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T Cell Generation in a Lymphopenic Environment Generates Disease when the Thoracic Thymus is Eliminated; Augmentation by IL-7/Anti-IL-7 Complexes

by

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ABSTRACT

Cervical thymus functionally mimics the thoracic thymus in supporting T cell development and exists in a subset of mice and humans. Importantly, it remains unknown whether the cervical thymus generates an overall repertoire of T cells that are self-tolerant similar to the thoracic thymus. Mice that developed T cells in the absence of the T cell output from the thoracic thymus developed a disease characterized by lethargy, swollen eyes and cachexia. Our data supports that the cervical thymus can function in the absence of the thoracic thymus; however, the T cell repertoire is not fully self-tolerant. Evidence suggests that disease occurs as a result of lymphopenia-driven autoimmunity similar to disease seen after transfer of thymocytes into lymphopenic hosts. Therefore, we used this model to demonstrate that IL-7, an important homeostatic cytokine, can augment disease although the mechanism remains to be elucidated.

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LIST OF SYMBOLS, NOMENCLATURE OR ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
°C	Degree(s) Celsius
Ag	Antigen
AOD	Autoimmune ovarian disease
APC(s)	Antigen presenting cell(s)
APS	Ammonium persulfate
B220	CD45R
BALB/c RAG	C.129S6(B6)-Rag2tm1FwaN12
BALB/c SCID	C.BySmN.CB17 BALB/c SCID/NCR
BCR	B cell receptor
BSA	Bovine serum albumin
BTLA	B and T cell Lymphocyte Attenuator
CD40L	CD40 ligand
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
d3Tx	Day three neonatal thymectomy
DC(s)	Dendritic cell(s)
ddH_2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DN	Double negative
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
EtOH	Ethanol
FasL	Fas ligand
FBS	Fetal bovine serum
FcγR	Fc-gamma receptor
FITC	Fluorescein isothiocyanate
FL(s)	Fetal liver(s)
FLC(s)	Fetal liver cell (s)
g	Acceleration due to gravity
g	Gram(s)
H&E	Hematoxylin and Eosin
ICS	Intracellular Cytokine Staining

IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobulin Alpha
IgG	Immunoglobulin Gamma
IgG1	Immunoglobulin Gamma 1
IgG2a	Immunoglobulin Gamma 2a
IgG2b	Immunoglobulin Gamma 2b
IgG3	Immunoglobulin Gamma 3
IgM	Immunoglobulin Mu
IL	Interleukin
I.P.	Intraperitoneal(ly)
I.V.	Intravenous(ly)
KO	Knockout
L	Litre
LN(s)	Lymph node(s)
LPS	Lipopolysaccharide
M	Molar (mol/L)
min	Minute(s)
MO	Macrophage
mo(s)	Month(s)
MS	Multiple sclerosis
NCI-Frederick	National Cancer Institute at Frederick
NK	Natural Killer cell
NKT	Natural Killer T cell
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PD-1	Programmed death-1
pH	Potential of hydrogen
PMA	Phorbol Myristate Acetate
Poly I:C	Polyinosinic:polycytidylic acid
RAG	Recombination activating gene
RPM	Revolution(s) per minute
RT	Room temperature
RTE(s)	Recent thymic emigrant(s)
SCID	Severe Combined Immunodeficiency
SEM	Standard error of the mean
SLE	Systemic Lupus Erythematosus
SP	Single Positive
SCID	Severe Combined Immunodeficiency
SDS	Sodium dodecyl sulfate

Sec	Second
SP	Single Positive
TCR	T cell receptor
TCR β	T cell receptor beta chain
TdT	Terminal deoxynucleotidyl transferase
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TLR	Toll like receptor
TRA(s)	Tissue restricted antigen(s)
Treg(s)	T regulatory cell(s)
Tx	Thymectomy
WB	Western Blot
WT	Wild type

Mathematical Prefixes Used

m	Milli 10^{-3}
μ	Micro 10^{-6}
n	Nano 10^{-9}

CHAPTER 1: INTRODUCTION

1.1 OVERVIEW OF THE IMMUNE SYSTEM

The immune system is essential for the elimination of foreign pathogens while avoiding a response to self-tissues[1]. Cancer cells are generated when normal self-cells undergo mutations in their genetic material and become immortalized. Mechanisms in the immune system have evolved to recognize and eliminate cancer cells[2] in addition to pathogens such as viruses, bacteria, parasites or fungi[1]. Although the immune system is a highly evolved, complex system encompassing many fail-safe mechanisms it still occasionally malfunctions. Dysregulated immune responses include the generation of autoimmunity (response against self-tissues), or hypersensitivity and allergic responses which are inappropriate immune responses to foreign molecules in the environment that are not pathogenic[3,4]. These responses offer no advantage to the host and can even lead to fatality; however, the reason for these responses remains to be elucidated.

There are two arms of the immune system, the innate and adaptive immune system. The innate immune system is essential to survival and is the first line of defence. It is comprised of physical barriers such as the skin, antimicrobial molecules and cells including natural killer cells (NK), dendritic cells (DCs) and macrophages (MO)[5]. These cells express germ-line encoded receptors that have evolved to recognize foreign pathogens[5,6]. The recognition and response are quite broad and generally recognize essential, conserved motifs that are found in

multiple pathogens. Memory of a response to a particular pathogen does not exist; therefore, if a pathogen tries to re-invade the host the innate immune response will react in the same manner every time[6]. The major benefit of the innate immune response is that the cells and defense mechanisms are able to respond immediately to an invasion.

The adaptive or acquired immune system is the other arm of the immune system. However, it is only present in jawed vertebrates[1]. There are two sub-arms of this immune system. First, there is the cell-mediated immune system comprised of T cells which are able to recognize and eliminate pathogens invading host cells. On the other hand there is the humoral immune system comprised of B cells that produce antibodies that bind and eliminate pathogens extracellular to the host's cells[7]. This response is delayed and can take up to seven days post-infection to generate an adequate immune response; nevertheless, memory is generated after the pathogen is eliminated[7]. Consequently, if the same pathogen tries to re-invade, the host the immune response is quicker and more efficient at eliminating the foreign invader. In addition, the response is highly specific but tremendously diverse (e.g. one T cell recognizes one specific pathogenic peptide).

a) *B cells*

B cells develop and mature in the bone marrow before being exported into the periphery. Each B cell expresses a specific membrane-bound B cell receptor (BCR) which recognizes a single epitope on carbohydrate or protein antigens. Naive B cells primarily express immunoglobulin (Ig)-D and IgM as their BCR[8].

After recognition of antigen by the BCR, the B cell becomes activated and undergoes clonal expansion and differentiation. The B cell can differentiate into memory B cells, effector B cells or plasma cells which secretes soluble antibody. There are several types of antibody isotypes including immunoglobulin mu (IgM), gamma (IgG), alpha (IgA), delta (IgD) and epsilon (IgE). Each isotype has a preferential location, functionality and biological activity[9]. IgG is the most abundant antibody and includes subclasses IgG1, IgG2a, IgG2b, IgG3 and IgG4.

i) Antibody functions

Soluble antibodies are comprised of a variable antigen binding region and a constant Fc portion. They can neutralize microorganisms by binding their cell surface or cause opsonization of the microorganism leading to phagocytosis via the Fc receptors[10]. In addition, antibodies can activate the innate pathway of complement which ultimately leads to the destruction of the microorganism by phagocytosis using complement-mediated receptors or activation of the membrane attack complex (MAC) which can insert and create holes in bacterial membranes[11]. On the other hand, NK cells can bind the antibody via an activating Fc-receptor enabling it to kill the pathogen. Alternatively, antibodies can mediate allergic reaction. IgE has a tendency to bind mast cells, an innate immune cell, by their high affinity Fc-receptors as opposed to remaining soluble in the serum[12]. If these antibodies recognize a non-pathogenic allergen it can lead to crosslinking of the bound IgE causing the mast cell to degranulate and release a multitude of factors including histamine, a major mediator of allergic reaction[13].

ii) Isotype class switching

Under the proper conditions of B cell activation with assistance of $CD4^+$ T cell help, B cells can undergo class switching and secrete a different isotype of antibody. Since different isotypes of antibodies mediate different functions in different locations class switching is essential to a favourable immune response and occurs depending on the cytokines present in the environment[9]. For instance, in the presence of interleukin (IL)-2, IL-4 and IL-5, IgM is generated[14]. However, in the presence of IL-4 alone, it mediates class switching from IgM to IgG1 or IgE[15]. Alternatively, interferon gamma (IFN-γ) leads to switching of IgM to IgG2a or IgG3[14]. Lastly, transforming growth factor beta (TGF-β) is involved in class switching of IgM to IgG2b or IgA[16]. Therefore, the cytokines present and class of antibody being produced can influence the overall immune response.

b) T cells

T cells are involved in the direct recognition of intracellular pathogens by cell-mediated immunity or they can provide assistance to other cell types such as B cells in the immune response[7]. T cell precursors are initially produced from hematopoietic stem cell progenitors in the bone marrow which then migrate into the thymus for further development and education[17]. T cells express a T cell receptor (TCR) involved in recognition of antigen presented in the context of the major histocompatibility complex (MHC)[18-20]. Generation and random re-arrangement of the TCR allows for a large diversity and potential recognition of

essentially any peptides[21]. MHC molecules are polymorphic between individuals. Therefore, T cells are MHC-restricted and can only recognize their cognate peptide in the context of self-MHC molecules.

i) T cell recognition of antigen and activation

Antigens recognized by the TCR are comprised of peptides that are processed and presented on the cell surface in the context of MHC. Classical T cells express either CD4 or CD8 co-receptor molecules in addition to the TCR. CD8 T cells are cytotoxic T cells involved in the recognition of intracellular peptides in the context on MHC I expressed on every nucleated cell[6]. However, CD4 T cells recognize antigen only in the context of MHC II expressed predominantly on antigen presenting cells (APCs) such as DCs[22]. Therefore, in order for a CD4 T cell to become activated the APC has to have picked up the foreign antigen from the environment, processed and presented its cognate peptide in the context of MHC II or pathogen must infect an APC. T cells are MHC restricted such that they can only respond to the MHC molecule expressed by the individual that they are generated in. An exception to this rule is recognition of allogeneic MHC molecules. Around 1-5% of the T cell population responds to a particular allogeneic MHC molecules from a different host (e.g. human donor transplant) or species (e.g. xenograft)[23].

The T cell receptor complex is composed of an array of subunits. The classical TCR itself is composed of an α and β chain which both possess a constant and variable region. The variable region is responsible for the T cell's

specificity to its cognate peptide. A transmembrane domain allows for association of the TCR with accessory molecules that form a TCR complex. The signal-transducing molecule, CD3, is also expressed in the complex in addition to the CD4 or CD8 co-receptor but these molecules have no role in antigen specificity. The CD3 contains an immunoreceptor tyrosine-based activation motif (ITAM) important in signal transduction after the TCR initiates interaction with its antigen[24].

A T cell requires two signals to become activated (**Figure 1.1**). The first signal is recognition of antigen in the context of MHC by the TCR. The second signal required is a co-stimulatory signal[25]. The molecules involved in this co-stimulation signal are CD28 on the T cell and B7 (CD80/CD86) on the APC. Activation of a naive T cell (e.g. a T cell that has never previously seen its antigen) must be by an APC. If a T cell only receives one of the two required signals it will become anergic or unresponsive. Accessory molecules on the T cell such as LFA-1 and CD2 are cell adhesion molecules that bind ligands on the APC to strengthen the interaction of the T cell with the APC. Other accessory molecules such as CD45R are involved in enhanced signalling of the T cell (**Figure 1.2**)[26-28]. Together the molecules involved in T cell signalling and recognition of antigen on the T cell and APC form the immunological synapse.

T cell activation leads to several different outcomes. First, the T cell enlarges and begins to undergo proliferation. In addition, IL-2, a cytokine essential to T cell survival, begins to be produced and the high affinity IL-2R (CD25) is upregulated on the T cell[29]. Along with proliferation, the T cell begins

to undergo differentiation. One cell type produced is effector T cells. $CD8^+$ effector T cells are cytotoxic and involved in the killing of infected target cells with the appropriate antigen presented in MHC I. However, $CD4^+$ effector T cells are involved in cytokine secretion, provide T cell help to B cells for responses such as isotype class switching, or can be directly pathogenic. These populations of T cells are important for eliminating foreign invaders but are short-lived cells. The second cell type produced through differentiation is memory T cells. These cells are long-lived and involved in a quicker, secondary immune response to protect against re-infection without the full requirement of co-stimulation. The two types of memory T cells that exist are effector and central memory T cells[30]. Central memory T cells are similar to naive T cells in the molecules they express and their location throughout the body. Alternatively, effector memory T cells are more likely to home to nonlymphoid tissues[31,32]. Effector memory T cells have been proposed to mediate the effector functions of a secondary T cell response whereas central memory T cells are thought to home to the lymphoid tissues to proliferate and produce new effector T cells upon re-activation[32].

ii) T helper subsets

$CD4^+$ T helper subsets exist that promote different effector functions. They have been designated Th1, Th2 and Th17 and have been grouped based on their cytokine secretion patterns and the presence of certain transcription factors. The initial observation of Th1 and Th2 subsets were made by Mosmann and Coffman (1986) based on the cytokine profiles these cells exhibited[33]. These subsets are

differentiated based on the environment they are in and can skew the immune system towards a certain type of response. For instance, Th1 responses are promoted in the presence of IL-12[34], promote a cell-mediated immune response and secrete cytokines such as IL-2 and IFN-γ. These cell types can promote the recruitment of macrophages and inflammation to the site of infection as well[33]. Th17 cells, on the other hand, differentiate and expand in the presence of IL-6, TGF-β and IL-23 and secrete cytokines such as IL-17, IL-22 and IL-21. These cells are important in inflammation, induce pro-inflammatory cytokine secretion of IL-1, IL-6 and TNF-α and promote the recruitment of neutrophils to the site of infection[35]. Lastly, Th2 cell types are involved in the promotion of humoral immunity to clear pathogens such as parasites and helminths that are extracellular within a host. This cell type is promoted in the presence of IL-4[34] and supports the cytokine production IL-4, IL-5 and IL-13[33].

These Th-subsets can be important and involved in disease as well. For example, the mouse model of Multiple Sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), was long thought to be a Th1-mediated disease. However, recent data has suggested that EAE is in fact a Th17-mediated disease which can be inhibited by neutralizing the Th17 response[36]. In addition, Th2 responses have been linked to allergic reactions and autoimmune disease such as Systemic Lupus Erythematosus (SLE) where disease is mediated by autoantibodies[37]. Therefore, disease can be mediated or promoted by an unbalanced Th1/Th2/Th17 response within the environment of the host. It has become evident that the cytokine secretion patterns of these Th-subsets can vary

according to the disease the host is presented with, and the Th-subsets mediating disease are not always clear[37].

1.2 T CELL TOLERANCE

T cell tolerance is necessary to retain an immune response to infection while preventing self-reactivity. Understanding the mechanisms that elicit tolerance has been at the forefront of research for quite some time now. Historically, Burnet and Fenner first proposed that early in the ontogeny of an organism they possess a tolerogenic window where antigen encounter leads to tolerance[38]. This idea was supported by findings from Owen[39], Hasek[40] and Medawar and colleagues[41]. Although simplistic in nature this proposed idea of an early window of tolerance lost favour based on the awareness that lymphocytes are continuously generated throughout life. Therefore, how would a T cell that is generated late in life become tolerant of self-antigens? As a result, Lederberg proposed that there is a window of tolerance early in development of the T cell as opposed to the organism (central tolerance)[42]. However, this theory did not address the requirement of tolerance in a mature lymphocyte (peripheral tolerance). Therefore, Bretscher and Cohn proposed a two-signal model of tolerance suggesting that activation required two signals: 1) engagement of a lymphocyte receptor with antigen and 2) help from an antigen-specific helper T cell[43]. Consequently, under this proposed theory all of the self-specific helper T cells would have been deleted early in life. Unfortunately, this theory suffered the

chicken and egg dilemma of who helped the first helper T cell? Thus, the mechanisms of peripheral tolerance remained obscured and continue to be at the forefront of research in the field of immunology and tolerance.

a) *Central Tolerance and T cell maturation*

The ability to discriminate between self and non-self is essential to maintaining the ability to mount an immune response against foreign invaders while avoiding immunodeficiency or autoimmunity[44]. Immature thymocytes migrate from the bone marrow, develop and are educated through central tolerance mechanisms in the thymus[45]. T cell precursors enter the thymic cortex and are termed double negative (DN) thymocytes because they do not express CD4 or CD8 co-receptors. These cells begin to slowly proliferate and express markers such as CD25 and $CD44^{low}$ at the DN2 stage. When the cells become DN3 they begin to re-arrange their β chain. The β chain then associates with the pre-Tα chain to form the pre-TCR. The formation of the pre-TCR complex signals that the cell has formed a successful β chain and the cell can continue on with maturation. At the completion of β chain re-arrangement the cell becomes double positive (DP) for CD4 and CD8 and signifies the cell as DN4. The α chain re-arrangement begins after this stage. The few cells that successfully undergo T cell development and express a functional αβ TCR that can signal upon interaction with self-peptide/MHC complexes become single positive (SP) for CD4 or CD8[46].

During development the immature thymocyte must undergo positive selection to ensure the re-arranged TCR is functional and capable of recognizing

self-MHC[45]. This involves interaction of the DP thymocyte with self-peptide/MHC complexes on cortical thymic epithelial cells (cTECs) in the cortex. T cells that are incapable of interacting with self-MHC undergo death by neglect at this stage in development. However, thymocytes express recombination activating genes (RAG)-1/2 and terminal deoxynucleotidyl transferase (TdT), involved in TCR re-arrangement and diversity, at this stage. Consequently, if the TCR is not capable of recognizing self-MHC the cell has a chance to re-arrange the TCR α chain again in an attempt to produce a viable and functional TCR[47,48].

Lastly, thymocytes must undergo negative selection to ensure self-reactive T cells that may have been generated through random TCR re-arrangement do not escape into the periphery. Negative selection occurs in the medulla and cortex and it can occur by macrophages or dendritic expressing MHC I or II or by cTECs and medullary thymic epithelial cells (mTECs)[44]. The mTECs express the transcription factor, autoimmune regulator (AIRE)[49], which allows the mTECs to express an array of tissue-restricted antigens that would not normally occur in the thymus to enhance deletion of self-reactive T cells[50-53]. In fact, mice that are deficient in AIRE develop several autoimmune disorders[54]. Moreover, if a T cell interacts with self-peptide/MHC with a high affinity interaction they undergo clonal deletion whereas low affinity interactions allow the T cell to differentiate and exit into the periphery[55]. If a T cell interacts with intermediate affinity they develop into T cells with a regulatory potential, termed "natural" Treg (**reviewed in peripheral tolerance section iv**).

Superantigens are proteins from bacteria, viruses, or their remnants that are involved in deletion of T cells in the thymus. Certain superantigens are present in the thymus during T cell maturation and induce negative selection of the T cells they bind[56,57]. Binding of a superantigen occurs depending on the specific β chain expressed on the T cell as opposed to being dependent upon antigen since it binds outside of the antigen binding region.

Once this process has been completed the T cell can then migrate into the periphery as a recent thymic emigrant (RTE). Until recently, a T cell exported into the periphery was thought to be fully functional and mature; however, recent evidence suggests that RTEs undergo continued maturation in the periphery[58] (**reviewed in section 1.9**).

b) Peripheral Tolerance

Despite mechanisms of central tolerance in the thymus, autoreactive T cells can escape deletion and enter the periphery. Therefore, mechanisms in the periphery are in place in order to prevent these autoreactive T cells from generating an immune response against self-antigens (Ag). Peripheral tolerance can be mediated by a number of mechanisms[59,60].

i) Co-stimulation

To begin, T cells must receive two signals to become activated as proposed by Lafferty and Cunningham's model[25]. Therefore, the APCs presenting antigen in the context of MHC I or II to the T cell is the first signal; however, a second co-

stimulatory signal received by the T cell from the APC is required. If the T cell receives signal 1 in the absence of signal 2 the T cell becomes unresponsive or anergic. Molecules involved in co-stimulation include B7, OX-40, 41BB and ICOS. However, it remained unclear what controlled these co-stimulatory signals until Janeway and Matzinger proposed solutions to this problem.

Janeway suggested that cells express pattern recognition receptors (PRRs) that recognize essential, conserved motifs or pathogen-associated molecular patterns (PAMPs) that are absent from the host and signify an infection inducing APCs to upregulate co-stimulatory molecules[61,62]. This model gained support with Hoffman's discovery of the function of Tolls in Drosophila followed by the independent discovery of their human homologs, Toll-like receptors (TLRs) that recognize different PAMPs[63,64]. On the other hand Matzinger proposed that instead of recognition of PAMPs by APCs, receptors instead recognize "danger signals" due to tissue damage and destruction from infection. This idea offered an explanation for observations such as transplant rejection which have no PAMPs, immunity versus tolerance to self-tumors and the ability to co-exist with our normal gut microbiota without a destructive immune response. This model became widely known as the "Danger model" and proposed that stress and injury causing the release of endogenous danger signals activate APCs rather than self/nonself discrimination playing a key role[65]. These molecules have been referred to as danger-associated molecular patters (DAMPs). But, neither of these models fully accounts for all situations of T cell activation and likely

encompasses aspects from both models since both PAMPs and DAMPs have been discovered.

ii) Co-inhibition

Models of co-stimulation propose that co-stimulatory molecules are required to activate a T cell by sending a positive signal. In the absence of these molecules engagement of the TCR with its antigen becomes a negative signal since the T cell become unresponsive. However, Sinclair proposed that instead there are co-inhibitory signals[66,67] and that activation or inhibition of the T cell is a balance of these positive and negative signals received[68]. While Sinclair's model was originally based on B cells and antibody-mediated inhibition through Fc-receptors numerous co-inhibitory molecules have been discovered in recent years involved in T cell regulation. For instance, co-inhibitory molecules such as Cytotoxic T Lymphocyte Antigen-4 (CTLA-4), Programmed Cell Death-1 (PD-1), B and T Lymphocyte Attenuator (BTLA) have been discovered along with a vast array of others. Many of these receptors share similar ligands as co-stimulatory molecules and vary in their expression patterns[69]. CTLA-4 on T cells, for example, recognizes the same ligands as CD28: B7-1 and B7-2 (CD80 and CD86). Although co-inhibitory molecules were initially proposed to send a negative signal through the T cell, evidence has emerged suggesting that this is only one mechanism of action of inhibition. In fact, CTLA-4 has been shown to remove the co-stimulatory molecules, CD80 and CD86, from the APC's surface rendering it less capable of activating naive T cells[70]. A number of co-inhibitory molecules

also play a role in regulation by regulatory T cells in addition to interactions between the T cell and APC[59].

iii) Ignorance

Zinkernagel put forth the idea that tolerance to antigens is largely a result of ignorance by the T cells[71]. If antigen does not reach the lymphoid tissue it will not be presented on MHC by APCs and thus T cells will remain ignorant to the antigen. However, if antigen reaches the lymphoid tissues infrequently at low amounts it will be loaded onto the APC where a T cell can generate an effective immune response against that antigen and exit into the periphery. Alternatively, if antigen is present in large, continuous quantities it will be presented by APCs for a sufficient amount of time to induce tolerance by deletion either centrally or peripherally.

iv) T regulatory cells

Regulatory T cells are responsible for mediating a suppressive form of tolerance. They maintain self-tolerance by suppressing autoreactive T cells that may be present in the periphery or to dampen the immune response[72]. Forkhead box P3 (Foxp3) is an important transcription factor present in Tregs[73-75]. Its importance is exemplified by the mutant *scurfy* mouse which lacks a functional *Foxp3* gene subsequently leading to fatal lymphoproliferative immune-mediated disease affecting multiple organs and tissues[76]. Tregs can be distinguished by their constitutive expression of the high affinity IL-2R (CD25) in addition to Foxp3[77].

Furthermore, a deficiency in Treg numbers has been implicated in several autoimmune diseases in both mice and humans[72,78,79]. "Natural" Tregs are generated in the thymus[72] by positive selection of intermediate affinity TCR:MHC/self-peptide interactions and then exported into the periphery[80-83]. On the other hand, "induced" Tregs are induced to express Foxp3 in the periphery, stemming from $CD4^+$ T cells that were exported from the thymus as naive, non-Tregs[59]. Suppression of effector T cells by Tregs can be mediated through cytokines such as IL-10 or TGF-β, or by cell contact via negative regulation through the APC or T cell[72]. Maintenance and expansion of Tregs in the periphery occurs through interactions with APCs expressing MHC molecules on their surface[84-86]

1.3 AUTOIMMUNITY

The immune system is tightly regulated to allow immune responses to foreign antigens while maintaining tolerance to self-tissues. However, under certain circumstances autoimmunity can occur and the host's own immune system recognizes and attacks self-tissues as foreign. There is a wide array of autoimmune diseases classified as systemic or organ-specific. Autoimmune diseases can be predominantly T cell or B cell-mediated, although there is a broad spectrum of cells and factors involved in each disease.

a) B cells

Autoreactive B cells and the antibodies they produce play an important role in multiple autoimmune diseases. These include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and idiopathic thrombocytopenic purpura (ITP). Several mechanisms are known by which autoreactive B cells can become pathogenic in the setting of autoimmunity. Antibodies can have direct cytotoxic effects where autoantibodies can bind complement leading to cell lysis, disrupt neuronal synapses causing impaired neuronal function, or form immune complexes that deposit in different tissues and generate an inflammatory response consequently leading to organ destruction[87]. B cells themselves can contribute to pathogenicity. For instance, the B cell can become activated upon recognition and crosslinking of T cell-independent antigens by the BCR, or can become activated by recognition of T cell-dependant antigens with $CD4^+$ T cell help. Since B cells express MHC II they can also act as APCs and activate T cells if their cognate peptide is presented[88]. Furthermore, B cells also have the ability to take part in forming ectopic lymphoid structures in organs that are not lymphoid in origin[87].

b) T cells

Similar to B cells, autoreactive T cells play a crucial role in autoimmune diseases such as Type 1 Diabetes (T1D) and MS. Both $CD4^+$ and $CD8^+$ can mediate damage during autoimmunity. How $CD4^+$ T cells mediate tissue destruction and autoimmunity without the requirement of $CD8^+$ T cells or B cells remains to be elucidated.

