between quorum sensing and coinhibitory receptors on the decision between tolerance and immunity as well as on the type (class) of immune response generated. This relationship can be tested *in vitro* and *in vivo* using polyclonal and/or TCR transgenic systems with constitutive vs. inducible genetic deficiency in coinhibitory receptors (Btla and/or PD-1).

For the *in vitro* system, titrated numbers of CD4 T cells could be stimulated with peptide-antigen or a polyclonal stimulus to determine the quorum required for generation of Th0, Th1 and Th2 cells (and where feasible Th17), and cellular proliferation. The effect of coinhibitor deficiency on the quorum number required for the various T cell functions could be examined. Also, the effect of T cell numbers on the expression of a several distinct coinhibitory receptors could be assessed. *In vivo* studies will determine the relationship between quorum and coinhibitor deficiency on tolerance vs. immunity to self. Titrated numbers of polyclonal newly generated CD4 T cells could be transferred to lymphopenic syngeneic recipients and these T cells will be wild type, constitutively coinhibitor deficient, or inducible coinhibitor deficient. The development of autoimmunity in the adoptive transfer recipient could be assessed as well as the phenotype of the transferred T cells; the latter will include the *in vivo* effect of T cell numbers on the expression of a several distinct coinhibitory receptors. Using a TCR transgenic system (e.g., anti-H-Y CD4 T cells), the relationship between T cell numbers and coinhibitor deficiency could be tested by adoptive transfer or titrated T cell numbers to recipients that are then challenged with peptide-antigen.

Overall, these studies will determine if quorum affects coinhibitor expression and whether the lack of a coinhibitor changes the number of T cells needed to achieve the quorum required for various T cell functions and for the development of autoimmune disease. Hypothetically, Btla or PD-1 might block autoimmunity by increasing the quorum required for T

cell activation, such that in the absence of these coinhibitors, T cell activation quorum is reduced, and pathogenic T cells can be easily activated. Examining if Btla or PD-1 affects T cell activation quorum size would provide more valuable information as to why many patients treated with anti-PD-1 mAb therapy develop immune-related adverse events (318,319).

5.3.2 Role of CD5 in the Btla$^{-/-}$ T cells

In Chapter 3, we showed that loss of Btla early in T cell ontogeny resulted in a long-term increase in CD5 expression level. Given that Btla is a T cell coinhibitory receptor, it is possible that CD5, also a coinhibitory receptor, compensates for the loss of Btla to limit autoimmunity in the Btla$^{-/-}$ mice. Although Btla$^{-/-}$ mice develop late-life autoimmunity (86), the CD5$^{-/-}$ mice remain relatively healthy in late-life (149). Investigating if CD5 plays a compensatory role for the loss of Btla signaling will help understand potential targets to improve the efficacy of the anti-Btla antibody currently in clinical trials for cancer treatment (326,327). To examine if CD5 plays a compensatory inhibitory role in the absence of Btla signaling, one could potentially cross the Btla$^{-/-}$ mice to the doxycycline-inducible CD5 knockdown (KD) mice or the CD5$^{flox/flox}$ x Cre-ER^{T2} [inducible CD5 knockout (CD5-iKO)] mice (150,338) to generate Btla$^{-/-}$ CD5-KD mice or Btla$^{-/-}$ CD5-iKO mice, respectively. Administration of doxycycline will reduce CD5 gene expression in the Btla$^{-/-}$ CD5-KD mice, and tamoxifen administration will eliminate the CD5 gene in the Btla$^{-/-}$ CD5-iKO mice. Should CD5 play a compensatory coinhibitory role in the absence of Btla signaling, we speculate that loss of CD5 signaling in the Btla$^{-/-}$ CD5-KD or Btla$^{-/-}$ CD5-iKO mice will [1] facilitate an early onset of autoimmunity typically present in late-life in the Btla$^{-/-}$ mice [2] cause more severe disease in lymphopenic recipients of Btla$^{-/-}$ CD5-

KD or Btla$^{-/-}$ CD5-iKO thymocytes [3] induce autoimmunity in lymphopenic recipients of adult Btla$^{-/-}$ CD5-KD or Btla$^{-/-}$ CD5-iKO splenocytes.

5.3.3 Investigating the fulcrum between tolerance and autoimmunity in the Btla$^{-/-}$ or PD-1$^{-/-}$ RTE

Our data support the hypothesis that coinhibitory receptors other than PD-1 enforce tolerance in newly generated T cells during LIP. Importantly, my work is the first to demonstrate that the coinhibitors (Btla and PD-1) are needed at the RTE stage. Prior work had only shown that the coinhibitors were needed at some unknown point for newly generated T cells. My data showed that even when the capacity to express coinhibitors is present throughout thymic selection, a loss of these coinhibitors after export from the thymus causes autoimmunity. While our group previously showed that Btla and PD-1 signals are dispensable in established T cells (113,268), we do not yet know at what point between the RTE stage and mature naïve T cell stage these coinhibitors become less important. GFP expression level is highest in the most recently exported RTE and absent in the established matured T cells of the B6.Rag2p^{GFP} mice (36), and our study showed that the CD5 expression level is higher in the RTE relative to the matured T cells (Fig. 3.1 D). To examine what stage between the RTE and mature T cell stage the coinhibitors, Btla and PD-1, becomes dispensable for establishing tolerance in lymphopenic mice, one could perform an adoptive transfer of splenocytes from the B6.Rag2p^{GFP}-Btla$^{-/-}$ mice (not yet generated) or B6.Rag2p^{GFP}-PD-1$^{-/-}$ mice sorted based on GFP and CD5 expression levels (e.g., GFP^{hi} CD5^{hi}, GFP^{hi} CD5^{lo}, GFP^{lo} CD5^{hi}, GFP^{lo} CD5^{lo}, GFP^{neg} CD5^{hi}, GFP^{neg} CD5^{lo}) and monitor recipient Rag$^{-/-}$ mice for disease. While this study and a previous study from our group showed that Btla$^{-/-}$ newly generated T cells caused autoimmunity in Rag$^{-/-}$ mice (268),

whether or not steady state periphery-derived Btla$^{-/-}$ newly generated T cells retained this heightened potential for autoimmunity is unknown. The experimental design proposed above could potentially answer this.

5.3.4 Investigating the role of thymic output in the prevention of EAE relapse

Our study showed that the elimination of thymic output before EAE induction and anti-CD5 treatment significantly increased EAE relapse, whereas euthymic mice remained relapse-free. In the euthymic B6 and NOD mice, the proportion of newly generated T cells populating the periphery peaked at about 2 – 3 weeks post the last dose of anti-CD52 (Fig. 4.7A, 4.9B, 4.12A). Given that the proportion of RTE significantly increases in euthymic mice post anti-CD52, one questions whether thymectomy post EAE induction and anti-CD52 treatment would delay or prevent EAE relapse. This is an important question because it helps answer if thymic output is only needed in EAE-diseased mice in the initial period following anti-CD52 treatment to prevent disease relapse or a continuous thymic output is needed to prevent disease relapse. To answer this question, one could perform thymectomy on EAE-diseased mice treated with anti-CD52 at about two weeks post anti-CD52 treatment, when the repopulating RTE population has peaked. Prevention of EAE relapse in these mice would suggest that thymic output in the initial period following anti-CD52 treatment is sufficient to prevent EAE relapse. In contrast, if these mice develop EAE relapse post thymectomy, it suggests that a continuous thymic output is required to prevent EAE relapse post anti-CD52 treatment.

In older mice, ageing is often accompanied by a significant decline in thymic output and a reduction in the proportion of naïve T cells in the periphery (30,438,439). Although thymic output is significantly reduced in aged mice relative to young mice, a small proportion of RTE

were still detectable in aged mice (30). Suppose it turns out that a continuous thymic output is required to prevent EAE relapse post anti-CD52 treatment. In that case, it then raises a question of how much thymic contribution is needed to prevent EAE relapse. It will be informative to know if the reduced thymic output in aged mice is sufficient to prevent EAE relapse post anti-CD52 treatment or whether these mice might need a thymus transplant post anti-CD52 treatment to prevent EAE relapse. This study is important as it will guide the clinical use of anti-CD52 in the geriatric population. Currently, there is a paucity of studies on the use of anti-CD52 in RRMS treatment in the geriatric population. However, anti-CD52 treatment of older patients (age ≥ 65 years) who were transplanted with kidneys is associated with a risk of death and early graft loss (440). It is possible that the reduced thymic output due to thymic involution in the geriatric population contributes to early graft loss in kidney transplant patients treated with anti-CD52. Based on this, one could speculate that the reduced thymic output in the geriatric population might not be sufficient to prevent MS relapse post anti-CD52 treatment. To address this, one could induce EAE in aged mice, followed by anti-CD52 treatment, and then monitor the mice for several weeks to see if they develop EAE relapse or not.