There have been several potential mechanisms put forth for how $CD4^+$ T cells could be mediating this action. Granzyme B is a potential mechanism of $CD4^+$ -mediated cytotoxicity[89]. Granzyme B is a cytolytic molecule delivered to the target cell inducing cell death by apoptosis[90]. Fas (CD95) and Fas ligand (FasL/CD95L) are expressed on the cell surface where interaction of the two molecules leads to apoptosis in the Fas-expressing cell[91]. FasL is transiently expressed on activated T cells and can act as a cytotoxic mechanism to kill target cells[91]. Organs such as the liver, heart and ovary can express Fas; therefore, these cells are potential targets of $CD4^+$-mediated cytotoxicity during autoimmunity[92]. Furthermore, aberrant expression of MHC II has been detected in the thyroid, pancreas, colon and stomach which could facilitate $CD4^+$ T cell responses[93-96]. Therefore, the role of Fas:FasL interactions have been studied as a potential mechanism of $CD4^+$-mediated autoimmunity. For instance, this interaction and Fas upregulation of gastric parietal cells is important in the pathogenesis of autoimmune gastritis in mice[97,98]. In addition, gastric parietal cells have been shown to be positive for MHC II and ICAM-I which are important for the interaction with $CD4^+$ T cells in the setting of certain autoimmune diseases[98].

Another possibility is that self-reactive $CD4^+$ T cells may be more prone to spontaneous activation compared $CD8^+$ T cells. This may be because of their TCR repertoire, MHC II restriction, or ability to respond to cytokines[99]. $CD4^+$ T cells can mediate autoimmunity through the release of cytokines such as IL-17 or IFN-γ leading to tissue damage[3]. Another possibility where $CD4^+$ T cells can be pathogenic is by providing T cell help directly to autoreactive $CD8^+$ T cells[100].

The interactions of CD40:CD40L, a set of co-stimulatory molecules, between a $CD4^+$ T cell and a DC can allow licensing of the DC[99,101] consequently leading to autoreactive $CD8^+$ T cells responding and becoming directly pathogenic.

1.3 HOMEOSTATIC PROLIFERATION

Homeostatic proliferation is important for regulating and maintaining the peripheral T cell pool[102]. It occurs when low numbers of T cells proliferate and expand to fill up the "empty space" in a lymphopenic environment within a host. Space in a lymphopenic host can be defined in several different ways. It could be defined as the total number of T cells in the environment, the physical space in the peripheral lymphoid organs, or the ratio of T cells to APCs or lymphoid stromal cells[103]. However, the space in a host during lymphopenia may encompass some or all of these factors. A decrease in T cell numbers causing lymphopenia can occur through mechanisms such as viral infection, irradiation, the use of cytotoxic drugs, or genetic mutations[104].

There are several requirements for homeostatic expansion to take place. It is widely accepted that there is a requirement for naive T cells to interact with self-peptide/MHC in the periphery to undergo homeostatic expansion[105-112]. These self- peptides that T cells interact with in the periphery are thought to be the same ligands mediating positive selection in the thymus[106,113]; however, some studies do not agree[108,112]. Furthermore, homeostatic expansion is absent or impaired in

$CD4^+$ T cells in the absence of MHC II and $CD8^+$ T cells in the absence of MHC I[114,115]. Unlike antigen-specific activation of naive T cells, the homeostatic expansion of a naive T cell does not require co-stimulation[106,116,117] or IL-2 signalling[116,118]. On the other hand, the cytokine interleukin (IL)-7 is required for homeostatic proliferation and/or survival[119].

Several mechanisms of proliferation exist. For example, $CD4^+$ T cells can homeostatically proliferate through an IL-7-dependant (slow proliferation) pathway or undergo spontaneous proliferation through an IL-7-independent but gut flora-dependant (rapid proliferation) pathway[120]. Naive T cells require IL-7 and "low affinity" self peptide/MHC interactions while memory T cells rely on IL-7 and IL-15 without the requirement for self peptide/MHC interations[104,106,121]. Normally, naive T cells that undergo homeostatic proliferation acquire a memory phenotype and function[122]. The memory phenotype and function gained is that of an effector memory T cell. These cells can have low expression of CD62L (L-selectin), required for entry into the high endothelial venules (HEV) in the LN, and high expression of CD44, an adhesion molecule required for entry into peripheral sites[103]. The fate of these memory T cells alters depending on the model system. For instance, in sublethally irradiated mice naïve T cells homeostatically expand, become effector memory T cells and ultimately revert back to a naïve T cell phenotype[123]. However, in $Rag^{-/-}$ mice the T cells retain their gained memory T cell phenotype[110].

Although homeostatic proliferation can maintain and restore the peripheral T cell pool, the addition of lymphopenia can lead to the generation of autoimmune

disease[124,125]. The expansion of T cells through homeostatic proliferation ultimately allows naive T cells to gain effector functions in the absence of antigen-specific interactions, co-stimulation or danger signals. Lymphopenia-induced autoimmunity is reviewed in **section 1.6**.

1.4 INTERLEUKIN-7

Interleukin-7 is a cytokine important in homeostatic proliferation and homeostasis of T cells[126,102]. It is produced mainly by stromal cells and in small amounts by dendritic cells, but T cells, B cells and NK cells are not capable of producing IL-7[127]. T cells require IL-7 as well as signalling through the TCR for survival in an environment filled with high competition for self-peptide/MHC interactions between T cells[102,128]. It is essential to the survival of naive T cells in the periphery whereas memory T cells are maintained by the presence of IL-7 and IL-15[102]. In the thymus, IL-7 is required for T cell development since the absence of IL-7 leads to the development of T cells that do not fully mature or exit into the periphery[129-131]. It is also important for the expansion of T cells in the periphery[129,132] as well as pre-B cells in mice[133-135]. In humans, however, IL-7 is only important for T cells and not B cells[136-139]. Furthermore, IL-7 is one of the few cytokines that has been shown to be effective in stimulating the proliferation of memory $CD4^+$ T cells along with naive $CD4^+$ and $CD8^+$ T cells compared to most other cytokines involved in T cell expansion that are limited to the

expansion of memory T cells or memory $CD8^+$ T cells[134]. Despite the effect of IL-7 on memory T cells, IL-7 preferentially expands recent thymic emigrants[140,141] over naive T cells[142,143] and naive T cells over memory T cell which allows for an increase in the TCR repertoire diversity. As opposed to the above populations, IL-7 has minimal effects on proliferation of mature B cells and thymocyte populations[135,144,144-147]. A schematic of IL-7R signalling is shown in **Figure 1.3**.

a) The IL-7 Receptor

The IL-7R is composed of the IL-7Rα subunit along with the common γ chain[148]. It is expressed on DN and SP thymocytes[149], naive T cells[150] where the majority of naive $CD4^+$ and $CD8^+$ T cells are IL-$7R^{hi}$ [151], activated and memory T cells[150], and low levels on RTEs[152] and Tregs[134,150,153]. However, for unknown reasons IL-7 has a more potent effect on $CD8^+$ T cells compared to $CD4^+$ T cells[154]. When IL-7 signals through the T cell it ultimately leads to proliferation. This can occur in the absence of TCR signalling in RTEs, or can act as a co-stimulatory signal in naive T cells inducing homeostatic proliferation of these T cells in presence of "low affinity" self-peptide/MHC complexes[106,113,154]. Finally, when T cells become activated the IL-7R gets down regulated from the cells surface to inhibit further IL-7 signalling[154]. Interleukin-7 receptor expression is shown in **Figure 1.4**.

b) Mechanism of Action

There are multiple effects of IL-7 on both $CD4^+$ and $CD8^+$ T cells. $CD8^+$ cytotoxic T lymphocytes were shown to have increased cytolytic activity, increased granzyme B expression, and faster rates of degranulation[155]. In contrast,

$CD4^+$ T cells favour Th17-skewing and secrete increased IL-17 when treated with IL-7[155]. Furthermore, the addition of IL-7 leads to increased serum levels of IL-6, IL-12, IL-17 and IFN-γ in mice[143].

The IL-7R on $CD4^+$ Tregs is expressed at very low levels[150,153,134]. Therefore, IL-7 may lead to an enhanced T cell response because of a lower expansion of Tregs during proliferation compared to other T cells decreasing their capacity to suppress[134,156]. Another reason for an increased T cell response could be that T cells become refractory to inhibition by Tregs when they are treated with IL-7[155]. In this case, when Tregs are pre-treated with IL-7 they are still capable of suppressing PBS-treated T cells[155]; therefore, the effect of IL-7 that is mediating the inability to suppress is a direct response to changes on the T cell as opposed to the Treg. In addition, $CD8^+$ T cells treated with IL-7 were shown to be resistant to TGF-β-mediated inhibition.[155] A decrease in PD-1 on T cells, and TGF-β II receptor and Cbl-b expression on $CD8^+$ T cells after IL-7 may also contribute to preventing their inhibition[155].

c) IL-7 as a Therapeutic Agent

Administration of IL-7 is capable of expanding T cells to re-establish homeostasis after a state of lymphopenia[134,144]. In different cases IL-7 has been shown to expand T cells through thymic-dependant or thymic-independent pathways[157]. However, many studies have found IL-7 to have a predominant effect on thymic-independent pathways leading to a large increase of T cells in the spleen and

lymph nodes (LN) in the absence of or with minimal effects on thymus and bone marrow in a lymphopenic environment[134,140,144,158].

IL-7 is becoming a favourable treatment option to restore T cells numbers in patients who have become lymphopenic from cancer treatment, Human Immunodeficiency Virus-1 (HIV-1) or when they undergo transplantation immunosuppression to alleviate their immunodeficiencies[149]. In fact, IL-7 treatment can increase the TCR repertoire diversity by preferential expansion of naive T cells subsets[142]. Therapeutic treatment using IL-7 has been shown to have the ability to enhance T cell recovery in HIV-1-infected patients[159,160]. Currently, clinical trials are under way for assessing the use of IL-7 in cancers such as melanoma, as well as HIV infection, Hepatitis C Virus (HCV) infection, and after hematopoietic stem cell transplantation[154].

The limited toxicity and low doses of IL-7 required makes it a favourable treatment option. This is compared to the use of IL-2 which has been approved by the United States Food and Drug Administration (FDA) for treatment in metastatic cancers, but has to be administered at very high doses to be effective which often leads to severe side effects[161,162,163]. However, IL-7 and the IL-7R have been shown to be involved in and linked to several diseases. Variations in the IL-7R have been implicated in MS[164], and IL-7 has been linked to immunopathology in arthritis[165], colitis[166], and dermatitis[167]. Prior to bone marrow transplantation, the patient is rendered lymphopenic, so treating the patient with IL-7 after transplantation could provide a quick and effective way of increasing T cell numbers after transplantation. However, the use of IL-7 in mice receiving

allogeneic bone marrow transplants actually worsens graft versus host disease[151]. In conclusion, the use of IL-7 as a therapeutic should be used with caution since it has the ability to benefit patients or be detrimental to their recovery.

d) IL-7/Anti-IL-7 Complexes

Using IL-7/anti-IL-7 antibody complexes (**Figure 1.5**) is more effective at lower doses in mice compared to recombinant IL-7 alone[134,168]. This is also the case for the use of cytokine/anti-cytokine antibody complexes for IL-2, IL-3, IL-4, or IL-6[162]. The reason for the increased biological activity of cytokine/anti-cytokine antibody complexes compared to the cytokine alone has not been fully elucidated. However, the specific clone of antibody and the presence of the Fc portion of the antibody are critical[134,169]. Although the Fc portion of the antibody is required for the increased biological activity the mechanism of action is not mediated through the Fc-gamma receptor (FcγR)[170,171]. Evidence suggests that the cytokine must first dissociate from the antibody to have an effect, and if there is excess antibody around it will neutralize the cytokine and render it ineffective[170]

1.5 INTERLEUKIN-15

IL-15 is important for the homeostatic proliferation and/or survival of memory T cells, NKT cells and NK cells[172,173]. IL-15 bound to an IL-15Rα complex can be presented and interact with the IL-2/IL-15Rβ and the common gamma chain on a

cell's surface[174] to induces expansion of $CD122^{hi}CD8^{+}$ memory T cells and NK cells in vivo but not naive T cells[175,176].

1.6 LYMPHOPENIA-DRIVEN AUTOIMMUNITY

Lymphopenia can occur when T cells become depleted in a host's immune system. It can occur due to viral or bacterial infection, stress, chemical induction such as anti-cancer treatments, immunosuppression after transplantation or age-dependent thymic involution[124,125]. Correlations between lymphopenia and increased risks of autoimmunity have been made in both humans and mice[125]. An example of lymphopenia-induced autoimmunity is after day 3 neonatal thymectomy (**reviewed in section 1.7**) where thoracic thymectomy[177-179] causes lymphopenia leading to homeostatic proliferation of T cells and autoimmunity in some cases[180,181]. Other diseases where lymphopenia can act as a factor in the generation of autoimmunity are inflammatory bowel disease[182] and type 1 diabetes[125]. By itself, lymphopenia is not sufficient to generate autoimmunity[125]; however, the preferential homeostatic expansion of T cells that interact with a higher affinity to "low affinity" self-peptide/MHC may selectively increase the proportion of autoreactive T cells in the T cell repertoire[113,124]. In fact, some data suggests that autoreactive $CD4^{+}$ T cells in the periphery recognize and proliferate in response to low affinity self-peptide/MHC ligands[183]. However, not all T cells are capable of undergoing homeostatic proliferation[106]. Of the T cells that are

capable of undergoing homeostatic proliferation the T cells with the highest affinity for self-peptide/MHC preferentially proliferate and survive[184]. Therefore, under certain circumstances homeostatic proliferation in a lymphopenic environment consequently leads to the development of autoimmunity.

1.7 DAY THREE NEONATAL THYMECTOMY

The phenomenon of day 3 neonatal thymectomy (d3Tx)-induced autoimmunity was first described in 1969 by Nishizuka and Sakakura as ovarian dysgenesia[185]. It occurs when mice are thoracically thymectomized between days 2 to 4 of birth leading to the generation of an autoimmune disease in a large proportion of these mice[179,186]. The highest incidence of autoimmune disease occurs when mice are thoracically thymectomized on day 3 after birth[187]. Autoreactive $CD4^+$ T cells mediate the disease[178,188-190], but the mice that develop an autoimmune disease and the organs affected after d3Tx are largely strain-dependant[191]. There is no correlation between susceptibility to autoimmunity induced by d3Tx and MHC haplotype, but data suggests that genetic susceptibility to disease may fall outside of the H-2 locus[192,191].

BALB/c and (C57BL/6 x A/J)F1 (B6AF1) mice are highly susceptible, but C57BL/6 mice are fairly resistant to d3Tx-induced autoimmunity[191]. The organs affected by disease include the stomach, ovary, thyroid, prostate, pancreas, lacrimal gland, and testis[191,193]. Autoimmune ovarian disease (AOD) and dacryoadenitis occur predominantly in B6AF1 female mice[189] whereas BALB/c

mice tend to generate gastritis[179,191]. In addition, environmental factors such as gut flora are not required for disease since germ-free mice are still susceptible to the development of autoimmunity[194].

The stomach is the main target of disease in BALB/c mice which generates gastritis characterized by submucosal mononuclear infiltrates and selective loss of parietal and chief cells[179,186,195]. The H+/K+ ATPase in the stomach are a main target of autoimmune gastritis[196,197]. Autoantibodies from B cells targeting the H+/K+ ATPase are generated during the disease process[186,198,199]; however, sera containing these autoantibodies alone cannot transfer disease to a new host. On the other hand, the transfer of splenocytes into a new immunodeficient, lymphopenic host can generate disease, although inefficiently (>10 weeks)[200,201]. In fact, T cells that respond to the H+/K+ ATPase are critical for the progression of disease[188,201], and a T cell clone that proliferates in response to a peptide derived from the H+/K+ ATPase α subunit was isolated in 1994[202].

There have been several theories proposed for why autoimmunity occurs after day 3 neonatal thymectomy. These ideas include: 1) thoracic thymectomy of a mouse between days 2 to 4 after birth enriches for autoreactive T cells that escape deletion in the thoracic thymus and 2) d3Tx depletes Tregs which would normally suppress disease.

a) Central Tolerance

A defect in central tolerance has been implicated in d3Tx-induced autoimmunity allowing autoreactive T cells to escape deletion in the thymus, enter the periphery and generate disease under the conditions of d3Tx. The neonatal thymus is inefficient at mediating clonal deletion allowing for the escape of potential autoreactive T cells[203-205]. For example, adult BALB/c and B6AF1 mice have minimal numbers of Vβ 11 T cells in the periphery; however, Vβ 11 T cells are readily detected in the thymus and spleen of neonatal mice for the first 10 days of life and d3Txed mice have a 10-fold increase in the proportion of these cells[203].These Vβ 11 cells found in the periphery may represent a potential population of autoreactive T cells. In neonatal mice and d3Tx mice these cells are nonresponsive to stimulation suggesting they may be undergoing anergy in the periphery[204]. A second example of a defect in deletion by the neonatal thymus is the premature escape of DP thymocytes into the periphery which could also represent a potential population of autoreactive T cells[206]. This is further supported by the finding that expressing a single autoantigen, the β subunit of the H+/K+ ATPase, under the MHC II I-E^{k}_{α} promoter in the thymus completely abrogates disease[188]. Therefore, a defect in central tolerance in the neonatal thymus may be a contributing factor to the development of d3Tx-induced autoimmunity.

Only under certain conditions does the normal thymus have the capacity to generate autoimmunity. A condition where this occurs is after the transplantation of a newborn thymus (days 0-1) or an adult thymus into an immunodeficient

BALB/c nude mouse[207] which lacks functional thymic disease due to a mutation in the *Foxn1* gene[208,209]. Transplantation of the newborn thymus generates autoimmunity similar to d3Tx within 5 months (mos), but the adult thymus generates autoimmunity less efficiently[207]. However, under both circumstances if the thymus is irradiated prior to transplantation it induces efficient autoimmunity[207]. In contrast, for the thymus to be capable of initiating autoimmunity in a normal immune sufficient host, the host first requires prior thymectomy and irradiation to induce lymphopenia[207]. Therefore, mature T cells or certain T cells in the normal T cell repertoire may be inhibiting activation of self-reactive T cells[207].

b) Regulatory T cells

There has been speculation that thymectomizing neonatal mice between days 2-4 depletes Tregs causing a deficiency in peripheral tolerance and allowing autoreactive $CD4^+$ T cells that escape thymic deletion to generate autoimmunity. The late ontogeny of Tregs[210] and inability to detect Tregs in the thymus or spleen of mice until 5 days after birth along with the ability of d3Tx to be suppressed by transfer of Tregs[180,211] supports this hypothesis. However, adoptive transfer of Tregs from normal mice is only effective at preventing d3Tx-induced autoimmunity when given prior to 10 days of age[212].

There has been controversy over the idea that d3Tx-induced autoimmunity is the result of a deficiency in Tregs. However, the functionality and presence of Tregs in the d3Tx model have been shown to be present in

several findings[213,214] even though there is a delayed ontogeny in their development[210]. Dujardin *et al*. (2004) found that the percentage of $CD4^+CD25^+$ regulatory T cells expressing Foxp3 in the spleens of d3Txed BALB/c mice was increased compared to untreated BALB/c mice (>20% compared to <10%, respectively)[213]. In accordance with this finding, Tregs have been shown to be capable of proliferating and out expanding effector T cells in a lymphopenic environment[215]. This group showed that the Tregs from d3Txed mice were capable of suppressing T cell proliferation during anti-CD3/CD28 stimulation comparable to Tregs from untreated mice, and were able to prevent the development of colitis in a disease model[213]. Unfortunately, they did not directly assess the ability of Tregs to suppress d3Tx-induced autoimmunity. Moreover, it has been argued that since colitis does not occur after d3Tx or in germ-free mice that it is not an ideal model for demonstrating suppression of autoimmunity by d3Tx-Tregs [216]. However, Samy *et al*. (2004) used B6AF1 mice that develop AOD and dacryoadenitis to show that when they undergo d3Tx, Tregs greatly out expand effector T cells and suppress disease when adoptively transferred to a new mouse that underwent d3Tx[214]. To support the premise that Tregs are both functional and present after d3Tx, while they are not detectable in the spleens of neonatal mice until 5 days after birth[210] they are detectable in the thymus and LNs of 3 day old mice[214]. Furthermore, Tregs that are disease-specific exist since adoptively transferred Tregs from the ovarian LN of mice that develop d3Tx-induced autoimmunity preferentially suppress AOD in a new d3Tx recipient and Tregs from the lacrimal gland LN preferentially suppress dacryoadenitis[214].

Functionality of these Tregs was further substantiated by the fact that in vivo depletion of Tregs by anti-CD25 enhances disease supporting the idea that these Tregs are in fact suppressing disease in vivo[214].

Samy *et al.* (2004) have suggested several reasons for why d3Tx-induced autoimmunity may or may not occur in the presence of Tregs. Although Tregs can out expand T effector cells there is recent evidence that a requirement for Tregs to interact with dendritic cells to control autoimmunity exists[217]. Therefore, the lymphopenic environment of a neonatal d3Txed mouse may reduce the effectiveness of Tregs due to the decreased T cell:DC ratio. Second, Aire is a transcriptional regulator of the expression of a multitude of tissue-specific autoantigens in the thymus[54,218]. *Aire* KO mice develop a similar autoimmune disease to d3Tx-induced autoimmunity[54,219,220] which may suggest that these disease mechanisms are similar as opposed to the very dissimilar disease of the *scurfy* mouse which lacks Tregs[221]. For that reason, d3Tx-induced autoimmunity is likely the result of more than just a defect in Treg control.

c) Lymphopenia

Neonatal mice represent a lymphopenic environment where T cells can undergo homeostatic expansion, which is enhanced when mice undergo thoracic thymectomy at day 3 of life[181]. This expansion is independent of IL-7 but requires the presence of MHC II and CD28[181]. A diverse Vβ repertoire of T cells is generated by the expansion[181] and occurs over a three week span in d3Tx mice [213]. At day 4 after thymectomy mice have 10^6 splenocytes and at four weeks post-Tx

mice have 10^8 splenocytes[201]. This suggests that homeostatic expansion increases the number of T cells since it occurs in the absence of T cell output from the thoracic thymus. To further support this, neonatal T cells are capable of undergoing homeostatic proliferation in an adult lymphopenic mouse similar to adult T cells[222].

Cyclosporin A is a drug which induces a state of lymphopenia by binding cyclophilins and inhibiting the activation of lymphocytes such as $CD4^+$ and $CD8^+$ T cells[223]. Daily administration for one week to newborn mice can lead to autoimmunity but not in adults, and disease is enhanced by thymectomizing these mice[224]. Supporting a role for the importance of lymphopenia in d3Tx-induced autoimmunity, inoculation of these mice with normal splenic T cells is capable of preventing disease[224]. Transplanting the thymus from mice treated with cyclosporin A into lymphopenic BALB/c nude mice, which have no functional thymic tissue, is capable of generating autoimmunity in the recipient[225]. Inoculation of these mice with normal splenocytes (25-30% T cells) prevented disease; however, inoculation with splenocytes from cyclosporin A treated mice (very few to no T cells) or neonatal splenocytes at days 1-3 after birth (less that 5% T cells) was not capable of suppressing the generation of autoimmunity[225]. Although cyclosporin A is not able to induce organ-specific autoimmunity in adults, treatment of BALB/c mice in addition to high dose or fractionated total lymphoid irradiation was able to break self-tolerance and lead to autoimmunity similar to d3Tx-induced autoimmunity[226]. This autoimmunity was prevented by the transfer of normal splenic T cells as well, and was strain-dependent[226].