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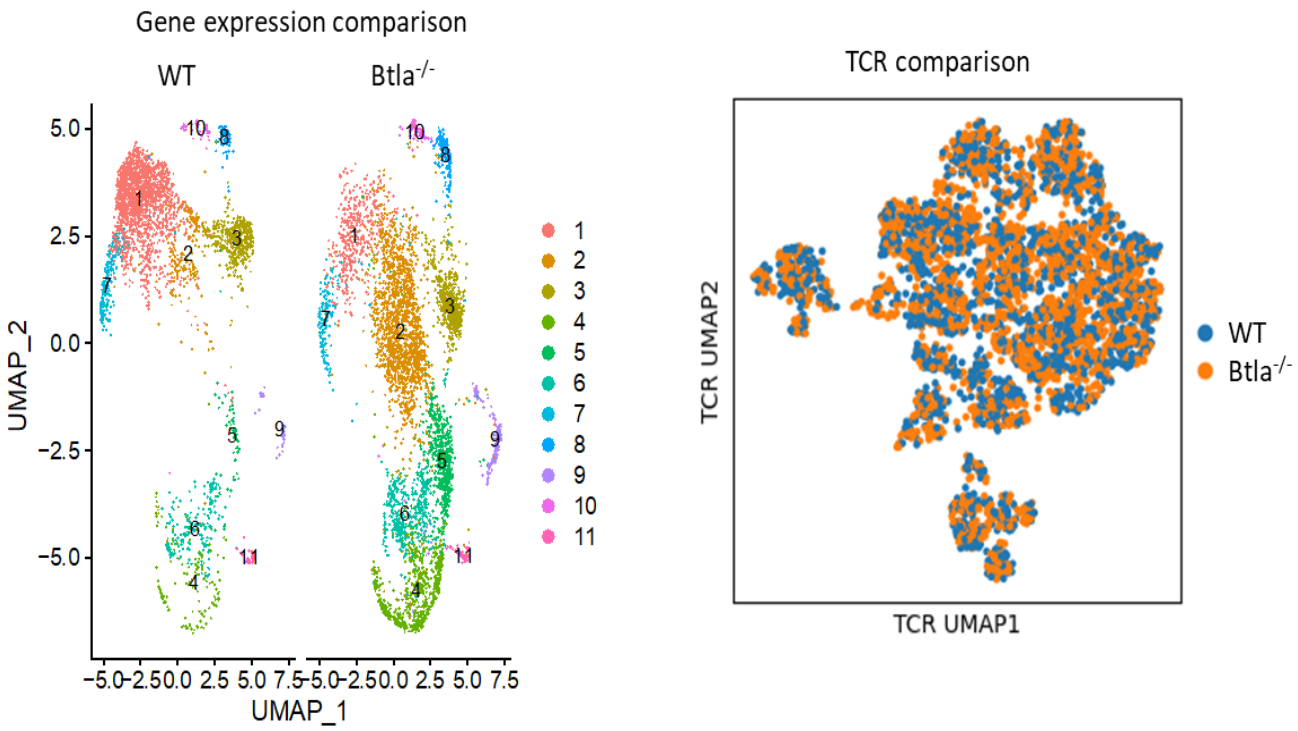
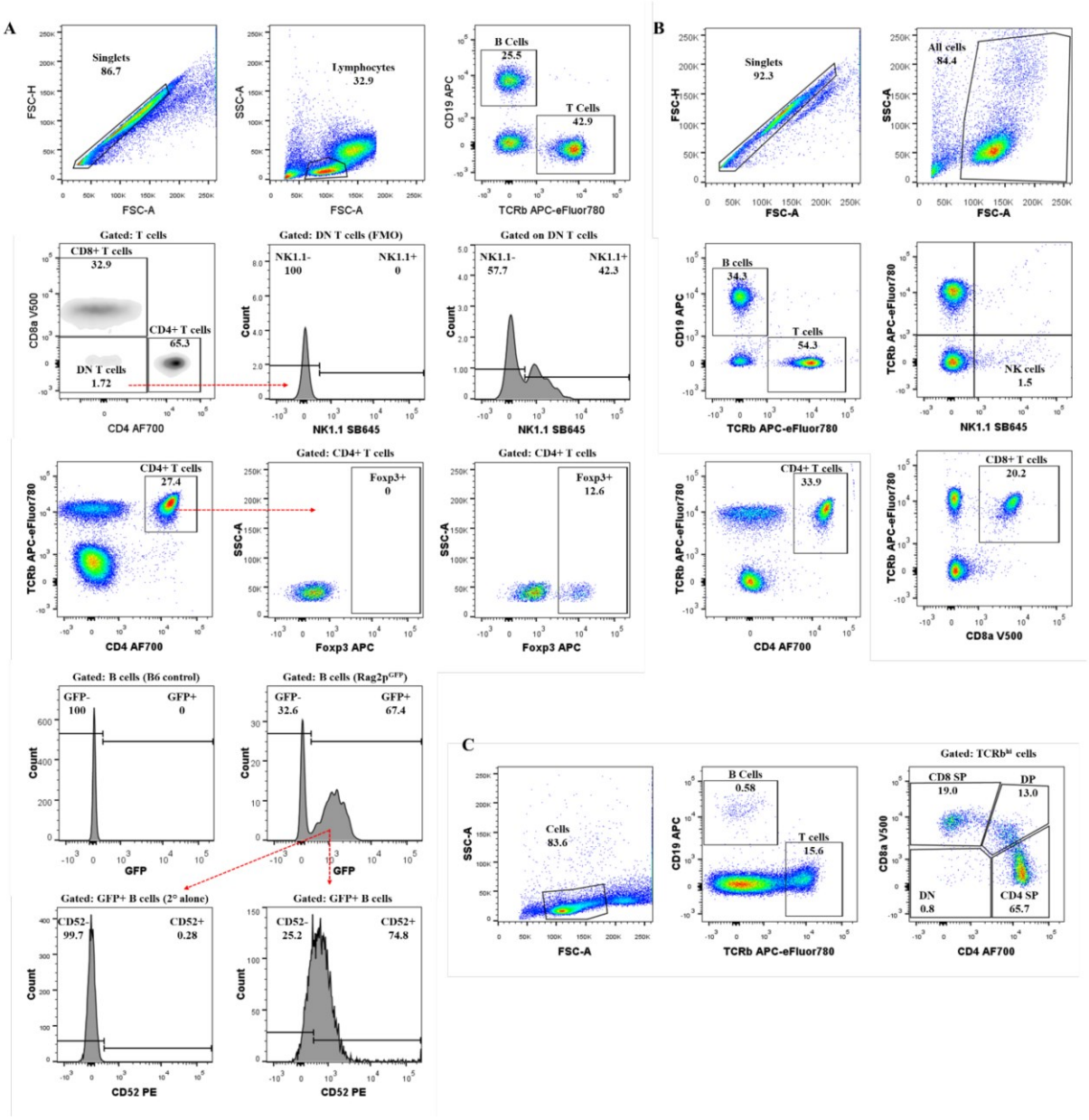
Appendices