A lymphopenic environment is not the sole explanation for d3Tx-induced autoimmunity since day 7 neonatal mice have a similar lymphopenic environment and Treg enrichment after Tx, yet they do not develop disease[227]. Furthermore, depletion of $CD25^+$ cells as a way to deplete Tregs in neonates that are 3 days old is not sufficient to induce autoimmunity. Although, transfer of CD25-depleted splenocytes from day 3 neonatal mice are capable of generating disease in a lymphopenic mouse, suggesting that a second signal, such as non-specific expansion of T cells or increased T cell activation, is necessary to generate disease[228]. Therefore, the lymphopenic environment of a neonatal mouse that has undergone thoracic thymectomy at day 3 after birth may act as a co-factor for autoimmune development.

d) Factors Affecting Disease

Several factors have been implicated in the prevention or augmentation of autoimmunity generated by day 3 neonatal thymectomy. Toll-like receptors (TLRs) are germ-line encoded innate immune receptors important for the recognition of PAMPs from microbial or host-derived sources during infection or tissue damage[229]. One such PAMP is polyinosinic:polycytidylic acid (Poly I:C) which mimics viral double-stranded RNA and is recognized by TLR 3 located on B cells, macrophages and DCs[230]. Recognition of poly I:C by TLR 3 can evoke an inflammatory response involving the production of IFN-γ[230]. BALB/c mice that are thymectomized at day 3 after birth and receive poly I:C during the first or second week post-Tx develop moderate to severe gastritis compared to PBS

treated mice that undergo d3Tx[231]. The administration of poly I:C leads to increased serum IL-6, IL-12p70, IFN-γ, TNF-α and causes a decrease in the frequency of Tregs[231]. This enhancement of disease may be attributed to the IFN-γ response that is important in disease. Treating mice with a neutralizing antibody to IFN-γ weekly for 6-12 weeks reduced the rate of gastritis from 69% to 16% in d3Txed BALB/c mice, and even a single dose of neutralizing IFN-γ antibody post-Tx could prevent disease[232]. On the other hand, although papers suggest that d3Tx-induced autoimmunity is a Th1-mediated disease Maity *et al*. (1997) suggest that it is a Th2-mediated disease and go on to show that splenic T cells from d3Txed mice have decreased levels of IFN-γ and increased levels of IL-4[233]. Therefore, it remains controversial as to whether disease is Th1- or Th2-mediated, but it could also potentially be mediated by a different cell type such as Th17 cells.

Many autoimmune diseases are due to a deficiency in regulation by Tregs. Cytokines such as IL-10 and TGF-β are important in Treg-mediated inhibition of effector T cells[234]. IL-10 can be produced by a multitude of cells including monocytes and T cells[235], and IL-10 knockout (KO) mice are more susceptible to certain autoimmune diseases[235]. Therefore, to determine the importance of IL-10 in the regulation of d3Tx-induced autoimmunity Suri-Payer *et al*. (2001) used IL-10 KO mice to show that these mice do not succumb to a more severe form of autoimmunity, and IL-10 is not required for protection from d3Tx-induced autoimmunity[236]. However, giving d3Txed mice IL-10-producing regulatory DCs

is able to alleviate disease, which may be attributed to a decrease in IFN-γ, IL-12p70 and an increase in IL-10-producing Tregs[237].

Activation of a T cell requires not only TCR:peptide/MHC interactions with an APC, but also a second signal of co-stimulation such as the interaction of CD40 ligand (CD40L) on the T cell and CD40 on the APC[238]. Targeting the CD40:CD40L interaction to prevent autoimmunity is therefore a potential treatment option. However, the mechanism of action of this treatment remains to be determined. The simplest explanation for the mechanism of action of CD40L blockade is that it blocks the co-stimulation signal required for full T cell activation[239]. However, in some cases CD40L blockade has been shown to enhance $CD4^+CD25^+$ Treg activity[240,241], and others suggest that it is an Fc-mediated mechanism of action which leads to the depletion of activated T cells[242]. Sharpe *et al.* (2003) determined that continuous CD40L blockade could prevent autoimmunity after d3Tx, blockade confined to the neonatal period was ineffective, and blockade starting at 4 weeks post-Tx caused regression of autoimmunity[243]. Blocking CD40L in mice that undergo d3Tx leads to a decrease in CD69 and CD25 activation markers as well as a decrease in IFN-γ production in splenocytes that were re-stimulated ex vivo[243]. Therefore, the interaction of CD40:CD40L is important in d3Tx-induced autoimmunity.

Natural Killer T cells (NKT) are an innate-type of T cell that recognizes foreign or self-peptides within CD1d-molecules[244,245]. These cells have been shown to have therapeutic potential in autoimmune diseases and defects have been associated with increased susceptibility to autoimmunity and cancer[244,245].

Furthermore, NKT cells have the potential to protect against Th1-mediated autoimmunity[246-248]. The role of NKT cells in d3Tx was studied in 1998 by Hammond *et al*. who found that NKT cells do not appear in the mouse until 7-14 after birth suggesting a potential role in protection from d3Tx-induced autoiummunity[249]. They found that d3Tx depletes NKT cells ($NK1.1^+$ T cells) in the liver, spleen and bone marrow of (BALB/c x C57BL/6)F1 more so than in C57BL/6 mice which are resistant to autoimmunity generated by d3Tx[249]. Therefore, NKT cells may be necessary in the neonate to protect against autoimmunity.

1.8 TRANSFER OF T CELLS INTO A LYMPHOPENIC ENVIRONMENT

Transfer of adult thymocytes, and neonatal thymocytes or splenocytes (less than 7 days of age), but not adult splenocytes are capable of generating autoimmunity (73% incidence) in lymphopenic BALB/c nude or severe combined immunodeficient (SCID) mice similar to that generated by d3Tx[189]. Disease induced by these transfers is dependent upon $CD4^+$ T cells but not $CD8^+$ T cells, and cotransfer of normal adult splenocytes is sufficient to prevent disease[189]. Previous data suggested that the inability of the neonatal thymus to delete Vβ 11 T cells which are normally deleted in the adult thymus may contribute to disease in d3Tx-induced autoimmunity. However, the transfer of neonatal thymocytes depleted of Vβ 11 cells into an immunodeficient, lymphopenic mouse is not

sufficient to prevent disease. This indicates that a lack of deletion of these cells either do not play a role in or does not fully explain the reason for d3Tx-induced autoimmunity[189].

Certain subsets of $CD4^+$ T cells are responsible for disease associated with the transfer of T cells into a lymphopenic host without a requirement for $CD8^+$ T cells or B cells (summarized in **Figure 1.6**)[236,250,251]. For instance, SCID mice inoculated with $CD45RB^{high}$ $CD4^+$ T cells generate chronic wasting disease[251-253] associated with mononuclear infiltrates in the colon and elevated levels of IFN-γ mRNA[252]. However, $CD45RB^{low}$ $CD4^+$ T cells are not capable of generating disease and when they were co-transferred with $CD45RB^{high}$ $CD4^+$ T cells were capable of suppressing disease[252]. This data indicated that there was a regulatory population of T cells within the $CD45RB^{low}$ $CD4^+$ T cell population capable of preventing disease. In terms of disease generated, intestinal inflammation was more severe at the more distal end of the colon suggesting that an increasing bacterial load may be associated with disease[252,254]. Furthermore, $CD25^-CD4^+$ T cells were shown to cause disease as opposed to $CD25^+CD4^+$ T cells which suppress disease[77]. Read *et al*. (2000) showed that suppression of disease mediated by the $CD45RB^{low}$ $CD4^+$ T cell population was dependent on $CD25^+$ T cells within the population since $CD45RB^{low}$ $CD4^+CD25^-$ T cells were not capable of disease suppression[255]. However, transfers of low numbers of $CD4^+$ T cells ($2x10^5$) from the LN or spleen into immunodeficient SCID recipients are still capable of causing a lethal bowel disease, yet non-separated splenocyte transfers

were not capable of causing disease[182]. Therefore, cells other than Tregs may be contributing to disease suppression in the splenocyte population.

a) Cytokine Requirements for Disease

Disease transferred by CD45RB^{high} CD4^{+} T cells has been examined to determine the factors necessary for disease to occur or be inhibited. For instance, transferring IFN-$\gamma^{-/-}$ CD45RB^{high} CD4^{+} T cells into an immunodeficient, lymphopenic host is unable to cause wasting disease or generate colitis[256]. In addition, CD4^{+}CD25^{-} T cells are capable of generating gastritis in a BALB/c *Rag*$^{-/-}$; however, when these cells are IFN-$\gamma^{-/-}$ they transfer less severe gastritis[236]. Therefore, IFN-γ is important but potentially not necessary in these autoimmune T cell transfer models similar to its importance in d3Tx-induced autoimmunity. However, the immunodeficient recipient used was not IFN-$\gamma^{-/-}$ in either model; therefore, determining the definitive role of IFN-γ could be hindered by the production of IFN-γ by innate immune cells in the recipient[236]. Furthermore, purified CD4^{+} T cells from the spleen can generate lethal bowel disease, and Bregenholt *et al.* (1999) report that purified IFN-$\gamma^{-/-}$ CD4^{+} T cells are still capable of generating colitis in an immunodeficient host[257]. IL-4, however, is important for the inhibition and suppression of disease since the transfer of CD4^{+}CD25^{-} T cells that are IL-4$^{-/-}$ results in more severe gastritis[236]. Furthermore, transferring IL-12$^{-/-}$CD4^{+}CD25^{-} T cells into an IL-12$^{-/-}$ BALB/c *Rag*$^{-/-}$ results in either prevention of disease or less severe disease[236]. This data suggests that disease may be the result of an unbalanced Th1/Th2 response.

The mechanism of suppression by $CD4^+CD25^+$ regulatory T cells remains unclear. Suri-Payer *et al.* (2001) assessed the ability of Tregs to regulate autoimmunity in a cytokine-dependant manner. They transferred either IL-$4^{-/-}$ or IL-$10^{-/-}$ $CD4^+CD25^+$ regulatory T cell populations with $CD4^+CD25^-$ T cell population and found that these cytokines were not necessary for Tregs to suppress gastritis[236]. IL-$10^{-/-}$ mice spontaneously develop colitis and transferring $CD4^+$IL-$10^{-/-}$ or $CD4^+CD25^+$IL-$10^{-/-}$ T cells still lead to spontaneous development of colitis; however, colitis was less severe when $CD4^+CD25^+$ IL-$10^{-/-}$ T cells were transferred compared to $CD4^+$IL-$10^{-/-}$ T cells[236]. Therefore, while IL-10 plays a role in colitis, there are still IL-10-independant mechanisms of suppression by Tregs[236]. In addition, BALB/c SCID and *Rag*$^{-/-}$ mice develop colitis in these transfer models as opposed to BALB/c nude mice which only develop gastritis, not colitis[236]. This may be due to the presence of IL-10-secreting B cells that protect the lamina propria in BALB/c nude mice compared to the BALB/c SCID and *Rag*$^{-/-}$ which are B-cell deficient[236].

1.9 RECENT THYMIC EMIGRANTS

T cell development occurs in the thymus where T cells are then exported into the periphery as recent thymic emigrants (RTEs). Neonatal mice have the highest proportion of RTEs in their periphery at around 100% from 0-3 weeks compared to 20% in adult mice[258]. Over the past few years evidence has emerged suggesting

that RTEs are still immature when they exit the thymus and undergo continued maturation in the periphery[259]. RTEs have been shown to be both phenotypically and functionally different from a naive T cell in the periphery of a mouse[152]. The phenotype of RTEs prior to maturation in the periphery was found to be $CD24^{hi}Qa2^{lo}CD45RB^{lo}IL\text{-}7R\alpha^{lo}TCR^{hi}CD3^{hi}CD28^{lo}$ (**Figure 1.7**) and they secrete lower amounts of IL-2 and IFN-γ when stimulated[152,260,261]. Continued maturation of RTEs in the periphery relies on contact with secondary lymphoid organs[262] but is independent of MHC[263]. However, MHC drives TCR repertoire shaping in RTEs[263]. The TCR repertoire of RTEs is altered compared to naive T cells; for instance, RTEs have a longer CDR3 which is involved in recognition of processed antigen[263].

a) Homeostatic proliferation

Recent thymic emigrants are most common in the lymphopenic neonate and after recovery from a state of lymphopenia. In a lymphoreplete host RTEs are at a disadvantage compared to mature naive T cells, and the chances of RTEs being incorporated into the peripheral T cell pool are low[264]. This inability to survive well can be altered by increased expression of the IL-7R and Bcl-2 in the RTE[264]. Nevertheless, in a lymphopenic host RTEs can out-compete mature naive T cells to repopulate the immune system[264]. The reasons for the ability of RTEs to preferentially expand in a lymphopenic environment over naive T cells remains unknown since there has been evidence that RTEs inefficiently transduce homeostatic and IL-7 signals[264]. For instance, RTEs have increased expression of

CD5 which negatively regulates TCR signalling and decreased GM1 expression which positively regulates responsiveness to homeostatic cytokines[264]. On the other hand, evidence suggests that responses to IL-7R signalling and homeostatic proliferation are increased in $CD5^{hi}$ T cells[265]. In addition, they conclude that IL-7R signalling in $CD5^{hi}$ T cells is heterogeneous and not dependent on T cell receptor affinity[265]. However, this does not completely exclude the possibility that RTEs may be more sensitive to TCR signals compared to naive T cells which could explain their differences in proliferation in a lymphopenic host. CD24 is another molecule that has been shown to be important for optimal homeostatic proliferation on T cells during lymphopenia[266]. Therefore, the increased proliferation rate of RTEs in a lymphopenic environment could encompass other molecules such as the high expression of CD24 on RTEs[152266].

b) Interleukin-7

Transfer experiments provide evidence that RTEs in the periphery are less likely to home to the spleen compared to naive T cells[140,140,264]. The addition of IL-7 increases the number of RTEs without enhanced thymic function as well as increased homing to the LNs, but not the spleen[140]. RTEs have an increased proliferative response compared to naive T cells in response to IL-7 even though they express lower levels of the IL-7R compared to their counterparts[140,141,267-272]. When RTEs are exposed to IL-7 at low doses it enhances their survival without requiring continuous presence of IL-7 but high dose IL-7 is necessary for proliferation of $CD4^+$ RTEs requiring its presence over at least a 5 day period[141].

c) Autoimmunity

RTEs must undergo continued maturation in the periphery and are not good effector cells. Therefore, this post-thymic maturation may represent a form of peripheral tolerance to prevent autoreactive T cells that have escaped thymic deletion from responding prior to becoming further tolerized in the periphery. RTEs have been shown to have a tendency to favour skewing towards a Th2-effector lineage as opposed to Th1 leading to increased amounts of IL-4, IL-5 and IL-13[273]. This Th2-skewing from $CD4^+$ RTEs has been associated with driving the production of IL-4 dependent IgG1 and causing inflammatory airway disease[273]. Therefore, the increased homeostatic proliferation in response to IL-7 of RTEs in a lymphopenic environment and preferential biased towards Th2 effector cells may represent a risk factor for the development of autoimmunity via lymphopenia-driven autoimmunity.

1.10 CERVICAL THYMUS

Cervical thymus exists in mice[274], humans[275] and many other species including chickens, sheep, pigs, koalas and possums[276]. It has been estimated that the occurrence of cervical thymus in humans is anywhere from rare to upwards of 50%[277]. The detection of cervical thymus in humans normally only occurs when it leads to difficulty breathing or is a noticeable lump removed for esthetical

purposes[275]. It is currently unknown if thymocytes from the cervical thymus play a pathogenic or protective role in mice or humans. However, a decrease in the frequency of remission in patients with Myasthenia Gravis who undergo thoracic thymectomy can occur due to the presence of cervical thymus which continues to generate T cells, suggesting a pathogenic role[278]. Cervical thymus was first described in mice in 1964 as a small, ectopic thymus that occurs in close proximity to the thyroid and parathyroid along the trachea as opposed to the thoracic thymus which occurs just above the heart (**Figure 1.8**)[274]. However, it was not until 20 years later, in 2006, when attention of the existence of cervical thymus was brought to the forefront again and was shown to be fully functional in the generation and exportation of T cells[279,280]. Consequences of the existence of a functional cervical thymus in thoracic Tx of mice and humans have been largely ignored, and the origin and contribution to the peripheral T cell pool remains unknown.

Presence of cervical thymus in mice has been detected in multiple strains, and the rate of occurrence is strain-dependant[279,280]. For example, the percentage of cervical thymus in BALB/c mice is 50-90%, and in C57BL/6 mice is 30-50%[279,280]. Furthermore, mice can have anywhere between one to three separate lobes of cervical thymus in a variety of locations along the trachea (**Figure 1.8**)[279]. The similarities and differences between the cervical and thoracic thymus are summarized in **Table 1.1**. Due to the size difference, the cellularity of the cervical thymus is drastically less (10^5) compared to the thoracic thymus (10^8)[279,280]. The cervical thymus displays normal medulla-cortex architecture; however, unlike the

thoracic thymus it only has a single medullary lobe (**Figure 1.9**)[279]. It has a regular distribution of CD40, CD80, CD86, MHC II, dendritic cells, TECs[280] and mTECs that are capable of expressing tissue restricted antigens (TRAs), and normal expression levels of Aire[279,280]. However, the variability in the expression of these TRAs can be variable between the different lobes of cervical thymus suggesting that what is considered as "self" may differ among cervical thymus lobes[280]. Thymocytes are capable of undergoing positive and negative selection in the cervical thymus although the proportion of mature thymocytes is slightly higher compared to the thoracic thymus[279,280]. In addition, thymocytes within the cervical thymus are capable of undergoing TCR re-arrangement and diversification signified by the expression of *Rag*1, *Rag*2, TdT and the pre-T cell receptor α chain, and it can produce a diverse T cell Vβ repertoire[279]. As well, $Foxp3^+$ thymocytes can be detected in the cervical thymus indicating that the thymus is capable of producing a regulatory T cell compartment[280]; however, the functionality of these Tregs has yet to be determined. Cervical thymus grafts under the kidney capsule of nude mice, which have no functional thymic tissue, have been shown to be capable of both exporting T cells[279,280] and generating new T cells leading to a CD4:CD8 ratio of 4:1 in peripheral blood[279]. T cells generated and exported from the cervical thymus are capable of mounting an immune response to viral infection and provide sufficient $CD4^+$ T cell help to B cells to produce an antibody response[279]. One of the major differences between the cervical and thoracic thymus occurs during embryogenesis; the cervical thymus has delayed thymopoiesis compared to the thoracic thymus indicated by late

Foxn1 expression[281]. Delayed thymopoiesis suggests that the cervical thymus may originate independently of the thoracic thymus rather than becoming detached from the thoracic thymus during development[276]. In addition, whether or not the cervical thymus undergoes atrophy with age like the thoracic thymus remains to be determined.

1.11 OVERVIEW OF MY THESIS

My thesis focuses on two main aspects. First, I looked at the ability of non-thoracic thymus tissue to generate a T cell repertoire after removal of the thoracic thymus. Second, I looked at the effect of IL-7 on lymphopenia-driven homeostatic proliferation and disease induced by thymocyte transfers into lymphopenic mice.

The contribution of non-thoracic thymic tissue such as the cervical thymus to the overall T cell repertoire remains to be elucidated. In addition, whether or not non-thoracic thymic tissue can support a fully competent and tolerant repertoire of T cells in the absence of contribution from the thoracic thymus is unknown. The use of thoracic thymectomy has been a common procedure employed in experimental research for many years without a noticeable contribution of T cells from non-thoracic thymic tissue. In addition, previous studies on the cervical thymus did not show that it was completely functional in the complete absence of signals from the thoracic thymus. Cervical thymus was only removed from wild type mice (that initially developed in the presence of the

thoracic thymus) and shown to be capable of exporting and producing new T cells that were immunocompetent. Therefore, we speculated that non-thoracic thymic tissue alone could not support the development of a complete T cell repertoire like the thoracic thymus. To explore this hypothesis, immunodeficient mice were thoracically thymectomized and reconstituted with hematopoietic stem cells to determine if non-thoracic thymic tissue could support a repertoire of T cells similar to the thoracic thymus. My data shows that T cells can develop after removal of the thoracic thymus, yet the T cells generated are not fully self-tolerant since these mice develop disease displayed by cachexia, lethargy, swollen eyes and hunching. The disease generated in this model lead us to speculate that continued output of T cells from non-thoracic thymic tissue after removal of the thoracic thymus may be important in d3Tx-induced autoimmunity although this has yet to be addressed.

My second study focused on several questions. First, we wanted to determine if the transfer of cervical thymocytes into an immunodeficient mouse could transfer disease similar to what had been shown in thoracic thymocyte transfer models (described in **section 1.8**)[189]. Cervical thymocytes were isolated from wild type mice and transferred as a whole thymocyte suspension into immunodeficient mice. Indeed cervical thymocytes generated disease similar to the transfer of thoracic thymocytes. However, transfer of whole splenocytes from wild type mice did not generate disease, similar to what was previously shown[189]. In the previous studies splenocytes were transferred as a whole cell suspension, but in our studies the splenocyte population was B cell-depleted prior to transfer.

Therefore, the presence of B cells does not prevent splenocytes from generating disease in immunodeficient mice. The differences in the populations of thymocytes and splenocytes that lead to this disparity in the generation of disease remain to be determined.

Lymphopenia-driven homeostatic proliferation and a defect in suppression by cell populations such as Tregs may be important factors in disease since the transfer of thymocytes into an immunocompetent mouse does not generate disease. Interleukin-7 is an important cytokine involved in homeostatic proliferation; therefore, we wanted to determine the impact of the addition of IL-7 on disease induced by thymocyte transfer. We utilized IL-7/anti-IL-7 complexes in our transfer model since these complexes were previously shown to have a greater biological effect on inducing homeostatic proliferation compared to IL-7 alone[134]. We found that the addition of these complexes did not enhance the severity of disease but decreased the time course to disease. The exact mechanism of the enhanced disease by IL-7 complexes has not been elucidated, but our data shows that IL-7 significantly increases the overall number of T cells in the spleens after 10 days post-transfer of mice who receive either thymocytes or splenocytes. One possible explanation for the difference in disease is that the thymocyte population has a greater number of autoreactive T cells that have not undergone peripheral tolerance mechanisms. Therefore, homeostatic proliferation may increase the number of autoreactive T cells during lymphopenia before undergoing peripheral tolerance. Alternatively, there could be a population of

cells within the splenocyte suspension that are capable of preventing disease. Therefore, more studies need to be done to elucidate the differences in disease.

Together this data demonstrates that thymocytes or T cells that develop in an immunodeficient mouse from non-thoracic thymic tissue after removal of the thoracic thymus during lymphopenia lead to a repertoire of T cells that are not fully self-tolerant. This process of disease by transfer of thymocytes can be augmented by the addition of IL-7/anti-IL-7 complexes.

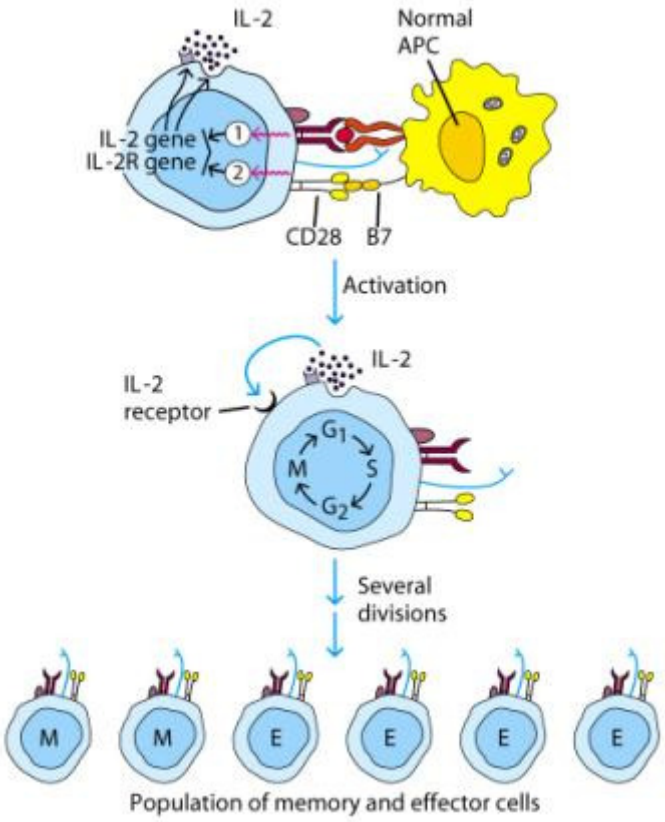


Figure 1.1. T cell activation.
T cell activation. Schematic of naive T cell activation from an antigen presenting cell (APC). Naive T cells must receive signal 1 and signal 2 in order to become activated, proliferate and differentiate. Figure adapted from Kuby (Immunology 6th Ed., 2007, W. H. Freean and Co.).