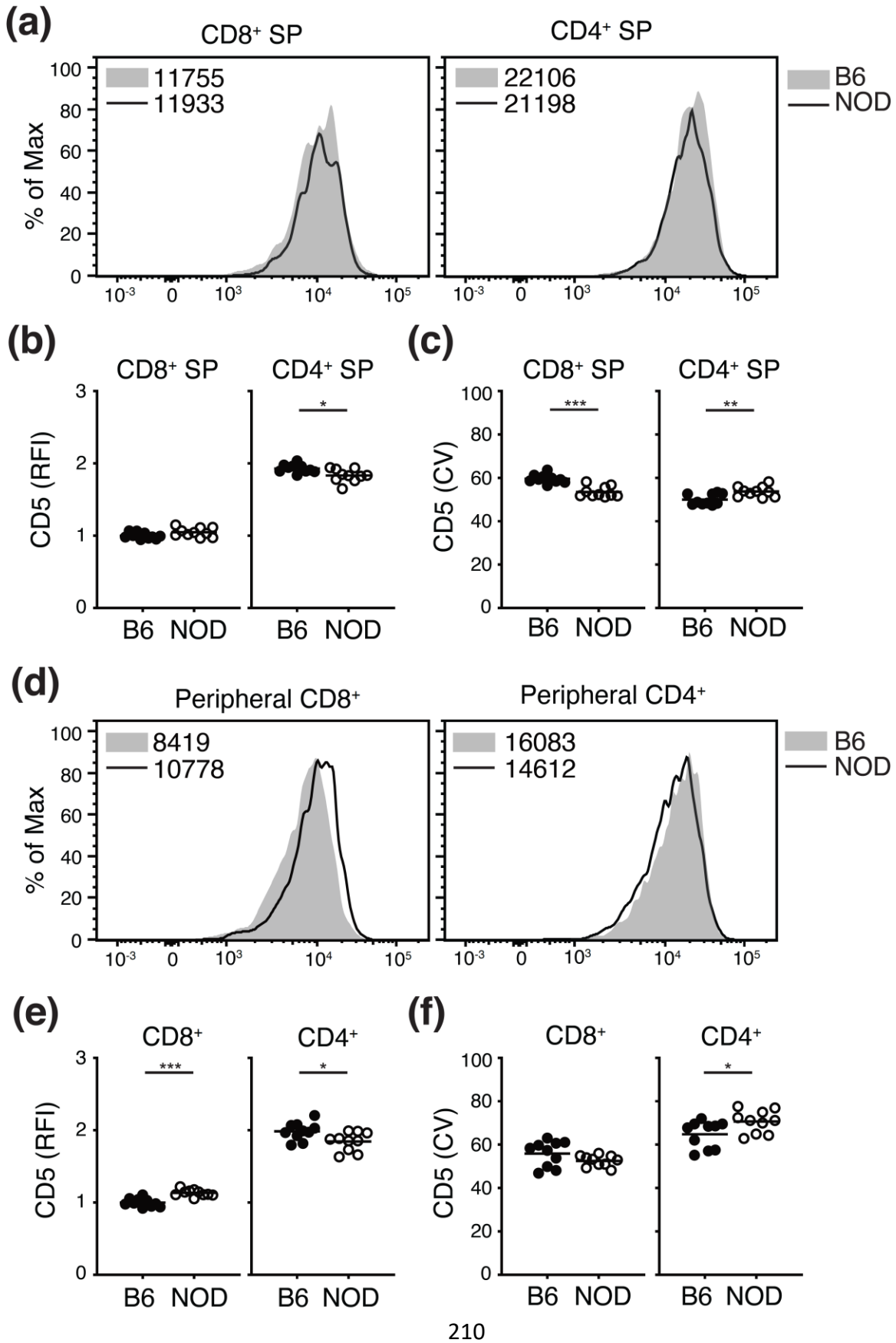
Appendix 1. *Gene expression and TCR comparison of WT and Btla$^{-/-}$ CD4 SP thymocytes.*