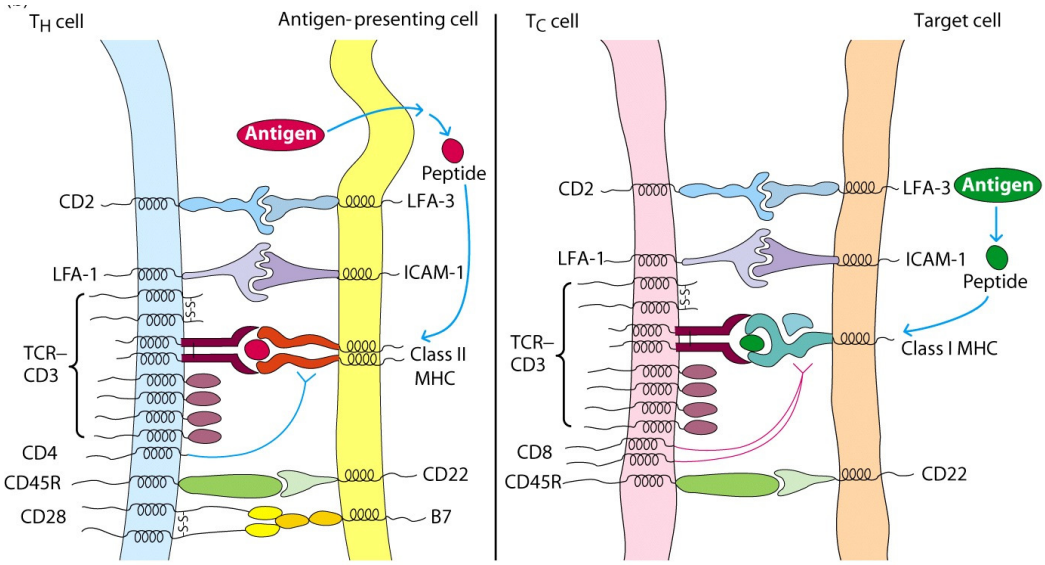


Figure 1.2. T cell activation and accessory molecules.
Schematic Diagram of the interactions and accessory molecules involved in T cell signalling of a CD4 T helper cell with an antigen presenting cell (APC; *left*) compared to the interaction of a CD8 cytotoxic T cell with a target cell (*right*). Figure adapted from Kuby (Immunology 6th Ed., 2007, W. H. Freean and Co.).

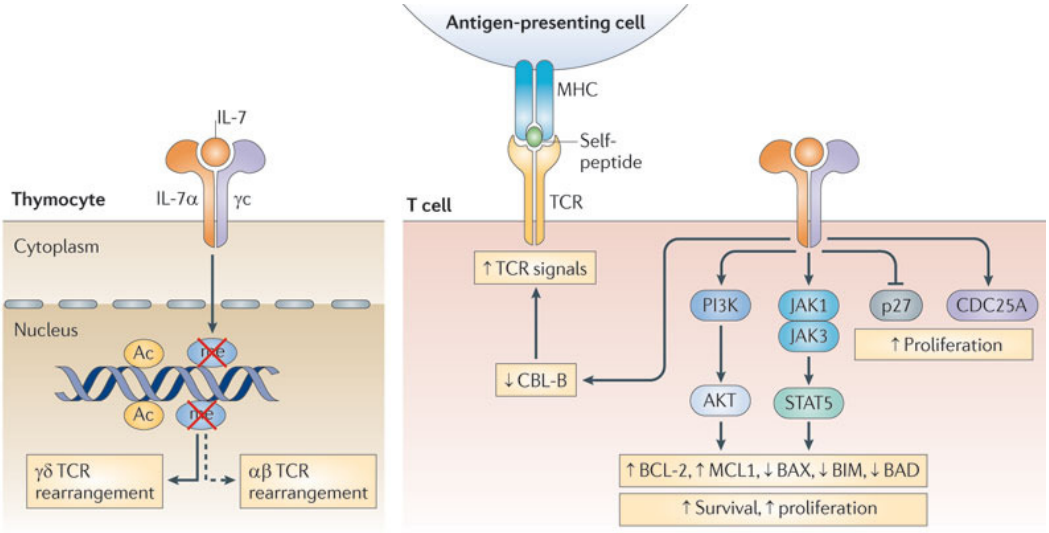


Figure 1.3. **Effects of interleukin-7 signalling in thymocytes and T cells in the periphery.**
Figure adapted from Mackell *et al.* (Nature Reviews Immunology, 2011, pp. 330-342)[149].

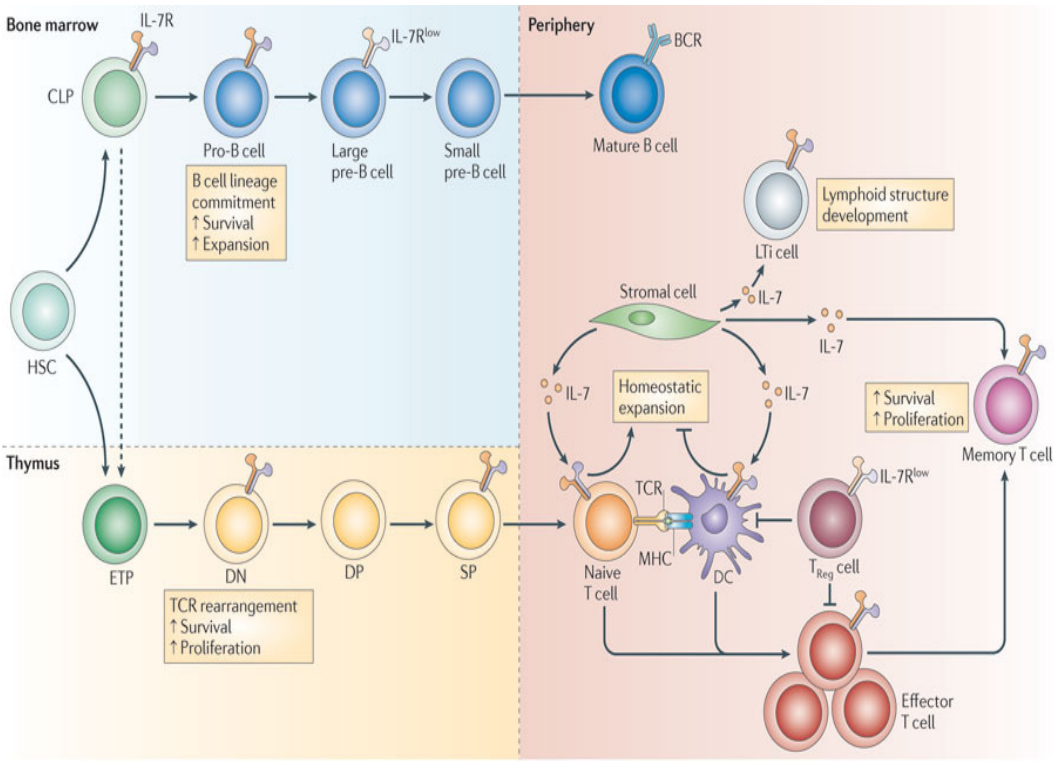


Figure 1.4. Expression of IL-7R during developmental stages of B and T cells and in the periphery.
Figure adapted from Mackell *et al.* (Nature Reviews Immunology, 2011, pp. 330-342)[149].

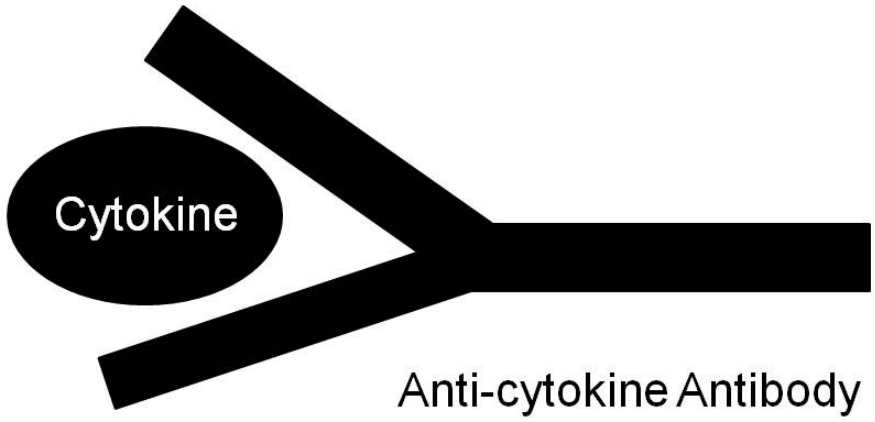


Figure 1.5. Cytokine/anti-cytokine antibody complex.

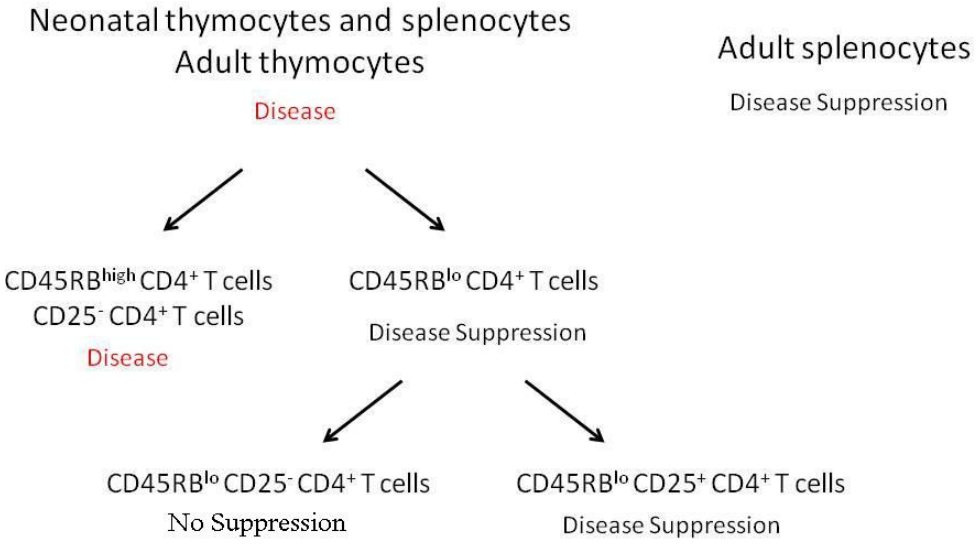


Figure 1.6. Cell types that mediate disease when transferred into a lymphopenic host.
Flow diagram of the different types of cells mediating disease or disease suppression when transferred into lymphopenic recipient.

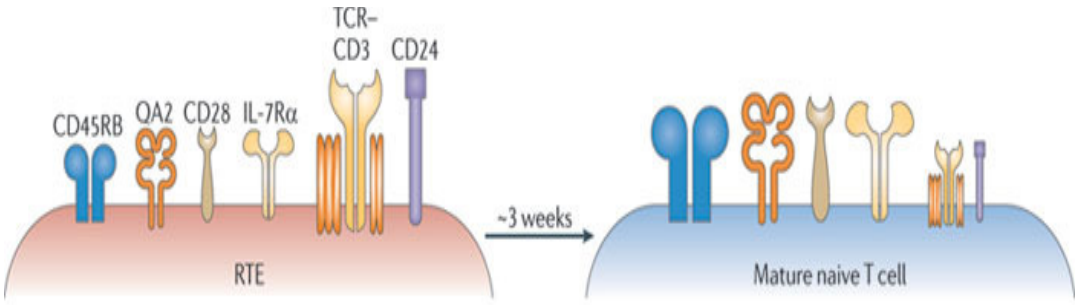


Figure 1.7. Expression markers on recent thymic emigrants compared to mature naive T cells.
The size of the marker is representative of its expression (high versus low). Figure adapted from Fink and Hendricks (Nature Reviews Immunology, 2011, pp. 544-549)[259].

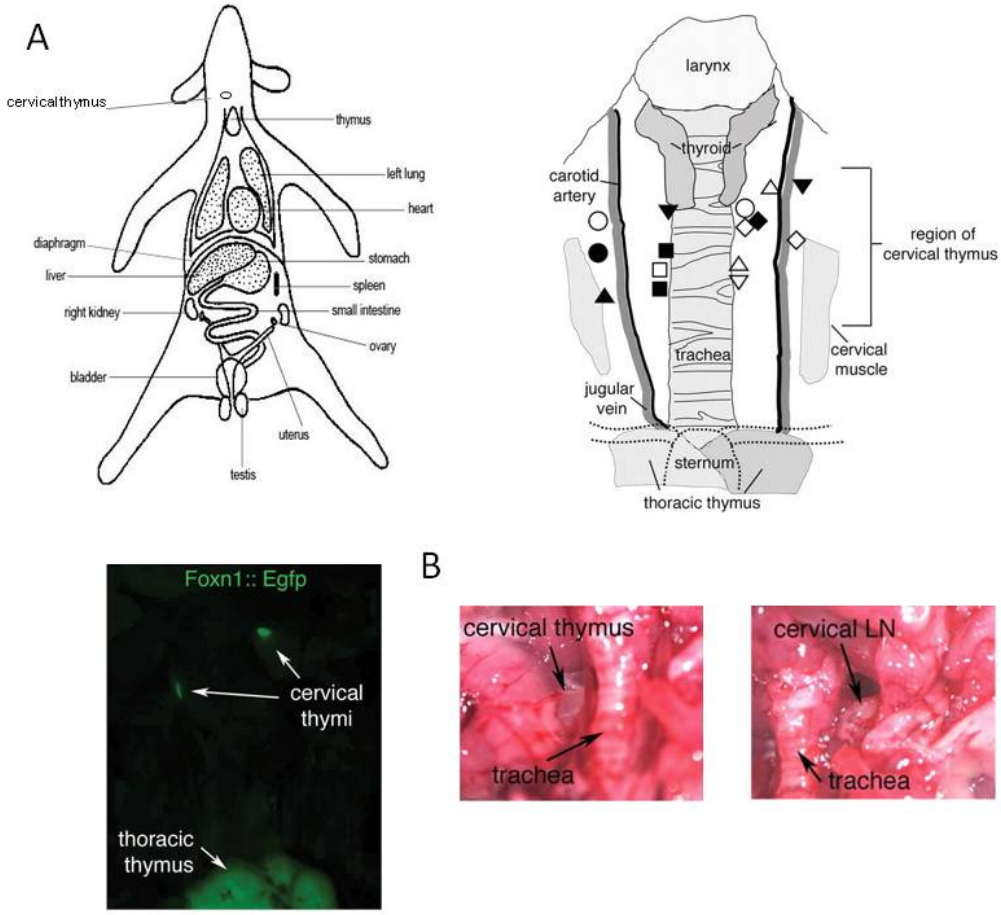


Figure 1.8. Location of the cervical thymus in mice.
(A) Location of the cervical and thoracic thymus in mice (*top left*; adapted from Anatomy and Physiology of Animals, 2008, J. Ruth Lawson). Circles, squares and triangles representing locations of cervical thymi along the trachea in mice (*top right*), and location of the cervical and thoracic thymus in a mouse that expresses green fluorescent protein (GFP) under the Foxn1 promoter (*bottom left*). (B) Cervical thymus compared to a cervical lymph node along the trachea of a mouse. Figure adapted from Terszowski *et al*. (Science, 2006, pp. 284-287)[279].

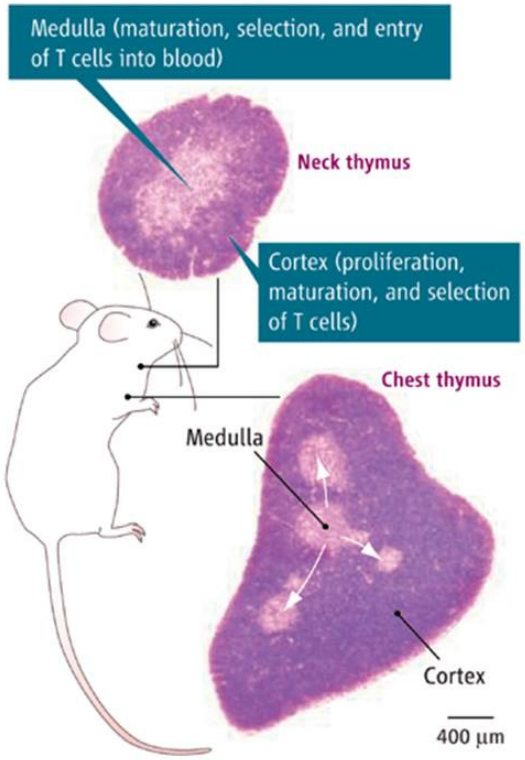


Figure 1.9. Thymic architecture of the medulla-cortex in the single-lobed cervical thymus compared to the bi-lobed thoracic thymus.
Figure adapted from H. Von Boehmer (Science, 2006 pp. 206-207)[282].

Table 1.1. Comparison of the cervical and thoracic thymus.
Data in table adapted from Terszowski *et al*. (Science, 2006, pp. 284-287)[279].

	Cervical Thymus	Thoracic Thymus
Medulla-Cortex Architecture	✓	✓
CD4 and CD8 SP and DP	✓	✓
Export T cells into periphery	✓	✓
Genes for TCR Rearrangement	✓	✓
Positive and Negative Selection	✓	✓
Amount of Thymocytes	10^5 - 10^6	~10^8
TCR Diversity	✓	✓
Expression of Self-Antigens	Slight differences (variable within group)	✓

CHAPTER 2: MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 ANIMALS

Adult immunodeficient BALB/c mice bearing the *SCID* mutation (BALB/c SCID; SCID/NCR; C.BySmn.CB17) and adult BALB/c wild type (WT) mice were obtained from Jackson Laboratory (Bar Harbor, ME) or from the National Cancer Institute of Frederick (NCI-Frederick; Frederick, MD). Immunodeficient BALB/c *Rag*$^{-/-}$ (C.129S6(B6)-Rag2tm1FwaN12) mice were obtained from Taconic Farms (Germantown, NY) and bred on-site at the University of Alberta. Timed pregnant BALB/c WT mice at day 13 of gestation were either obtained from NCI-Frederick, Charles River Laboratory or bred on-site at the University of Alberta. Immunodeficient mice were housed and bred under specific pathogen free conditions. All other animals were housed under standard conditions. Food and water were provided *ad libitum*. Immunodeficient mice received Novotrimel in their drinking water prior to use and for three weeks post-Tx. All protocols on care and handling of animals were carried out in facilities accredited by and in accordance with the Canadian Council on Animal Care (CCAC).

2.2 FLOW CYTOMETRY

Peripheral blood samples were obtained by tail bleeding and mixed at a 1:1 ratio with sodium heparin. Splenocytes were obtained by harvesting the spleen and

homogenized by mechanical disruption using frosted glass slides. Homogenate was passed through a 70 µm cell strainer (BD Biosciences) to remove tissue and collect splenocytes. Fluorescent anti-mouse TCR-beta chain (TCRβ; H57-597), CD4 (RM4-5), CD45R (B220; RA3-6B2), CD19 (ebio1D3), CD8α (53-6.7), CD44 (IM7), CD62L (MEL-14), CD25 (PC61), CD24 (M1/69), CD127 (A7R34; IL-7Rα), Foxp3 (FJK-16s), IFN-γ (XMG1.2), IL-4 (11B11), IL-17A (eBio17B7), IL-10 (JES5-16E3), rat $IgG_1\kappa$, rat $IgG_2b\kappa$, and rat IgG_2a were purchased from eBioscience (San Diego, CA), and anti-mouse CD45R (B220; RA3-6B2) was purchased from Caltag (Burlingame, CA). An anti-mouse TCR Vβ panel was purchased from BD Biosciences (San Diego, CA). Cells were incubated at a 1:1 ratio with a cocktail containing anti-CD16/32 antibody (2.4G2; BioXCell, West Lebanon, NH) and mouse, rat and hamster sera (Equitech-Bio; Kerrville, Tx) to prevent Fc-receptor binding of the flow cytometry antibody for 5 min at room temperature (RT). Cells were then incubated with the appropriate antibodies diluted in 1% dialyzed bovine serum albumin (BSA) for 15 minutes (min) at 4°C then washed in 1x phosphate buffered saline (PBS) or red cell lysis buffer in the case of peripheral blood staining and centrifuged at 1200 RPM (300x*g*) for 10 min. Cells were resuspended in 1x PBS.

For intracellular cytokine staining, surface molecules were stained using the above method then cells were fixed with BD IC Fixation buffer (BD Biosciences) for 10 min at room temperature (RT). Cells were then washed 2x with 0.1% saponin permeabilization buffer. Cells were incubated with intracellular antibodies or isotype control antibodies diluted in 0.1% saponin

permeabilization buffer for 30 min at 4°C and washed 2x with permeabilization buffer. Cells were then resuspended with 1x permeabilization buffer for analysis on the flow cytometer. Intracellular detection of Foxp3 was done using a Foxp3 Staining Kit purchased from eBioscience and staining was done according to the manufacturer's instructions. Antibodies were either pre-titrated or used according to the manufacturer's instructions. Cells were analyzed on either a 4-color FACSCalibur™ flow cytometer (BD Biosciences) using Cell Quest™ Pro Software for acquisition and analysis or a 15-color BD™ LSR II flow cytometer (BD Biosciences) equipped with FACSDiva™ Software for acquisition and analyzed using FCS Express 3 (De Novo Software; Los Angeles, CA).

2.3 FETAL LIVER CELL TRANSPLANTATION

Fetal livers (FLs) from fetuses contain hematopoietic stem cells that can generate mature T and B cells[283]**.** Fetuses from pregnant mice at days 14-16 of gestation were isolated and the FLs were extracted and homogenized by pipetting up and down in 1x sterile PBS to obtain a single cell suspension, pooled and counted. Prior to pooling, each FL was stained for anti-mouse TCRβ, CD45R, CD4 and CD8α (as described under 'flow cytometry') prior to use to ensure no mature T or B cells were present prior to injection. For immune reconstitution of BALB/c SCID or BALB/c *Rag*$^{-/-}$ mice 10-20 million fetal liver cells were injected intravenously (I.V.) into the tail vein.

2.4 ADOPTIVE TRANSFER OF THYMOCYTES OR SPLENOCYTES

The thymus or spleen was isolated from adult BALB/c WT mice in cold 1x PBS and homogenized by mechanical disruption between frosted glass slides. Tissues were then put through a 70 µm cell strainer (BD Bioscience) to obtain a single cell suspension and counted. Splenocytes were depleted of B cells using an anti-mouse CD45R (B220) microbeads and MACS® LD cell separation columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Splenocyte populations contained less than 3% B cells ($CD19^+$) after depletion as measured by flow cytometry (described in 'flow cytometry' sections). The percentage of SP CD4 or CD8 T cells was determined by flow cytometry and applied to the absolute cell counts, and a suspension containing $5x10^5$ SP T cells from B cell-depleted splenocytes or thymocytes were transferred I.V. into BALB/c *Rag*$^{-/-}$ or BALB/c SCID mice.

2.5 SPLENOCYTE RE-STIMULATION

Splenocytes were obtained by harvesting the spleen from mice and homogenized by mechanical disruption using frosted glass slides. Homogenate was then passed through a 70 µm cell strainer to remove tissue debris and collect a single cell suspension of splenocytes. Splenocytes were cultured at $1x10^6$ cells/mL in 2 mL of tissue culture media (described in appendix) with 50 ng/mL of phorbol myristate acetate (PMA) and 1 µg/mL of ionomycin. After 1 hour 2 µM monensin and 3 µg/mL brefeldin A were added to each well and cells were incubated for a total of 5 hours at 5% CO_2 and 37°C. Cells were then collected by pipetting from

the well, washed 2x with PBS and stained for intracellular cytokines following the procedure under "flow cytometry."

2.6 SERUM IMMUNOGLOBULIN ISOTYPE QUANTITATION

At the time of sacrifice, mice were anaesthetized by inhalation of isofluorane. They were exsanguinated using a 3 mL syringe and 23-gauge needle and blood was transferred into a 1.5 mL microcentrifuge tube and allowed to clot at RT for at least 20 min. Blood was then centrifuged for 20 min at >2000 x g for serum recovery. Serum was stored at -80°C until analysis was performed. Immunoglobulin isotyping was performed using an Ig-specific multiplex antibody bead assay for IgG1, IgG2a, IgG2b, IgG3, IgA and IgM (Millipore, Billerica, MA) according to the manufacturer's instructions. Data was acquired using a Luminex 100^{TM} System (Applied Cytometry Systems, Sheffield, UK) and analyzed using STarStation 2.0 software.

2.7 THYMECTOMY

All complete thymectomies and some sham thymectomies were performed by Dr. Lin Fu Zhu in the Department of Surgery, University of Alberta, Edmonton, AB. Some sham thymectomies and all half lobe thymectomies (**Appendix**) were performed by Dr. Dave Al-Adra, Department of Surgery, University of Alberta, Edmonton, AB. Mice at 5-6 weeks of age were pre-medicated with 0.05 mg/kg of Atropine intramuscularly and then anaesthetized by inhalation under isofluorane at 5% until under surgical plane then reduced to 1.5% to maintain surgical plane.

Ophthalmic ointment was applied to the eyes of the mice. Mice were injected with 0.5 mL of warm lactated Ringer's solution to maintain blood pressure during the procedure. Surgeries were carried out using aseptic microvascular techniques. A 1 cm incision above the sternum on the neck was made and then following underneath the sternum, the whole thymus was exposed and excised with caution to avoid pneumothorax. In the case of the sham thymectomies no thymic tissue was removed and in the case of the half lobe thymectomies only one of the two lobes of thoracic thymus was removed. The incision was closed in two layers with absorbable suture. The animal's body temperature was supported using a heating blanket, a warmed fluid infusion in the abdominal cavity and an overhead heating lamp after the procedure was completed. The mice were maintained in a warmed nursery incubator for the first 48 hours after the procedure where the mouse had unrestricted access to food and water. The mice were monitored and euthanized if signs of lethargy, or dehydration occurred. Surgical removal was used as opposed to another thymectomy technique using a vacuum pipette to suction out the thymus because surgically exposing and removing the thoracic thymus visually ensures complete removal.

2.8 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

These protocols were adapted from Dr. Gina Rayat's Lab or the Alberta Diabetes Institute Histology Core.

a) Frozen Sections

Organs were placed in histology moulds, embedded in OCT compound and frozen at -80°C. Frozen sections were cut at 5 µM at -20°C and placed on Fisher Brand Superfrost Plus microscope slides (Fisher Scientific, Ottawa, ON)**.** Slides were stored at -20°C.

b) Immunohistochemistry for CD4

The frozen sections on the slides were air dried frozen for 1 min then fixed in cold acetone for 4 min at 4°C. Slides were then air dried for another min and any remaining acetone was suctioned off. Tissues were encircled with a Pap pen (Invitrogen, Burlington, ON) and then blocked in 2% FBS for 20 min before it was flicked off. Tissues were then blocked with 1 drop of avidin (Vector Laboratories, Burlingham, CA) for 10 min, flicked off then blocked with 1 drop of biotin (Vector Laboratories) for 10 min and flicked off. The primary antibody, anti-CD4 (L3T4; 1:500)(BD Biosciences), was added to the tissues and incubated for 1 hour. The 2° biotin-conjugated anti-rat IgG (H+L) (1:500) (Jackson Immunoresearch, West Grove, PA) was added and incubated for 30 min. Control sections were incubated with secondary alone in the absence of the primary antibody. Tissue sections were washed 3x in 5 min. Next, VECTASTAIN® ABC Standard Complex (Vector Laboratories) was added to all tissue sections and allowed to incubate for 40 min. Once again, tissue sections were washed 3x in 5 min. The DAB substrate (Vector Laboratory) was then added to the tissues and allowed to incubate 5 min in the dark. To stop the substrate reactions slides were

rinsed for 10 min under tap water. Slides were then counterstained with Harris' hematoxylin and coverslipped (see appendix for protocol).

2.9 IL-7/ANTI-IL-7 COMPLEXES

Mice were intraperitoneally (I.P.) injected with IL-7/anti-IL-7 complexes (at approximately a 1:1 ratio) on days 1, 4 and 7[134]. Each injection per mouse contained 15 µg of anti-IL-7 (M25; BioXcell), and 1.5 µg carrier-free recombinant mouse IL-7 (eBioscience) in 200 µl sterile PBS. Complexes were incubated for 30 min at 37°C prior to injection.

2.10 STATISTICAL ANALYSIS

All statistical analyses were calculated using GraphPad Prims 4 software (GraphPad Software, San Diego, CA). A two-tailed student's t-test was used to compare the means between two groups with statistical significance defined as $p<0.05$. Means are reported along with standard error of the mean (SEM).

CHAPTER 3: T CELLS GENERATED AFTER THE REMOVAL OF THE THORACIC THYMUS IN LYMPHOPENIC MICE ARE NOT FULLY SELF-TOLERANT

3.1 INTRODUCTION

Based on the premise that the presence of cervical thymus has been largely ignored over the years even in the presence of many experimental designs incorporating thoracic thymectomy, we hypothesized that the cervical thymus could not function in the absence of signals from the thoracic thymus. Although the cervical thymus to date is the only non-thoracic thymus tissue known to exist in mice there could potentially be other non-thoracic thymus tissue contributing to T cell development. For this thesis the use of the term cervical thymus will include cervical thymus and any other non-thoracic thymus tissue that may have yet to be discovered.

In order to determine if the cervical thymus could function in the complete absence of the thoracic thymus we used immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mice which lack functional T and B cells and subjected them to thoracic thymectomy (Tx) or a sham thoracic thymectomy. We then adoptively transferred BALB/c wild type day 14-16 of gestation fetal liver cells (FLCs) into these mice that contain hematopoietic stem cells that can develop into T cells if the thymus is present and B cells. Therefore, mice that receive a thoracic Tx will only generate a T cell repertoire if cervical thymus is present, and if the cervical thymus is absent then these mice will only develop B cells. The mice that receive a sham

thoracic Tx will be able to generate T cells via the thoracic thymus and the cervical thymus, if present. The experimental design is diagrammed in Figure 3.1..

3.2 RESULTS

a) Disease induced by T cell development in the absence of the thoracic thymus

Mice that develop T cells in the absence of the thoracic thymus fail to develop a repertoire of T cells that are fully self-tolerant. However, mice that develop T cells in the presence of the thoracic thymus remain healthy (**Figure 3.2A**). Disease incidence in mice that receive a thoracic thymectomy and fetal liver (thoracic Tx + FL) is 75%, and 16/21 mice developed disease compared to 0/14 mice who received a sham thoracic thymectomy and fetal liver (Sham Tx + FL; **Figure 3.2B**). Disease was determined to be present and their endpoint was reached when mice had 2 or more of the following symptoms: weight loss, swollen eyes, scruffy, unhealthy looking fur, hunching or lethargy. Disease was quite a long process since it took anywhere from 100-400 days; however, the majority of mice developed disease by 200 days post FL. A few of the mice that developed disease after thoracic Tx + FL were examined for the presence of cervical thymus but no cervical thymus tissue was recovered. This could be due to the technical difficulty of finding the cervical thymus. In addition, it is not known whether or not the cervical thymus undergoes thymic atrophy and involution

similar to the thoracic thymus. If thymic involution occurs in the cervical thymus it could make locating the cervical thymus more difficult. Therefore, we can only presume that these mice develop T cells via the cervical thymus in the absence of the thoracic thymus. It is unlikely that these T cells would develop through extra-thymic T cell development[284] since 12 mice that received a thoracic Tx + FL developed B cells only. The lack of T cells is most likely due to the absence of cervical thymus (which is not present in all mice) since extra-thymic T cell development would not require the presence of thymic tissue. As disease progresses mice that receive a thoracic Tx + FL and develop T cells lose weight (g) over time. **Figure 3.3A** depicts the weight of mice post-transfer that receive a thoracic Tx + FL (n=8) or a sham Tx + FL (n=12) and **Figure 3.3B** depicts the percent change in the mouse's weight gained or lost over time compared to its initial weight. For the mice that receive a thoracic Tx + FL the dotted line indicates mice that did not develop disease based on the above symptoms and the solid line indicates mice that did develop disease. This shows that the mice that develop disease lose a significant amount of weight compared to mice that receive a sham Tx + FL or mice that receive a thoracic Tx + FL and do not develop disease.

b) T cell phenotype in the peripheral blood

The peripheral T cell phenotype was monitored in mice to determine if there were differences in the phenotype between mice that develop disease in the absence of the thoracic thymus compared to when T cells develop in the presence of the

thoracic thymus. The appearance of T cells in the periphery in mice that receive a thoracic Tx + FL is quite variable and can occur anywhere between 40 to over 100 days which may correlate with the differences in time course for disease whereas T cells show up in the periphery at around 30-50 days in mice that have thoracic thymus (**Figure 3.4A**). The delayed detection of T cells in thoracic Tx + FL mice may be due to the smaller size of the cervical thymus and the number of cervical thymic lobes within a mouse. This size difference would contribute to the difference in detection of T cells in the periphery because export of T cells from the thymus is directly proportional to the size of the thymic tissue[285]. In addition, the percentage of T cells within the lymphocyte population in mice that receive thoracic Tx + FL is lower and more variable compared to sham Tx + FL mice (**Figure 3.4A**). Furthermore, the percentage of T cells in the periphery is maintained over time compared to sham Tx + FL and BALB/c WT mice where their T cell percentages reach 65-75% at day 100 and then begin to decline over time. There were no substantial differences in the CD4:CD8 ratio in the periphery although mice that received thoracic Tx + FL tended to have a slightly higher amount of CD4 T cells (**Figure 3.4B**). Next, we looked at the percentage of CD4 T cells that were Tregs determined by presence of both CD25 and Foxp3 to determine if a lack or deficiency of Tregs may play a role in disease. However, mice that received thoracic Tx + FL did not have an absence or lower percentage of Tregs in the periphery and had a slightly higher percentage compared to sham Tx + FL or WT (**Figure 3.4C**). Nevertheless, there did appear to be a trend in mice that generated disease at around 150-200 days when there was a steep

decline in the percentage of $CD25^{+}Foxp3^{+}$ CD4 T cells in the periphery which may be a contributing factor to disease or predict the onset of disease. The percentage of T cells that were of an effector memory T cell phenotype ($CD62L^{lo}CD44^{hi}$) was also assessed. There was a large increase in the percentage of T cells that were of the effector memory phenotype in mice that had thoracic Tx + FL compared to sham Tx + FL or WT (**Figure 3.4D**). This could suggest that these cells are interacting with their Ag in the periphery and may be causing tissue damage or that these cells are rapidly undergoing non-specific expansion via homeostatic proliferation to acquire a sufficient peripheral T cell pool[286,287] due to lower T cell export from the cervical thymus. Homeostatic proliferation in a lymphopenic environment leads to non-specific division of T cells and an acquired memory T cell phenotype[110,122,123].

c) T cell phenotype and repertoire in the spleen

We next analyzed the T cell phenotype and repertoire in the spleen to determine if there were any differences that could explain or contribute to the disease process. First, we analyzed the T cell number (**Figure 3.5A**) and percentage (**Figure 3.5B**) in mice. We found that mice that received thoracic Tx + FL had both a lower number ($p<0.0001$) and percentage ($p=0.0006$) of T cells compared to sham Tx + FL and BALB/c WT mice. The average number of T cells in mice that had a thoracic Tx + FL was under $10x10^6$ compared to sham Tx + FL and BALB/c WT which had over $20x10^6$ T cells.

Similar to the peripheral blood, mice that received a thoracic Tx + FL had a higher percentage of effector memory T cells compared to sham Tx + FL or BALB/c WT mice. Naive T cells are defined as $CD62L^{hi}CD44^{lo}$, central memory as $CD62L^{hi}CD44^{hi}$ and effector memory T cells as $CD62L^{lo}CD44^{hi}$. A representation of what the peripheral memory phenotype looks like in the spleen is shown for thoracic Tx + FL mice compared to sham Tx + FL and BALB/c WT (**Figure 3.6A**). Mice that receive thoracic Tx + FL have a lower percentage of both naive T cells ($p<0.0001$) and central memory T cells ($p=0.0027$). However, they have a significantly higher percentage of effector memory T cells in the spleen ($p<0.0001$) compared to sham Tx + FL and WT.

A decrease in Tregs could allow for the activation and response of autoreactive T cells in the periphery due to a decreased capacity for suppression. Therefore, we sought to determine if there was a lower number of Tregs in the spleen even though there was no decrease in the percentage in the peripheral blood of mice that received thoracic Tx + FL. There was an increase in the percentage of CD4 T cells that were $CD25^{+}Foxp3^{+}$ compared to sham Tx + FL and BALB/c WT (**Figure 3.7 A and B**; *$p=0.0112$); however, there was no significant difference in the percentage of Tregs in the entire T cell population. There was a decrease in absolute Treg numbers in the spleen of mice that received a thoracic Tx + FL compared to sham Tx + FL and BALB/c WT although it was not statistically significant (**Figure 3.7B**). In addition, it is not surprising that there is a small decrease in absolute Treg numbers since there is a decrease in total T cell numbers in mice that receive thoracic Tx + FL.

We wanted to determine if T cell development in the absence of the thoracic thymus could generate a diverse repertoire of T cells. In addition, we wanted to determine if there was any skewing in the Vβ repertoire in mice that generated disease. A Vβ flow cytometry panel (BD Biosciences) was used to assess the presence and prevalence of TCR Vβ 2, 3, 4, 5.1 and 5.2, 6, 7, 8.1 and 8.2, 9, 10[b], 11, 12, 13, 14, and 17[a]. We found that mice that develop disease do not have any major skewing except for a slight increase in Vβ 6 and decrease in Vβ 2 compared to sham Tx + FL and BALB/c WT (**Figure 3.8**). In addition, a diverse Vβ repertoire of T cells was present in the absence of the thoracic thymus. Finally, negative selection in the absence of the thoracic thymus appeared to be functional due to the absence of the following Vβ's normally deleted in the thoracic thymus by superantigens: Vβ 3, 5.1, 5.2, 11, and 12[57]. Furthermore, there was no major Vβ skewing in the repertoire of either CD4 or CD8 T cells and they contained a diverse Vβ repertoire within the CD4 and CD8 subsets of T cells (**Figure 3.9 A and B**). However, the increase in Vβ 6 was mainly limited to CD4 T cells as opposed to CD8 T cells. But, overall a diverse T cell repertoire is capable of being generated in the absence of T cell development in the thoracic thymus.

d) Intracellular cytokines

It is possible that the development of disease in mice that generate T cells after they are thoracically thymectomized and given fetal liver could be partly due to differences in cytokine secretion and Th skewing in T cells. Therefore,

splenocytes or pooled cells from the inguinal, mesenteric and periaortic LNs were obtained from thoracic Tx + FL, sham Tx + FL or BALB/c WT mice and stimulated for 5 hours with PMA and ionomycin in the presence of brefeldin A and monensin. Cells were then analyzed for the presence of intracellular IFN-γ, IL-17A, IL-4 and IL-10 in CD4 or CD8 T cells by flow cytometry.

The intracellular detection of cytokines by thoracic Tx + FL, sham Tx + FL and BALB/c WT mice are depicted in **Figure 3.10**. There were no significant differences in intracellular detection of IL-4 in mice that generated disease after thoracic Tx + FL compared to sham Tx + FL or WT. However, there was a significant increase in IFN-γ positive CD4 T cells in mice that received a thoracic Tx + FL and developed disease in both the spleen and LNs (**Figure 3.10B**; * $p=0.0017$, ** $p<0.0001$). Mice that received a sham Tx + FL and BALB/c WT mice had on average 10% of their CD4 T cells containing intracellular IFN-γ, however, that percentage increased to 30-40% in mice that developed disease. In addition, IL-17A was detected in CD4 T cells in mice that developed disease from thoracic Tx + FL (**Figure 3.10D**; * $p=0.0054$, ** $p=0.0083$) whereas IL-17A was not detected in sham Tx + FL or BALB/c WT mice. Lastly, an increase in IL-10 was detected in thoracic Tx + FL mice that developed disease compared to minimal amounts of CD4 T cells containing IL-10 in sham Tx + FL or BALB/c WT (**Figure 3.10E**; * $p=0.0038$, ** $p=0.0382$). The only cytokine detected in CD8 T cells was IFN-γ which was only significantly increased in the LNs of mice that developed disease (**Figure 3.11** A and B; * $p=0.002$).

e) Immunoglobulin isotypes

Autoantibodies are often detected in and associated with autoimmune diseases and isotype class switching can occur based on the presence of certain cytokines within the environment[14]. We used a multiplex bead assay to determine the type and quantity of Ig isotypes in mice that received thoracic Tx + FL and developed T cells, thoracic Tx + FL and developed B cells only, sham Tx + FL or BALB/c WT. The isotypes analyzed were IgG1, IgG2a, IgG2b, IgG3, IgA and IgM (**Figure 3.12**). Mice that received thoracic Tx + FL and developed B cells only, had minimal amounts of the above isotypes in their serum, likely because immunoglobulin class switching is dependent on T cell help[288]. There was a significant increase in IgG1 (* p=0.0191) and IgG2b (** p=0.0253) in mice that received thoracic Tx + FL compared to sham Tx + FL which usually occurs in the presence of IL-4 or TGF-β, respectively[14-16]. There was also a slight increase in IgG2a and IgA in some of the mice that received a thoracic Tx + FL but not statistically significant.

f) Recent thymic emigrants and homeostatic proliferation

Interleukin-7 receptor is expressed on the majority of naïve and memory T cells and IL-7 is important in survival and homeostatic proliferation of T cells[149]. Immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mice have a lymphopenic environment which can lead to increased serum levels of IL-7 because there are no T cells using the cytokine in the environment[127]. The recognition of IL-7 through the IL-7R can act as a co-stimulatory molecule for naïve T cells or activate RTEs in the

absence of low affinity/MHC interactions leading to a decrease in the IL-7R[106,113,149]. Therefore, we analyzed the expression of the IL-7R on both CD4 and CD8 T cells. There was no difference in expression of the IL-7R on CD4 T cells in thoracic Tx + FL, sham Tx + FL or BALB/c WT, but there was a decrease in IL-7R expression on CD8 T cells in thoracic Tx + FL mice that develop disease (**Figure 3.13**; * p=0.0083), consistent with the finding that CD8 T cells are more responsive to IL-7[149].

Recent thymic emigrants have been implicated in autoimmune and inflammatory disease models[289,290]. Therefore, we wanted to determine if there was a higher percentage of RTE's in mice that received thoracic Tx + FL. Traditionally, RTEs have been distinguished by the presence of CD24[259]. In the spleen of mice that receive thoracic Tx + FL and get disease there is an increased percentage of both CD4 and CD8 T cells expressing CD24 compared to sham Tx + FL mice (**Figure 3.14 A and B**; * p=0.0066, ** p=0.0371).

3.3 DISCUSSION

In summary, T cell development in the absence of the thoracic thymus in a lymphopenic mouse leads to the generation a T cell repertoire that is not fully self-tolerant. The majority of mice that underwent thoracic Tx + FL developed disease outwardly characterized by weight loss, swollen eyes, scruffy fur, hunching and/or lethargy. On average, disease took 200 days from time of initial

FL transfer to disease; however, appearance of T cells in the periphery of mice in the absence of the thoracic thymus took upwards of 60-80 days.

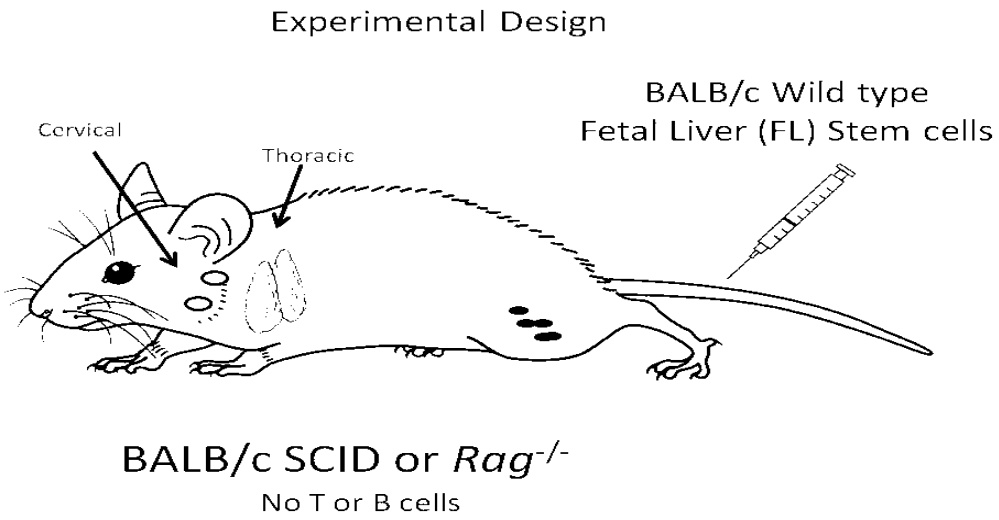
We found differences in the T cell phenotype in mice that developed disease in the absence of the thoracic thymus as opposed to mice that developed T cells in the presence of the thoracic thymus. For instance, the percentage of T cells in the peripheral blood was much more variable and lower compared to mice that developed T cells in the presence of the thoracic thymus (sham Tx + FL) or BALB/c WT. As well, there was a large increase in the percentage of effector memory T cells suggesting that these cells are either becoming activated or undergoing homeostatic proliferation. There was the possibility that disease induced in a large number of thoracic Tx + FL mice was due to a defect or deficiency in Treg numbers; however, there was a slight increase in the percentage of CD4 T cells that had a Treg phenotype and a similar absolute number in mice that generated disease compared to sham Tx + FL mice. Therefore, our data suggests that a defect in the presence of Tregs may not play a major role in disease. Alternatively, the decrease in absolute numbers of T cells in mice that generated disease could make suppression by Tregs inefficient assuming suppression is linked and the Treg and T cell must interact with the same APC for suppression to occur. During lymphopenia the chances of a Treg interacting with the same APC as the T cell to be suppressed could be lower. Furthermore, the functionality of the Tregs was not determined, so differences in functionality could be attributed to the differences in disease. There was a significantly lower number of T cell numbers in the spleens of mice that underwent thoracic Tx + FL.

This could be due to the inability of the cervical thymus or non thoracic thymic tissue to export and maintain sufficient numbers of T cells, or T cells could be accumulating in the LNs or infiltrating organs during disease. Overall, the reasons why T cell numbers in mice lacking thoracic thymus do not reach similar numbers in the spleen as in the presence of thoracic thymus remains unclear. In addition, there was a decrease in the amount of CD8 T cells expressing the IL-7Rα and an increase in the number of CD4 and CD8 T cells expressing CD24 implying an increase in RTEs. The increase in potential RTEs could suggest that the cervical thymus is continuously exporting T cells compared to the thoracic thymus which may decrease thymic output once a full, competent T cell repertoire is generated. However, analyzing other markers expressed on RTEs would be required to definitively conclude that these are "true" RTEs[152]. Furthermore, we determined that in the absence of the thoracic thymus a diverse T cell repertoire could be generated as assessed by the TCR Vβ's present, although not fully tolerant to self.

Next, we wanted to assess differences in cytokines to determine if any cytokines could be mediating disease. We assessed serum isotype classes since isotype switching can indicate which cytokines are present in the environment of mice. There was an increase in serum IgG1 and IgG2b suggesting an increase in IL-4 and TGF-β, respectively. Second, we looked at intracellular IFN-γ, IL-4, IL-17A and IL-10 in the spleen and LN of mice that generated disease (thoracic Tx + FL) compared to healthy mice (sham Tx + FL and BALB/c WT). There was an increase of CD4 T cells positive for IFN-γ, IL-17A and IL-10 in the spleen and

LN of thoracic Tx + FL mice. However, the mechanism for disease in mice that develop T cells in the absence of the thoracic thymus remains to be determined.

A.



B.

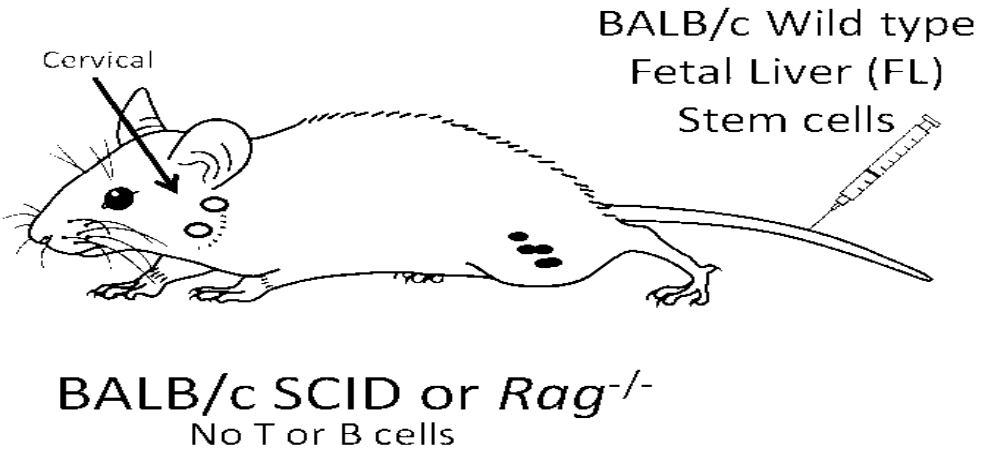


Figure 3.1. Experimental procedure used to determine the contribution of the cervical thymus to the peripheral T cell pool.
Immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mice received either a thoracic thymectomy or sham thoracic thymectomy and 10-20x10^{6} BALB/c wild type fetal liver cells from day 14-16 gestation timed pregnant BALB/c wild type mice.