CD4SP thymocytes were purified from WT or Btla$^{-/-}$ mice for single-cell gene expression or TCR repertoire analysis using the 10X Genomics 5' RNA Gene expression and TCR sequencing kits. UMAP plots were generated from the Seurat R package (left) or Clonotype Graph Neighbor Analysis (CoNGA) package (right) following filtering and normalization of sequencing reads.



Appendix 2. *Flow cytometry gating strategy*. **[A]** For blood or spleen or lymph node cells, gate on singlets and then lymphocytes and then T or B cells is shown. T cell gated DN T cells were analyzed for NK1.1 expression (control FMO also shown). Also depicted: Isotype control or Foxp3 staining in CD4 T cells; GFP expression in the B cell gate of B6 control or Rag2p^{GFP} cells; CD52 expression on GFP+ gated B cells (gating was similar for GFP+ T cells) with 20 alone (no anti-CD52) also shown. **[B]** Gating strategy for subpopulations in the all cell gate is

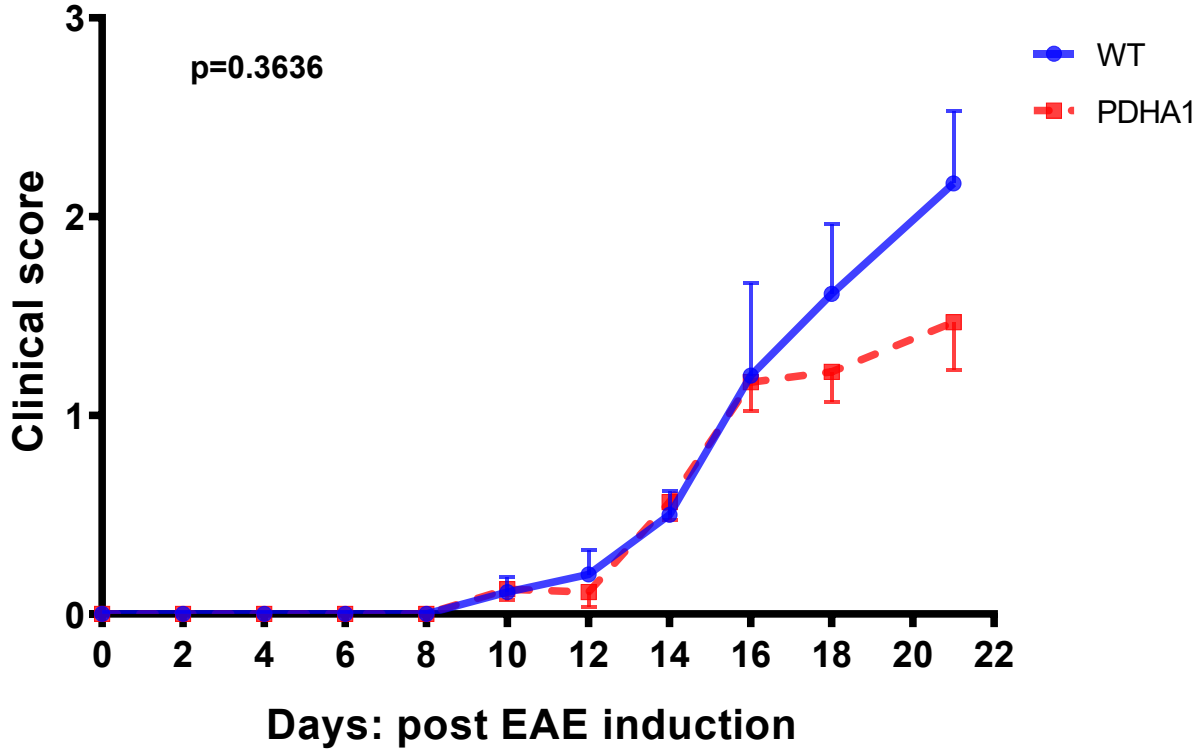
depicted (gating on singlets, then all cells). **[C]** Gating strategy for thymocytes in lymphocyte gate (for all cell gate, gating also included high FSC cells). Only TCRβ^{hi} cells were gated in order to restrict analysis to more mature T cells within the thymus (i.e., to exclude immature DN and DP T cells).


(a)
CD8+ SP
CD4+ SP
11755
11933
22106
21198
B6
NOD
% of Max
(b)
CD8+ SP
CD4+ SP
CD5 (RFI)
B6 NOD
(c)
CD8+ SP
CD4+ SP
CD5 (CV)
B6 NOD
(d)
Peripheral CD8+
Peripheral CD4+
8419
10778
16083
14612
B6
NOD
% of Max
(e)
CD8+
CD4+
CD5 (RFI)
B6 NOD
(f)
CD8+
CD4+
CD5 (CV)
B6 NOD

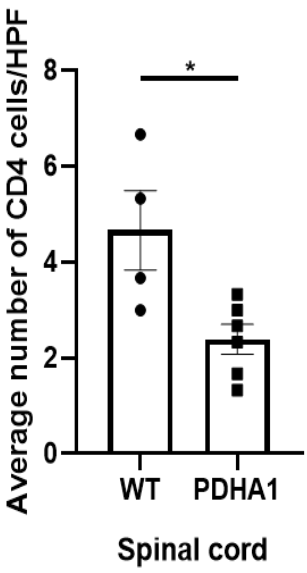
Appendix 3. *Peripheral T cells from NOD mice express differential CD5 levels compared to their B6 counterparts.* [**A**] Representative histograms of CD5 expression on TCRβ^{hi} CD8^{+} SP and CD4^{+} SP thymocytes from B6 (grey-shaded) and NOD (solid line) mice. [**B**] CD5 RFI and [**C**] CD5 CV on CD8^{+} SP and CD4^{+} SP from B6 and NOD mice. [**D**] Representative histograms of CD5 expression on TCRβ^{hi} CD8^{+} and CD4^{+} splenic T cells from B6 (grey-shaded) and NOD (solid line) mice. [**E**] CD5 RFI and [**F**] CD5 CV on CD8^{+} and CD4^{+} splenic T cells from B6 and NOD mice. The RFI is calculated by normalizing to the average of the CD5 MFI on CD8^{+} SP thymocytes or CD8^{+} splenic T cells from B6 mice. Each dot depicts data from an individual mouse from two independent experiments; B6 (n=10) and NOD (n=10). Unpaired two-tailed student t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This data has been published in reference (151).