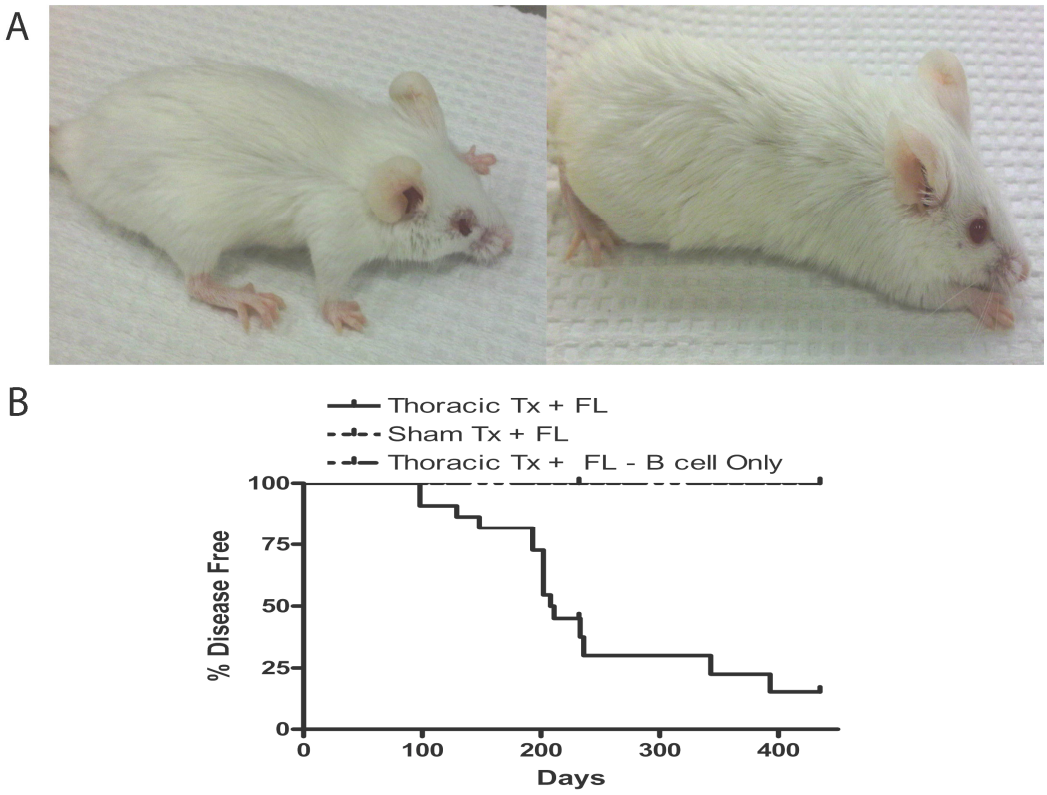


Figure 3.2. Disease induced in mice that develop T cells in the absence of the thoracic thymus.
Disease occurs in mice that develop T cells in the absence of the thoracic thymus. Mice were considered diseased when they had two or more of the following symptoms: weight loss, hunching or lethargic, swollen eyes or scruffy fur. (A) *Left Panel*: A diseased mouse that received a thoracic Tx and FL and developed T cells. *Right Panel*: A healthy mouse that received a sham thoracic Tx and FL and developed T cells. (B) The percent of mice that remained disease free after thoracic/sham thoracic Tx and FL. The solid line represents mice that received thoracic Tx and FL and developed T cells (n=21), the dotted line represents mice that underwent a sham Tx and FL and developed T cells (n=14), and the dashed line represents mice that received thoracic Tx and FL but developed B cells only (n=12). Data shown include the results of four independent experiments.

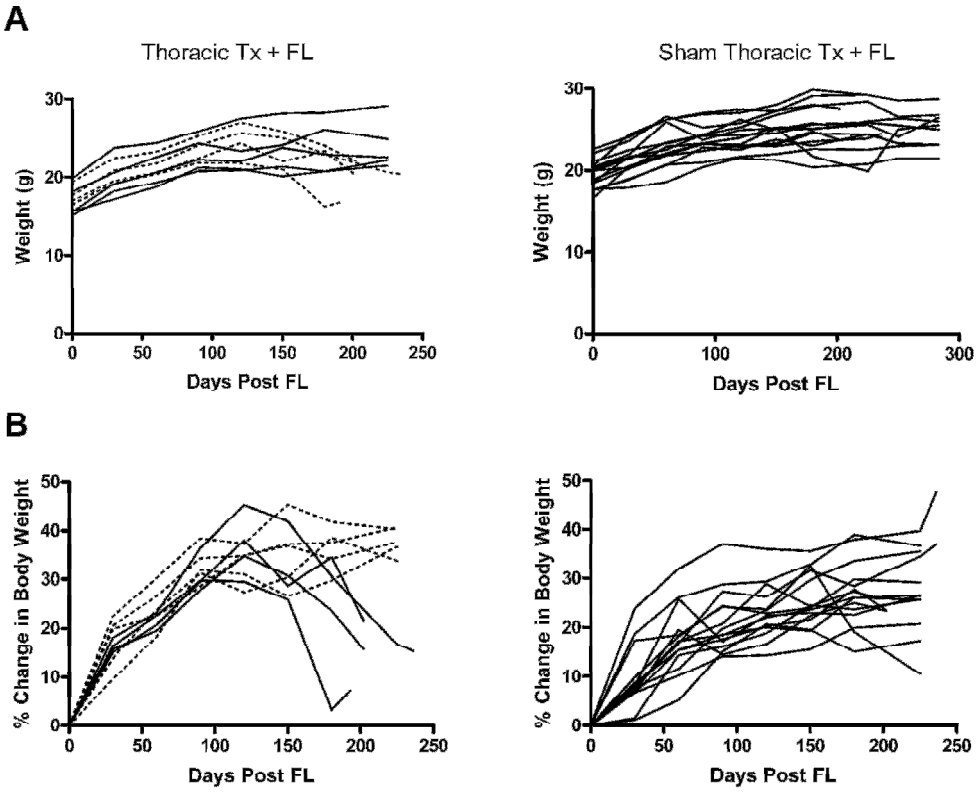


Figure 3.3. Decreased weight in mice that develop disease.
Mice that receive thoracic thymectomy, fetal liver cells, develop T cells and become diseased lose weight over time. The *left panel* represents mice that received thoracic Tx and FL (n=8). The dotted line represents mice that developed T cells but did not develop disease after thoracic Tx + FL and the solid line represents mice that underwent thoracic Tx + FL, developed T cells and developed disease. The *right panel* represents mice that received a sham Tx and FL (n=14). (A) Actual weight (g) over time. (B) Weight expressed as percent of initial body weight lost or gained over time. Data are representative of four independent experiments. Each line is representative of one mouse and weight measurements end when mice are euthanized due to disease or as controls.

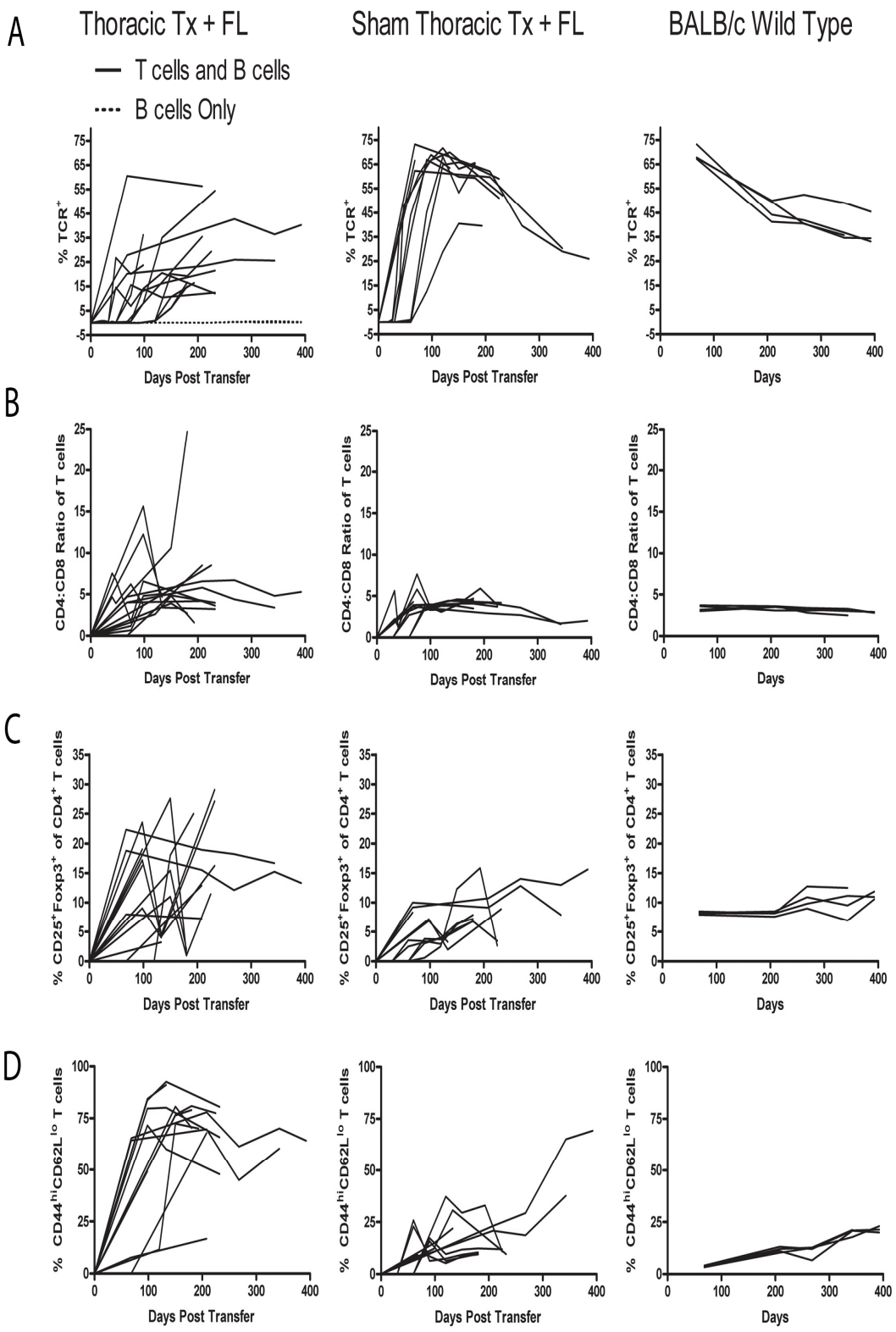

A
Thoracic Tx + FL
Sham Thoracic Tx + FL
BALB/c Wild Type
T cells and B cells
B cells Only
% TCR+
Days Post Transfer
Days
B
CD4:CD8 Ratio of T cells
C
% CD25+Foxp3+ of CD4+ T cells
D
% CD44hiCD62Llo T cells

Figure 3.4. Peripheral T cell repertoire over time in mice that develop T cells in the absence or presence of the thoracic thymus compared to wild type. Mice that receive a sham thymectomy and fetal liver have a peripheral T cell repertoire that mimics BALB/c wild type mice but mice that receive thoracic thymectomy and fetal liver have a slightly altered phenotype. All cells are gated on lymphocytes in the peripheral blood. In the Thoracic Tx + FL (*left panel*) the sold lines represent mice that underwent thoracic Tx, received fetal liver cells and generated T and B cells (n=14), and the dotted line represents the mice that did not develop any T cells but did develop B cells (n=12). The *middle panel* represents mice that underwent a sham thoracic thymectomy and received fetal liver cells (n=10) and the far *right panel* represents a group of BALB/c WT mice that were followed starting at 6 weeks of age for 393 days (n=4) (A) Percentage of cells in the periphery that are T cell determined by TCR β. (B) CD4:CD8 ratio of T cells. (C) Percentage of $CD4^+$ T cells that are Tregs based on $CD25^+$ and $Foxp3^+$ expression. (D) Percentage of T cells that are effector memory T cells ($CD62L^{lo}CD44^{hi}$).

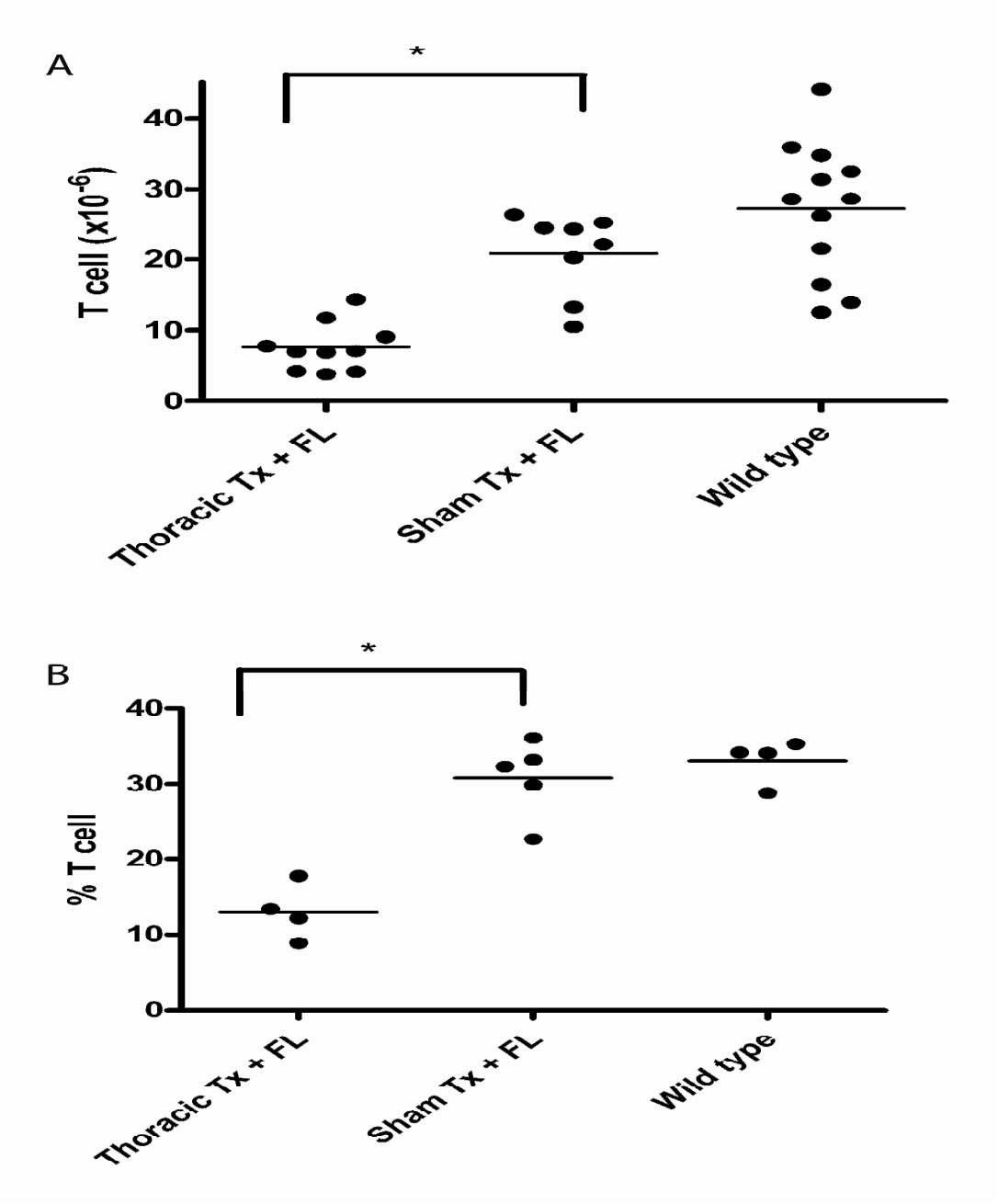


Figure 3.5. Peripheral T cell counts and phenotype in mice that get disease compared to mice that develop T cells in the presence of thoracic thymus or wild type.

Mice that received a thoracic thymectomy and fetal liver cells have a decreased number and percentage of T cells compared to sham thoracic thymectomy and fetal liver mice, and BALB/c wild type. (A) Absolute number of T cells ($x10^{-6}$) in the spleen (* $p<0.0001$). (B) Percentage of T cells (TCR $V\beta^{+}$) in the spleen. Gated on all live cells. (* $p=0.0006$). Each dot is representative of one mouse.

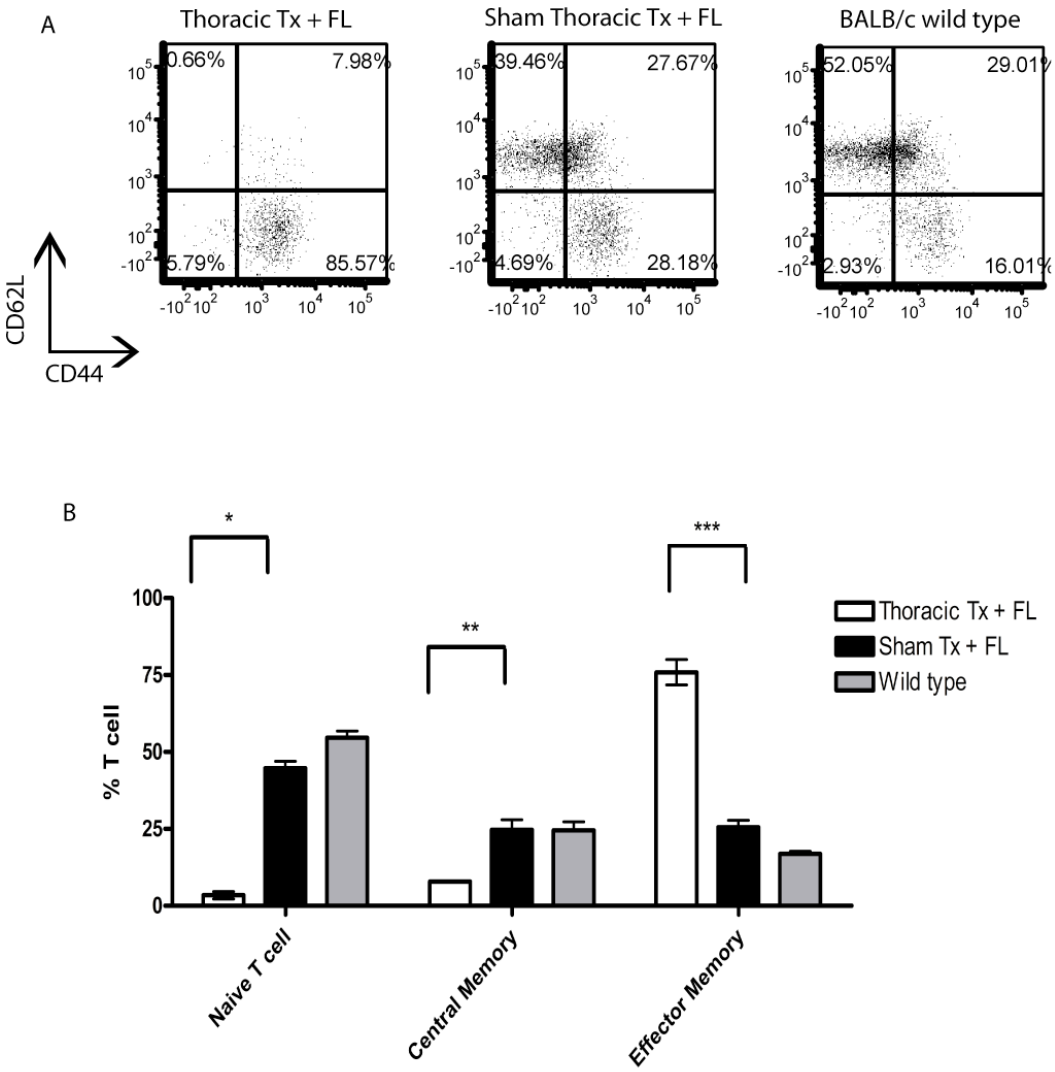


Figure 3.6. Peripheral memory T cell phenotype differs in mice that develop disease.

Mice that receive thoracic thymectomy and fetal liver have an increased percentage of effector memory T cells in their spleen. Naive T cells were defined as $CD62L^{hi}CD44^{lo}$, central memory T cells were $CD62L^{hi}CD44^{hi}$ and effector memory was $CD62L^{lo}CD44^{hi}$. (A) Representative dot plots of memory T cells gated on TCR β^{+} lymphocytes. (B) Percentage of naive, central or effector memory T cells in the spleen (mean + SEM). The white bar represents mice that received thoracic Tx + FL (n=4), the black represents sham thoracic Tx + FL (n=5) and the grey bar represents BALB/c WT (n=4). (* $p<0.0001$, ** $p=0.0027$, *** $p<0.0001$).

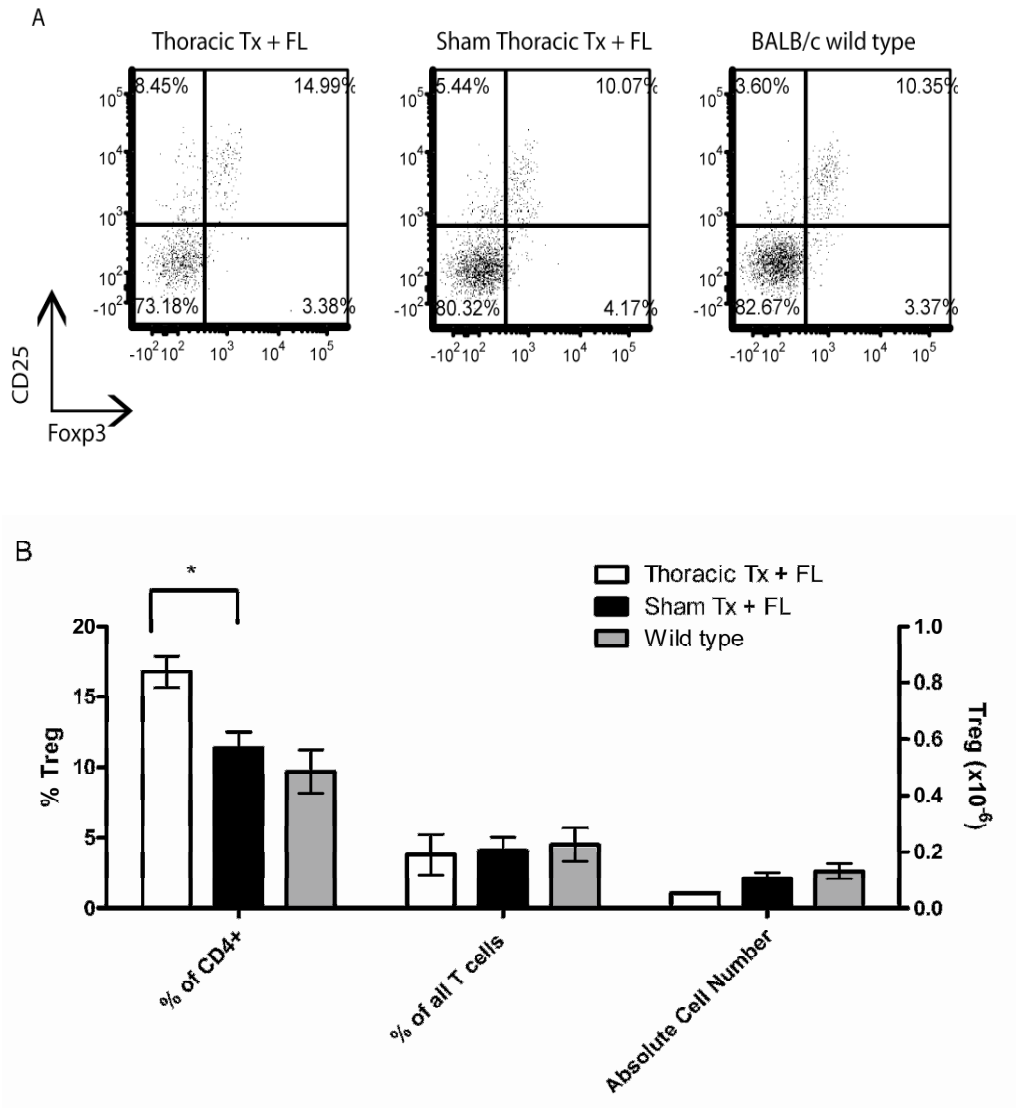


Figure 3.7. Peripheral regulatory T cell phenotype and numbers differ in mice that develop disease.

Mice that undergo thoracic thymectomy, receive fetal liver cells and generate T and B cells have an increased percentage of $CD4^+$ T cells that are Treg ($CD25^+Foxp3^+$) in their spleen. (A) Representative dot plots of Tregs in the spleen. Cells are gated on $CD4^+$ T cells. (B) Regulatory T cells in the spleen (mean + SEM). The white bar represents mice that received thoracic Tx + FL (n=4), the black represents sham thoracic Tx + FL (n=5) and the grey bar represents BALB/c WT (n=4). When analyzing Treg percentages cells are first gated on lymphocytes. The "% $CD4^+$" is gated on $CD4^+TCR\ \beta^+$ cells, "% total T cell" is gated on TCR β^+ cells alone, and "cell count" represents the total number of $CD25^+Foxp3^+$ T cells in the spleen. (* p=0.0112).

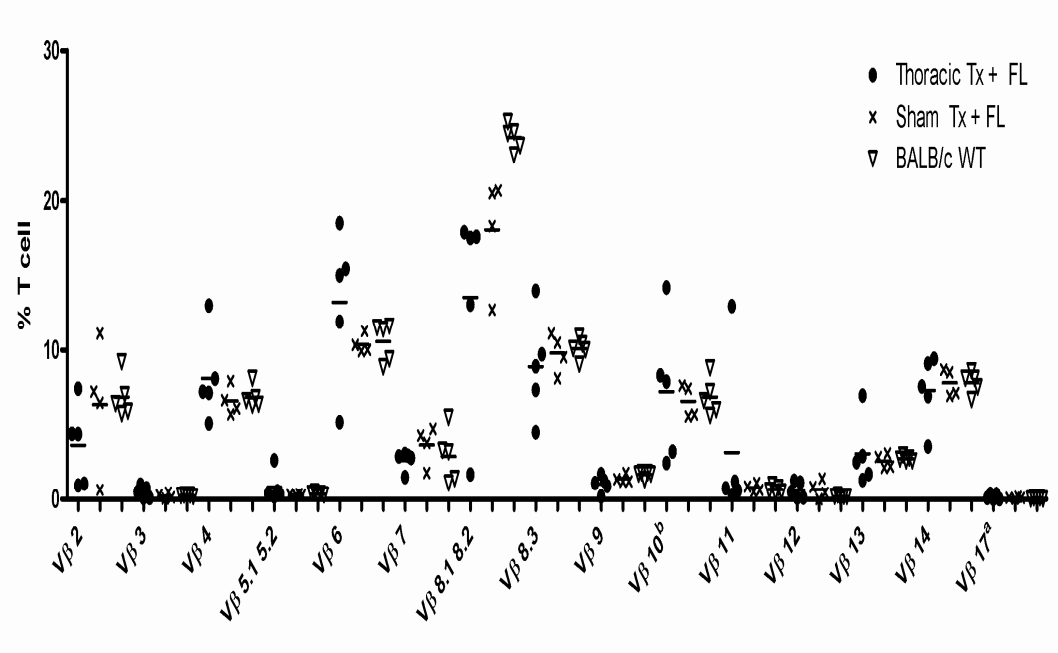


Figure 3.8. A diverse T cell Vβ repertoire is generated even in the absence of T cell development by the thoracic thymus.
Mice have a diverse TCR Vβ repertoire even in the absence of the thoracic thymus. The graph represents the percentage of T cells that are positive for each Vβ in the panel. Cells were first gated on lymphocytes in the spleen and the percent of cells positive for each Vβ was determined. From that the percentage of T cells that are positive for each Vβ was determined based on the percentage of total TCR $V\beta^+$ cells in each mouse. The black dots represent mice that received a thoracic thymectomy, fetal liver cells, generated T and B cells and got disease (n=5), the "x's" represents mice that received a sham thoracic thymectomy and fetal liver cells (n=4) and the upside down triangles represents BALB/c wild type mice (n=5). Each symbol is representative of one mouse. The mean is displayed by a black horizontal bars.

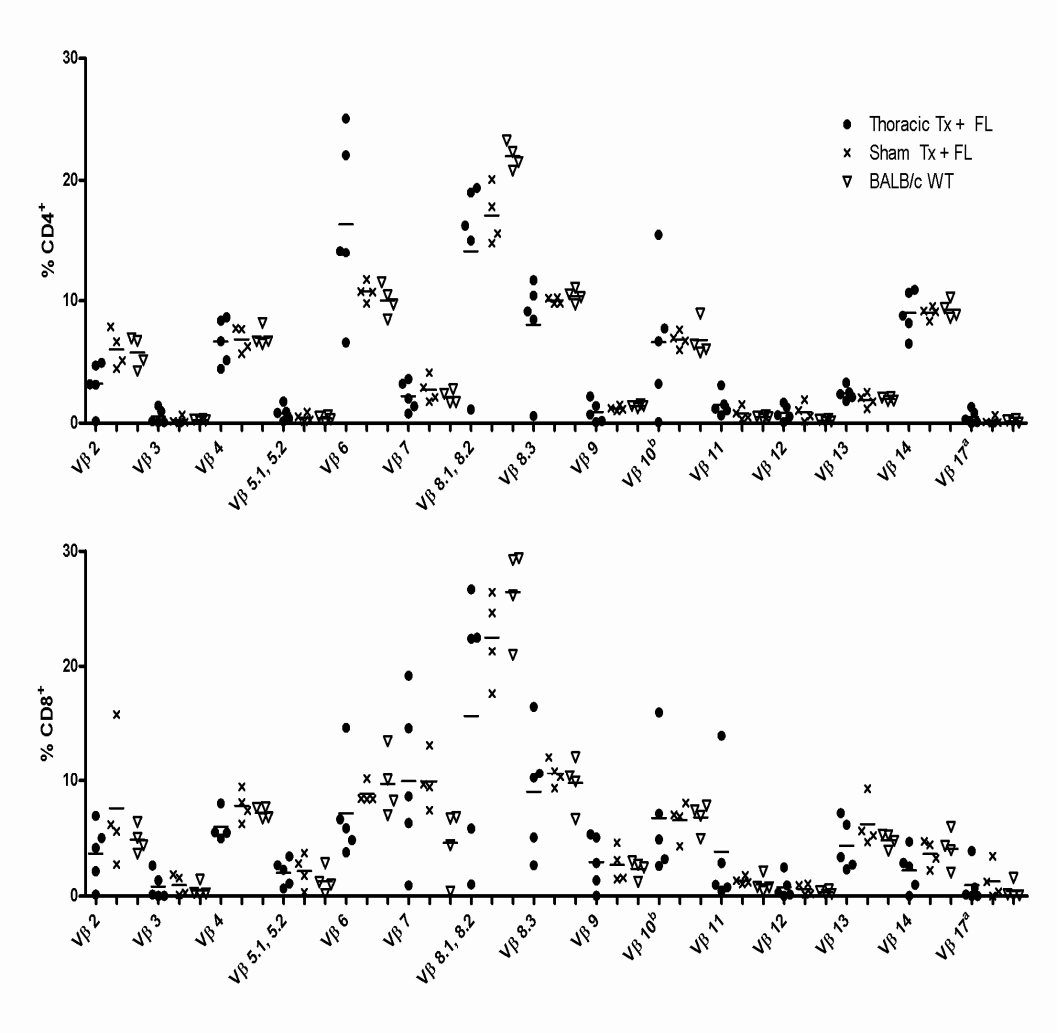


Figure 3.9. A diverse CD4 and CD8 Vβ repertoire is generated even in the absence of T cell development by the thoracic thymus.
Mice that get disease when T cells are generated in the absence of the thoracic thymus have a diverse $CD4^+$ and $CD8^+$ Vβ repertoire. (A) Percent of $CD4^+$ cells in the spleen that are positive for each Vβ. (B) Percent of $CD8^+$ cells in the spleen that are positive for each Vβ. Percentages were determined by gating on lymphocytes in the spleen then subsequently gating on $CD4^+$ or $CD8^+$ cells. The black dots represent mice that received a thoracic thymectomy, fetal liver cells, generated T and B cells and got disease (n=5), the "x's" represents mice that received a sham thoracic thymectomy and fetal liver cells (n=4) and the upside down triangles represents BALB/c wild type mice (n=5). Each symbol is representative of one mouse. The means are displayed by black horizontal bars.

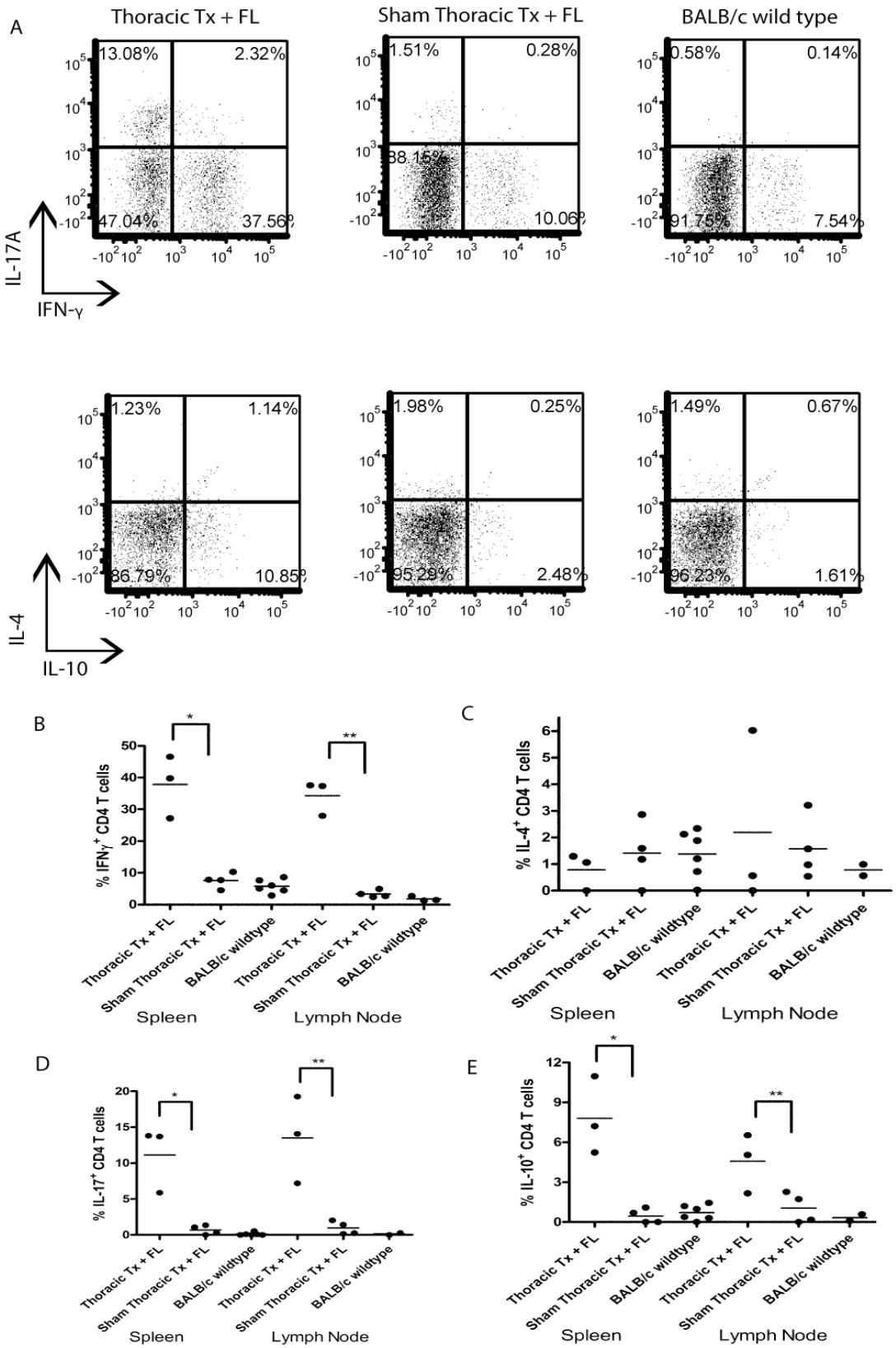
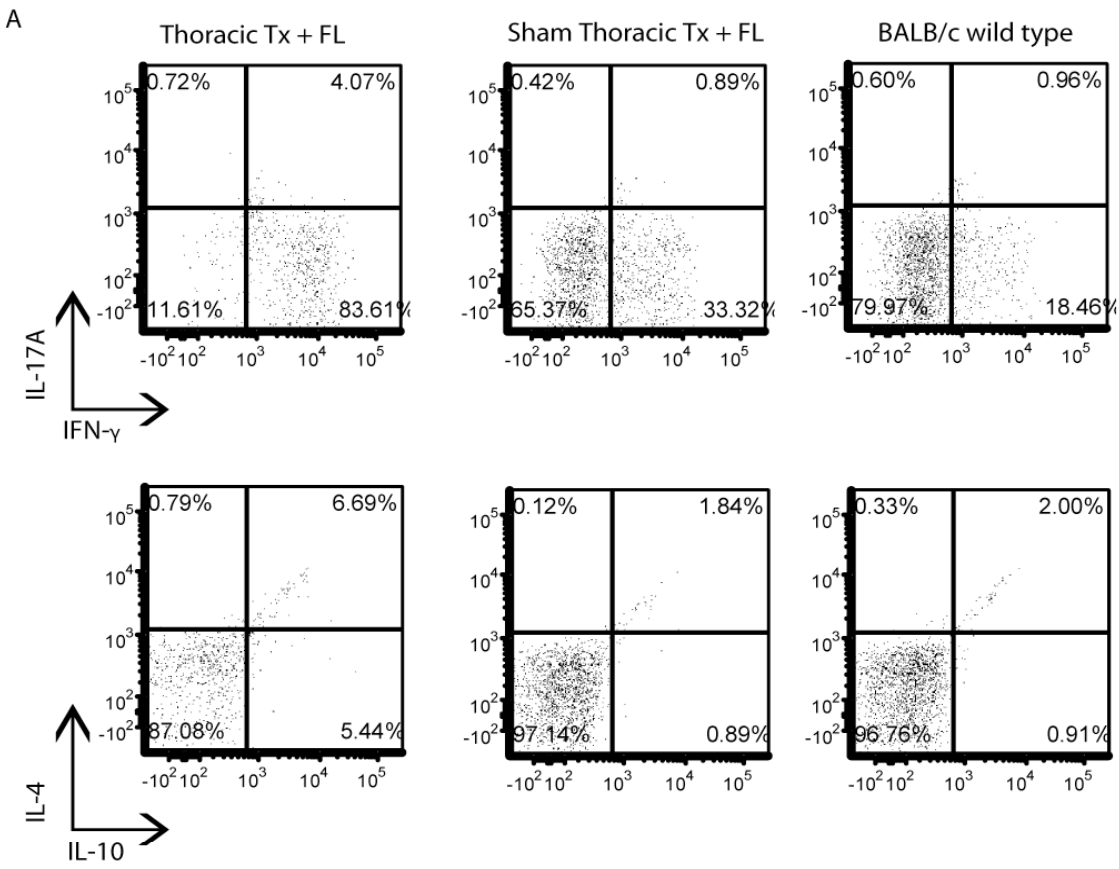

A
Thoracic Tx + FL
Sham Thoracic Tx + FL
BALB/c wild type
13.08%
2.32%
47.04%
37.56%
1.51%
0.28%
38.15%
10.06%
0.58%
0.14%
91.75%
7.54%
IL-17A
IFN-γ
1.23%
1.14%
86.79%
10.85%
1.98%
0.25%
95.29%
2.48%
1.49%
0.67%
96.23%
1.61%
IL-4
IL-10
B
% IFNγ+ CD4 T cells
C
% IL-4+ CD4 T cells
D
% IL-17+ CD4 T cells
E
% IL-10+ CD4 T cells
Thoracic Tx + FL
Sham Thoracic Tx + FL
BALB/c wildtype
Spleen
Lymph Node

Figure 3.10. Differences in intracellular cytokine profiles of CD4 T cells in mice that develop disease.
Mice that get disease after generating T cells from thoracic thymectomy and fetal liver have increased percentages of CD4+ T cells with IFN-γ, IL-17A and IL-10. Splenocytes or pooled mesenteric, inguinal and renal lymph node cells were re-stimulated for 5 hours with PMA and ionomycin in the presence of brefeldin A and monensin. (A) Representative flow cytometry dot plots for IFN-γ, IL-17A, IL-10 and IL-4 from thoracic thymectomy and fetal liver mice (n=3), sham thoracic thymectomy and fetal liver mice (n=4) and BALB/c wild type mice (n=6) gated on $CD4^+TCR\ \beta^+$ cells. (B-E) Percentage of $CD4^+$ TCR β^+ cells in the spleen (*left*) or pooled lymph nodes (*right)* that are positive for (B) IFN-γ (* p=0.0017, ** p<0.0001), (C) IL-4, (D) IL-17A (* p=0.0054, ** p=0.0083) or (E) IL-10 (* p=0.0038, ** p=0.0382). Each dot is representative of one mouse.


A
Thoracic Tx + FL
Sham Thoracic Tx + FL
BALB/c wild type
0.72%
4.07%
11.61%
83.61%
0.42%
0.89%
65.37%
33.32%
0.60%
0.96%
79.97%
18.46%
IL-17A
IFN-γ
0.79%
6.69%
87.08%
5.44%
0.12%
1.84%
97.14%
0.89%
0.33%
2.00%
96.76%
0.91%
IL-4
IL-10

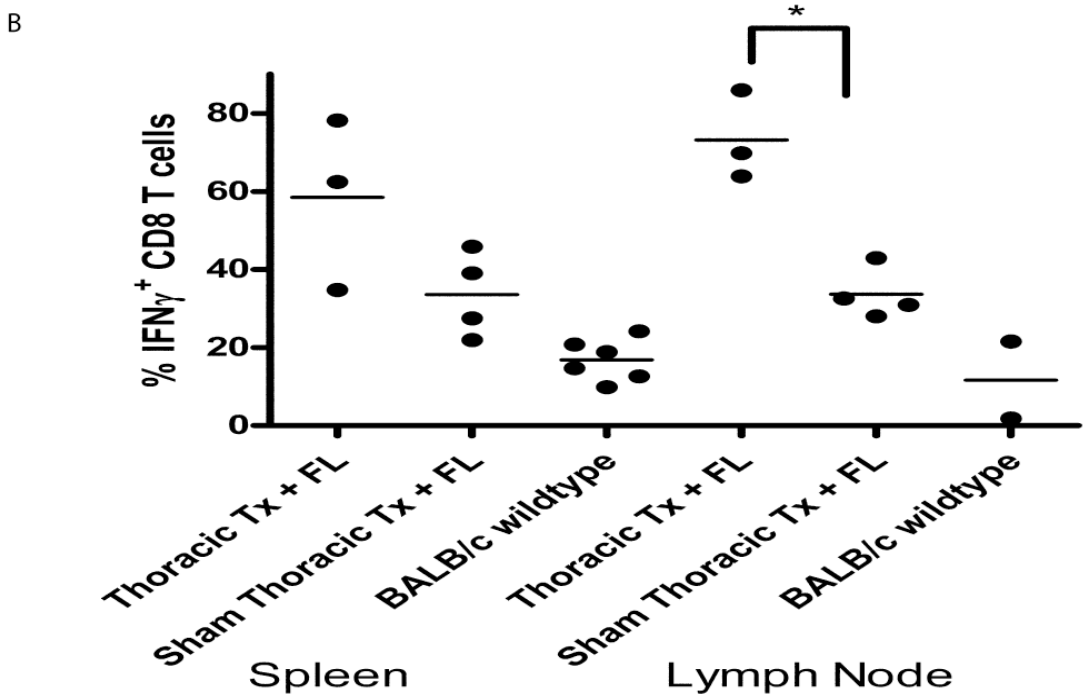

B
*
% IFNγ+ CD8 T cells
80
60
40
20
0
Thoracic Tx + FL
Sham Thoracic Tx + FL
BALB/c wildtype
Thoracic Tx + FL
Sham Thoracic Tx + FL
BALB/c wildtype
Spleen
Lymph Node

Figure 3.11. Differences in intracellular cytokine profiles of CD8 T cells in mice that develop disease.
Mice that get disease from generating T cells after thoracic thymectomy and fetal liver have increased percentages of $CD8^+$ T cells with IFN-γ in their lymph nodes. Splenocytes or pooled mesenteric, inguinal and renal lymph node cells were re-stimulated for 5 hours with PMA and ionomycin in the presence of brefeldin A and monensin. (A) Representative flow cytometry dot plots for IFN-γ, IL-17A, IL-10 and IL-4 from thoracic thymectomy and fetal liver mice (n=3), sham thoracic thymectomy and fetal liver mice (n=4) and BALB/c wild type mice (n=6) gated on $CD8^+$TCR β^+ cells. (B) Percentage of $CD8^+$ TCR β^+ cells in the spleen (*left*) or pooled lymph nodes (*right*) that are positive for IFN-γ (* p=0.0020).

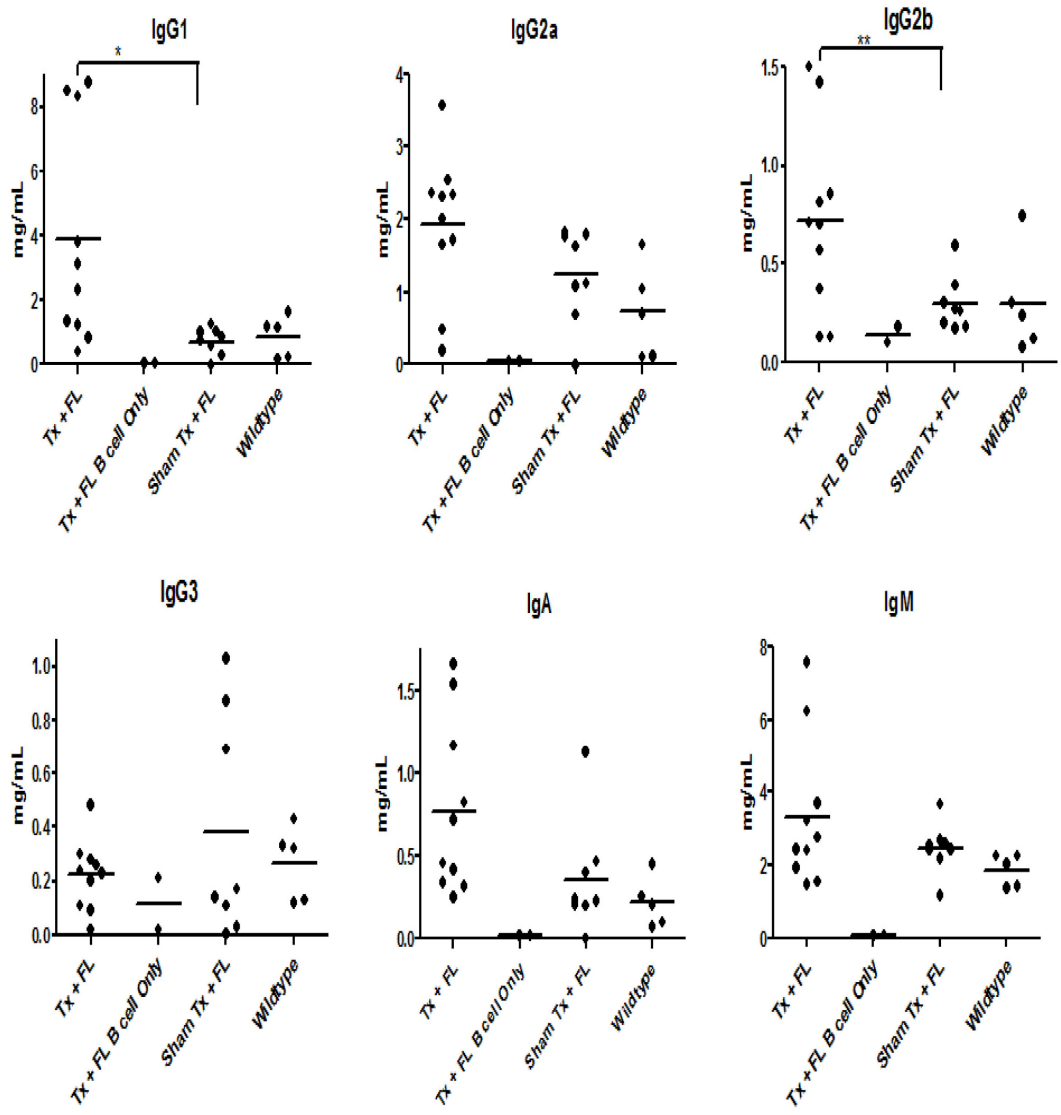


Figure 3.12. Mice that develop disease have increased IgG1 and IgG2b serum immunoglobulin isotypes.
Mice that receive a thoracic thymectomy, fetal liver and generate T cells have increased amounts of IgG1 and IgG2b in their serum. Immunoglobulin isotypes were quantified using a multiplex bead assay. Thoracic Tx + FL (n=10), thoracic Tx + FL – B cell only (n=2), sham thoracic Tx + FL (n=7), and BALB/c wild type (n=5) (* p=0.0191, ** p=0.0253). Each dot is representative of one mouse.

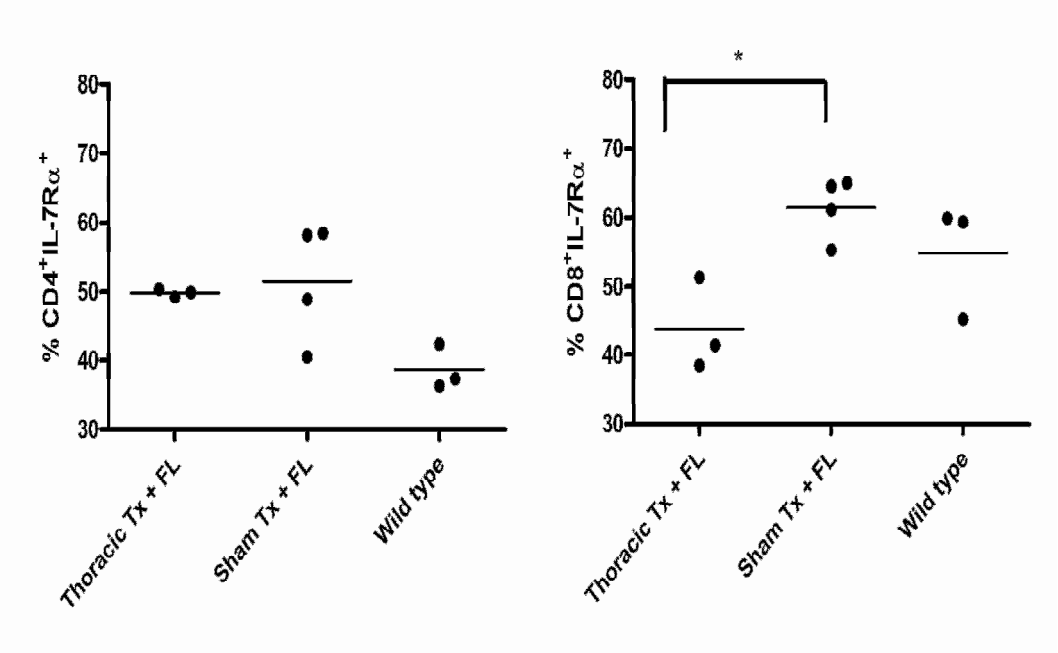


Figure 3.13. Peripheral T cell IL-7 receptor expression is lower in CD8 T cells but similar in CD4 T cells of mice that develop disease compared to mice that develop T cells in the presence of thoracic thymus.
Development of disease in mice that are thoracically thymectomized and receive fetal liver cells have a decreased percentage of $CD8^+$ T cells expressing IL-7Rα. All cells are gated on lymphocytes in spleen. The *left panel* is representative of $CD4^+$ cells and the *right panel* is representative of $CD8^+$ cells. Thoracic Tx + FL (n=3), sham thoracic Tx + FL (n=4) and BALB/c wild type (n=3). (* p=0.0083). Each dot is representative of one mouse.