In a collaborative study, we examined the effect of deleting pyruvate dehydrogenase (PDH), an enzyme that catalyzes the conversion of pyruvate to acetyl-coA, in $CD4^+$ T cells on peripheral T cell responses. We utilized the EAE model, which is mediated by the $CD4^+$ T cells. Here, WT ($CD4^{CreERT2}$) or mice with T cell-specific deletion of PDHA1 ($CD4^{CreERT2}$ $PDHA1^{fl/fl}$) were induced with EAE, and clinical progression of the paralysis was scored during 21 days. Mice lacking PDHA1 in $CD4^+$ T cells had diminished severity of disease progression compared with $CD4^{CreERT2}$ littermates (Appendix 4A) and reduced $CD4^+$ T cell infiltration of the spinal cord (Appendix 4B&C) at the later time points, consistent with PDHA1-deficient $CD4^+$ cells having reduced functionality. This suggests an essential role for PDH activity in driving $CD4^+$ T cell activation and effector function. This data has been submitted for publication (revision requested) and is available in a preprint (441).

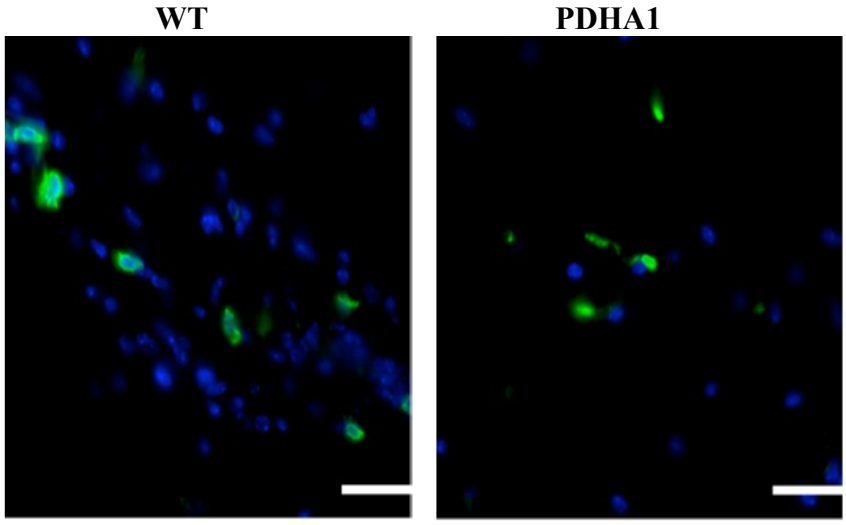
A


p=0.3636
WT
PDHA1
Clinical score
0
1
2
3
0 2 4 6 8 10 12 14 16 18 20 22
Days: post EAE induction

B


Average number of CD4 cells/HPF
0
2
4
6
8
*
WT
PDHA1
Spinal cord

C


WT
PDHA1

Appendix 4. *Clinical score and histology of EAE-induced PDH$^{-/-}$ mice* **[A]** Clinical scores of mice and induced with EAE ($CD4^{CreERT2}$ $PDHA1^{fl/fl}$, n= 16; $CD4^{CreERT2}$, n= 9). **[B]** Average number of $CD4^{+}$ cells infiltrating the spinal cord of EAE-induced mice ($CD4^{CreERT2}$ $PDHA1^{fl/fl}$, n= 6; $CD4^{CreERT2}$, n= 4). **[C]** Representative micrograph of immunohistochemistry staining of spinal cord sections from EAE-induced $CD4^{CreERT2}$ $PDHA1^{fl/fl}$ mice and their WT counterparts. Data are from two separate experiments. All graphs represent mean and SEM. Statistical significance was measured by Student's T-test or Mann-Whitney test of the AUC for EAE clinical score. *$p < 0.05$.