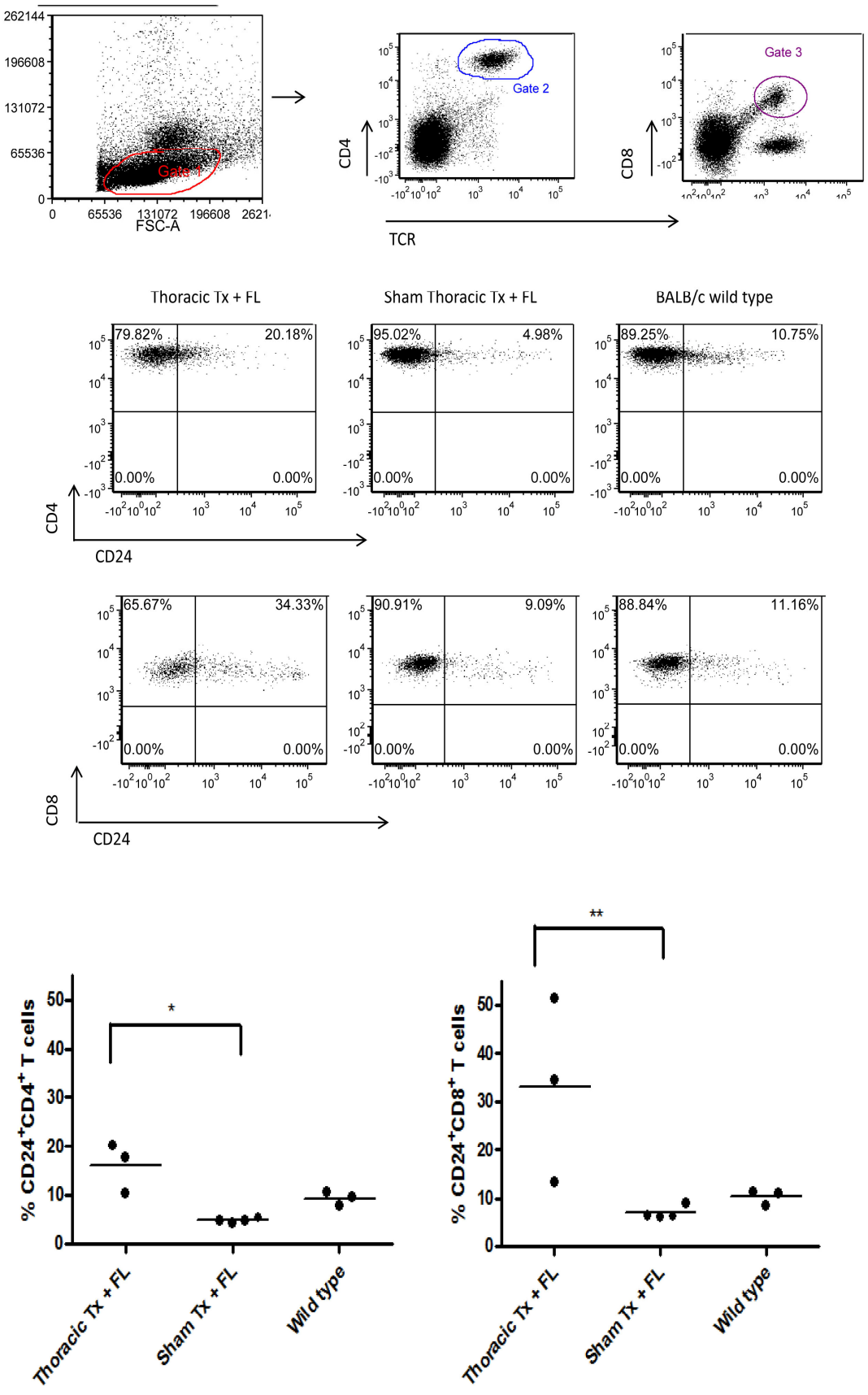

262144
196608
131072
65536
0
Gate 1
0
65536
131072
196608
2621
FSC-A
Gate 2
CD4
TCR
Gate 3
CD8
Thoracic Tx + FL
Sham Thoracic Tx + FL
BALB/c wild type
79.82%
20.18%
0.00%
0.00%
95.02%
4.98%
0.00%
0.00%
89.25%
10.75%
0.00%
0.00%
CD4
CD24
65.67%
34.33%
0.00%
0.00%
90.91%
9.09%
0.00%
0.00%
88.84%
11.16%
0.00%
0.00%
CD8
CD24
% CD24+CD4+ T cells
*
Thoracic Tx + FL
Sham Tx + FL
Wild type
% CD24+CD8+ T cells
**
Thoracic Tx + FL
Sham Tx + FL
Wild type

Figure 3.14. Peripheral T cell CD24 expression is increased in mice that develop disease.
Mice that develop disease after thoracic thymectomy and fetal liver have increased percentages of $CD4^+$ and $CD8^+$ cells expressing CD24. (A) All cells are gated on lymphocytes in spleen then subsequently gated on $CD4^+$ or $CD8^+$ cells. The *top panel* is representative of $CD4^+$ and $CD24^+$ and the *bottom panel* is representative of $CD8^+$ and $CD24^+$ cells from the spleen of Thoracic Tx + FL (n=3), sham Tx + FL (n=4) or BALB/c WT (n=3) mice. (B) The *left panel* is representative of $CD4^+$ cells and the *right panel* is representative of $CD8^+$ cells (* p=0.0066, ** p=0.0371). Each dot is representative of one mouse.

CHAPTER 4: DISEASE INDUCED BY TRANFER OF THYMOCYTES IN LYMPHOPENIC CONDITIONS IS AUGMENTED BY IL-7/ANTI-IL-7 COMPLEXES

4.1 INTRODUCTION

There could be several factors involved in disease generated when mice undergo thoracic thymectomy and develop T cells after fetal liver. First, the non thoracic thymic tissue such as the cervical thymus could be present and may not be as efficient or effective at deleting autoreactive T cells compared to the thoracic thymus[188,204,206]. Second, the RTEs exiting the non thoracic thymic tissue could be undergoing a higher rate of homeostatic proliferation and increase T cell numbers in a lymphopenic environment leading to the potential of lymphopenia-driven autoimmunity developing[264]. Furthermore, RTEs may escape central tolerance and may have the potential to be more autoreactive compared to "mature" naive T cells since they must undergo continued maturation in the periphery[152,262].

If the first possibility was correct then transfer of cervical thymocytes but not thoracic thymocytes into a lymphopenic, immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mouse should lead to disease. However, if homeostatic proliferation and lymphopenia-driven autoimmunity is a major factor for disease then mice that receive thoracic or cervical thymocytes should both develop disease. Lastly, if mice that receive splenocytes do not develop disease it may suggest that RTEs in the thymus have a higher potential for causing lymphopenia-driven autoimmunity.

However, there is the potential that thymocyte populations are capable of generating autoimmunity because of thymocytes being transferred that would normally have been deleted rather than entering the periphery. In addition, it was previously shown that thoracic thymocytes but not splenocytes could generate disease in a lymphopenic mouse[189]. The experimental design is diagrammed in **Figure 4.1**.. Furthermore, we wanted to determine factors that may affect disease. Since IL-7 is important in homeostatic proliferation of T cells[149] we wanted to determine if the addition of this factor would enhance disease.

4.2 RESULTS

a) Disease incidence in thymocyte transfer mice

Initially we hypothesized that cervical thymocytes, but not thoracic thymocytes would generate disease when transferred into an immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mouse. Therefore, we transferred whole cervical or thoracic thymocytes containing $5x10^5$ SP CD4 and CD8 T cells. To our surprise both cervical and thoracic thymocytes were capable of generating disease. We later came across a paper by Smith *et al*. (1992) which had previously shown that adult thoracic thymocytes but not splenocytes were capable of generating disease in an immunodeficient mouse[189]. Therefore, we repeated the experiment using transfer of B cell-depleted splenocytes containing $5x10^5$ T cells into immunodeficient mice and they also did not develop disease in our hands. We depleted B cells from the splenocyte population to eliminate any potential effect that B cells may be

having on the T cell population within the splenocyte population although other cell types in the population other than B cells may be having an effect. **Figure 4.2A** shows a picture of a mouse that received cervical thymocytes (*left panel*), thoracic thymocytes (*middle panel*) or B-cell depleted splenocytes (*right panel*). Mice were determined to be diseased after presenting with 2 or more of the following symptoms: weight loss, swollen eyes, scruffy fur, or hunching and lethargy. The development of disease was not very efficient and took anywhere from 120-180 days post transfer. **Figure 4.2B** shows the prevalence of disease, and 2/3 mice that received cervical thymocytes and 5/10 mice that received thoracic thymocytes generated disease. However, all of the mice that received B cell-depleted splenocytes remained healthy. Mice that developed disease lost weight over time similar to mice that received thoracic Tx + FL in the previous chapter (**Figure 4.3**). Thymocyte recipients had a tendency to lose or maintain weight whereas splenocyte recipients had a tendency to gain weight. Even though not all thymocyte recipients developed disease it is possible that if the time point was extended that they too would develop disease.

b) Augmentation of disease in thymocyte transfer mice using IL-7/anti-IL-7 complexes

Based on the previous chapter it appears that generation of disease in mice that develop T cells in the absence of the thoracic thymus and presumably through the cervical thymus (thoracic Tx + FL) was unlikely due to increased escape of autoreactive T cells. This is because thymocyte transfers from either the thoracic

or cervical thymus into an immunodeficient, lymphopenic mouse caused disease. Therefore, it seems more likely that increased homeostatic proliferation in the absence of the thoracic thymus may be increasing the autoreactive T cell clone frequency of T cells that escape central deletion in the thymus and enter into the periphery. The resulting disease may be a form of lymphopenia-driven autoimmunity. Since IL-7 is an important cytokine in homeostatic proliferation we wanted to determine if the addition of IL-7 had an effect on disease. However, it was possible that IL-7 might have no effect in the already lymphopenic environment of an immunodeficient mouse since the lack of T and B cells to consume available IL-7 already increases the basal levels of IL-7[127,291]. On the other hand, the addition of IL-7 could increase the amount and dose of IL-7 available to each T cell in the lymphopenic environment.

Therefore, we transferred $5x10^5$ SP CD4 and CD8 T cells from whole thoracic thymocytes or $5x10^5$ T cells from B-cell depleted splenocytes into an immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mouse. On days 1, 4 and 7 the mice were given IL-7/anti-IL-7 complexes I.P.[134]. Thoracic thymocytes were used instead of cervical thymocytes in the experiment since the previous experiment did not indicate any significant differences between the two populations in the disease model. Similar to the previous experiments, mice that received thymocytes and IL-7/anti-IL-7 complexes developed disease (5/9) (**Figure 4.4A**; *left panel*), but mice that received B cell-depleted splenocytes and IL-7/anti-IL-7 complexes (n=9) still remained healthy (**Figure 4.4A**; *right panel*). We found that IL-7/anti-IL-7 complexes given to thymocyte recipients did not increase the

severity of disease, but it accelerated the onset of disease (**Figure 4.3 compared to 4.4**). Mice that generate disease lose weight as disease progresses similar to without the IL-7/anti-IL-7 complexes and splenocyte and IL-7/anti-IL-7 recipients maintain their weight (**Figure 4.5**).

c) T cell phenotype in the peripheral blood

There were two questions we attempted to answer in the following experiments. First, why do thymocytes but not splenocytes cause disease when transferred into a lymphopenic host? Secondly, what is the effect of IL-7/anti-IL-7 complexes that causes increased onset of disease?

To begin, we monitored the peripheral repertoire in the blood of mice over time after transfer. **Figure 4.6** shows the peripheral T cell repertoire after thymocyte transfer on the *left panel* and after splenocyte transfer on the *right panel*. The solid line indicates mice that have not received IL-7/anti-IL-7 complexes and the dotted line indicates mice that have received these complexes. There were no drastic differences in any of the populations for mice receiving thymocytes or splenocytes and/or IL-7/anti-IL-7 complexes when it came to the peripheral phenotype. All mice had similar percentages of T cells (**Figure 4.6A**), CD4:CD8 ratio (**Figure 4.6B**), and effector memory T cells (**Figure 4.6C**). However, there was a slight increase in the percentage of CD4 T cells that were Tregs ($CD25^+Foxp3^+$) in the majority of thymocyte recipients with or without IL-7/anti-IL-7 complexes compared to splenocyte recipients (**Figure 4.6D**). Many of the thymocytes recipients had over 20% of their CD4 T cells being $CD25^+Foxp3^+$

whereas splenocyte recipients had only 10-20% of their CD4 T cells expressing a Treg phenotype.

d) T cell phenotype in the spleen

Next, we assessed whether or not there were any differences in the T cell phenotype in the spleen of thymocytes or splenocyte transfer recipients with or without IL-7/anti-IL-7 complexes. There were no significant differences in cell number or percentage of T cells, CD4 T cells, CD8 T cells or Tregs in the spleen (**Figure 4.7**). Although not significant, there was a small increase in cell number and percentage of T cells in thymocyte recipients receiving IL-7/anti-IL-7 complexes compared to thymocytes alone (**Figure 4.7 and 4.8A**). Furthermore, there was also a slight increase in total numbers of CD4 and CD8 T cells likely due to the small increase in total T cell numbers of thymocyte recipients receiving IL-7/anti-IL-7 complexes, but the percentage of CD4 and CD8 remained the same. We wanted to determine the absolute number of Tregs in the spleens of mice with or without IL-7/anti-IL-7 complexes since there has been indications that IL-7 may selectively "deplete" Tregs due to their low expression of the IL-7R[150,153]. This low expression of the IL-7R may leave Tregs at a disadvantage to respond and proliferate to IL-7 compared to other T cells that express higher levels of the IL-7R. There were similar percentages of CD4 T cells that were Tregs in the spleens of thymocyte recipients with or without IL-7/anti-IL-7 complexes. Nevertheless, there was a slight increase in absolute numbers of Tregs in the mice that received thymocytes and IL-7/anti-IL-7 complexes again likely

due to the increase in absolute numbers of T cells (**Figure 4.7 and 4.8B**). Changes in the CD4:CD8 ratios have been a suggested as a potential effect of IL-7 on T cells[146]. However, in our mice the addition of IL-7/anti-IL-7 complexes does not alter the CD4:CD8 ratio in thymocyte or splenocyte recipients with or without IL-7/anti-IL-7 complexes (**Figure 4.9**).

e) Intracellular cytokines

The addition of IL-7 in mice can promote Th17 skewing of CD4 T cells[155]. Therefore, we analyzed T cells for intracellular cytokine presence of IL-17A as well as IFN-γ, IL-4 and IL-10 in transfer mice. **Figure 4.10A** shows a representative dot plot of the intracellular cytokines detected in mice that receive thymocytes or splenocytes along with IL-7/anti-IL-7 complexes. A decrease in CD4 T cells expressing IFN-γ was detected in splenocyte populations with IL-7/anti-IL-7 complexes compared to splenocytes alone, but neither of these populations generates disease in mice (**Figure 4.10B**; *top left panel*, p=0.36). A decrease in IL-4 was detected in thymocyte recipients compared to splenocyte recipients which could be a factor in disease (**Figure 4.10B**; *top right panel*, * p=0.038). However, there was no significant difference in IL-4 between thymocytes or splenocytes with IL-7/anti-IL-7 complexes, suggesting that this difference may not be a significant factor in disease. There were also no differences in IL-10 in any of the CD4 T cell populations of transfer recipients (**Figure 4.10**; *bottom right panel*). Lastly, we looked at the presence of IL-17A in CD4 T cells. The addition of IL-7/anti-IL-7 complexes generated an increased

percentage of CD4 T cells expressing IL-17A in splenocytes populations (** p=0.0105), but there were no significant differences between thymocytes recipients with or without IL-7/anti-IL-7 complexes (**Figure 4.10B**; *bottom left panel*). There was, however, an increase in IL-17A in the splenocyte compared to the thymocyte recipients in mice that received IL-7/anti-IL-7 complexes (* p=0.0207). But, since the splenocyte recipients remain healthy this increase in IL-17A does not explain the differences seen in disease although it does not completely exclude the potential importance of IL-17A in disease or as a protective cytokine. The only intracellular cytokine detected in CD8 T cells was IFN-γ (**Figure 4.11**). Unexpectedly, there was a decrease in the percentage of CD8 T cells that contained IFN-γ with the addition of IL-7/anti-IL-7 complexes, although only the difference between the two splenocyte populations were significantly different (* p=0.0119).

f) CD4 T cell organ infiltration

Several organs were analyzed for CD4 T cell infiltration to determine if there were differences between mice that received thoracic thymocytes and IL-7/anti-IL-7 complexes compared to mice that received B cell-depleted splenocytes and IL-7/anti-IL-7 complexes. Organs from mice that received thymocytes or B cell-depleted splenocyte without IL-7/anti-IL-7 complexes were not examined. The following organs were assessed for CD4 cells: heart, lung, liver, stomach, large intestine, small intestine, ovary, and pancreas. **Figure 4.12** shows a representative image of the organs from thymocyte + IL-7/anti-IL-7 mice on the *left*, splenocyte

+ IL-7/anti-IL-7 in the *middle* and BALB/c WT on the *right.* Surprisingly, there was CD4 staining detected in several organs of the mice that received either thymocytes or splenocytes + IL-7/anti-IL-7 complexes, yet only the mice that received thymocytes developed disease. Consequently, there were no differences between CD4 staining of organs from thymocyte or splenocyte that could explain disease. Nevertheless, the phenotype of these CD4 T cells was not determined; therefore, it is possible that a difference in phenotype of CD4 infiltrating cells rather than the presence of CD4 cells in the organ could explain a difference in disease susceptibility. Moreover, it is possible that the major organ affected by disease was not assessed (e.g. thyroid). **Table 4.1** summarizes the number of mice from each group that had CD4 cells present in the organ evaluated. Overall, there were major differences in presence of CD4 cells between both groups of transfer recipients compared to BALB/c WT mice in the lung, liver and pancreas. In these organs, there were no CD4 cells detected in WT but they were detected in the transfer mice.

g) Initial effect of IL-7/anti-IL-7 complexes on thymocytes and splenocytes

Previous experiments suggested that the addition of IL-7 had little effect on T cell numbers and phenotype after transfer. Therefore, we sought to determine if there was an initial effect of IL-7/anti-IL-7 complexes on thymocytes or splenocytes. As in the previous experiments, BALB/c SCID mice were given whole thymocytes I.V. containing $5x10^5$ SP thymocytes or B cell-depleted splenocytes containing $5x10^5$ T cells then given IL-7/anti-IL-7 complexes on days 1, 4 and 7

or PBS as a control. At day 10 post-transfer, mice were sacrificed and their spleens were evaluated for total T cell numbers and expression of IL-7Rα.

We looked at the absolute number of T cells along with the total number of CD4 T cells compared to CD8 T cells. The addition of IL-7/anti-IL-7 complexes lead to an increase in total number of T cells after 10 days in recipients of thymocytes or splenocytes (**Figure 4.13A**; * p=0.0174, ** p=0.0276). The average number of T cells in mice that were recipients of thymocytes alone was under $5x10^5$ compared to mice that received IL-7/anti-IL-7 complexes in addition to thymocytes and had numbers almost double at around $1x10^6$ T cells. T cells from the splenocyte population appear to have either increased proliferation or survival since even without IL-7/anti-IL-7 complex their numbers were $1x10^6$ T cells on average after 10 days post transfer. Once the IL-7/anti-IL-7 was given these numbers increased to almost $2x10^6$, however. The addition of IL-7/anti-IL-7 complexes in either thymocyte or splenocyte recipients lead to increases in CD4 T cells in some mice, but this increase was not statistically significant. Comparing CD8 T cell numbers revealed that with the addition of IL-7/anti-IL-7 complexes an increase in numbers in only a few splenocyte recipient mice occurred, but the majority of the recipients had no differences in their CD8 T cell numbers. On the other hand, there was an increase in CD8 T cell numbers in thymocyte recipients that received IL-7/anti-IL-7 complexes compared to without (* p=0.022).

Next, we wanted to determine the effect of IL-7/anti-IL-7 complexes on the IL-7Rα expression on T cells in the spleen of thymocyte or splenocyte recipients 10 days post transfer. CD4 T cells from splenocyte, thymocyte + IL-

7/anti-IL-7 or splenocyte + IL-7/anti-IL-7 recipients had similar IL-7Rα expression compared to splenic T cells from BALB/c WT (**Figure 4.14**; *left panel*). However, IL-7Rα expression on thymocyte recipients alone was significantly lower compared to thymocyte recipients given IL-7/anti-IL-7 complexes (**Figure 4.14**; *p=0.0172). Whether or not the IL-7Rα is being upregulated in CD4 T cells, by the addition of IL-7/anti-IL-7 complexes, remains to be determined. However, in the CD8 population of T cells the IL-7Rα seems to by downregulated in thymocyte recipients given IL-7/anti-IL-7 complexes (**Figure 4.14**; *right panel*; ** p=0.0172).

4.3 DISCUSSION

Transfer of thymocytes but not splenocytes into immunodeficient, lymphopenic mice leads to generation of disease in mice similar to thoracic Tx + FL mice. Disease in thymocyte transfer mice was augmented by the addition of IL-7/anti-IL-7 complexes; however, splenocyte recipients still remained healthy. The generation of disease was inefficient and took anywhere from 120-180 days post transfer. In addition, only around 50% of thymocyte transfer mice with or without IL-7/anti-IL-7 complexes developed disease. Disease severity was not increased by the use of IL-7/anti-IL-7 complexes; however, the time to disease was largely decreased with these complexes. Mice that developed disease had similar symptoms as in the previous set of experiments. They become hunched and lethargic, developed scruffy fur, swollen eyes and/or weight loss.

We examined the T cell phenotype to determine if there were any differences between thymocyte or splenocyte transfer mice or if the addition of IL-7/anti-IL-7 complexes leads to any differences. However, the addition of IL-7/anti-IL-7 complexes had no significant effect on T cells from either thymocyte or splenocyte transfer mice in the periphery. There was no decrease in the percentage of CD4 T cells with a Treg phenotype between thymocyte and splenocyte transfer mice, and if anything there was a slight increase in Tregs in thymocyte transfer mice with and without IL-7/anti-IL-7 complexes. Furthermore, there was a slight increase in Tregs in the spleens of thymocyte transfer mice with the addition of IL-7/anti-IL-7 complexes, but there was no difference in the splenocyte recipient groups. The ratio of CD4:CD8 T cells were similar in all mice in the periphery and spleen. In terms of the cytokines generated there were no substantial differences that could explain why thymocyte recipient mice get disease but not splenocyte recipients, or why the addition IL-7/anti-IL-7 complexes have an effect in disease.

In order to determine the initial affect of IL-7/anti-IL-7 complexes on both thymocytes and splenocytes transfer recipients we euthanized mice at day 10 post transfer to assess the splenic T cell phenotype. We found that IL-7/anti-IL-7 complexes enhanced the number of T cells in both splenocyte and thymocyte transfer recipients. There was an increase in the number of CD4 T cells in some of the mice in both groups receiving IL-7/anti-IL-7 complexes although not statistically significant. In terms of CD8 T cells, there was an increase in the overall numbers of CD8 T cells in thymocyte transfer recipients with the addition

of IL-7/anti-IL-7 complexes. However, there were only a small minority of splenocyte transfer recipients that had increased CD8 T cell numbers with the addition of IL-7/anti-IL-7 complexes. Overall, the majority of the mice had similar CD8 T cell numbers as splenocyte recipients without IL-7/anti-IL-7 complexes. The overall effect of IL-7/anti-IL-7 complexes on the IL-7Rα was also analyzed. The expression of IL-7Rα in thymocyte recipients on CD4 T cells was higher with the complexes; however, the expression on CD8 T cells was lower with the complexes. On the other hand, there were no significant differences on the expression of the IL-7Rα on CD4 or CD8 T cells in splenocyte transfer recipients with or without the IL-7/anti-IL-7 complexes.

Overall, the reasons for the differences in disease seen in thymocyte compared to splenocyte transfer recipients remains to be determined. There may be differences in the frequency of autoreactive T cells in these populations, or there may be a subset of cells within the splenocyte population suppressing disease. The addition of IL-7/anti-IL-7 complexes decreased the onset of disease and initially lead to an increase in T cell numbers compared to in the absence of the complexes after 10 days. If the thymocyte population contains an increased frequency of autoreactive clones the addition of the IL-7 complexes could simply be increasing their frequency at a faster rate. In conclusion, more experiments need to be done to really elucidate the role of IL-7/anti-IL-7 complexes in the disease seen after thymocyte transfer.

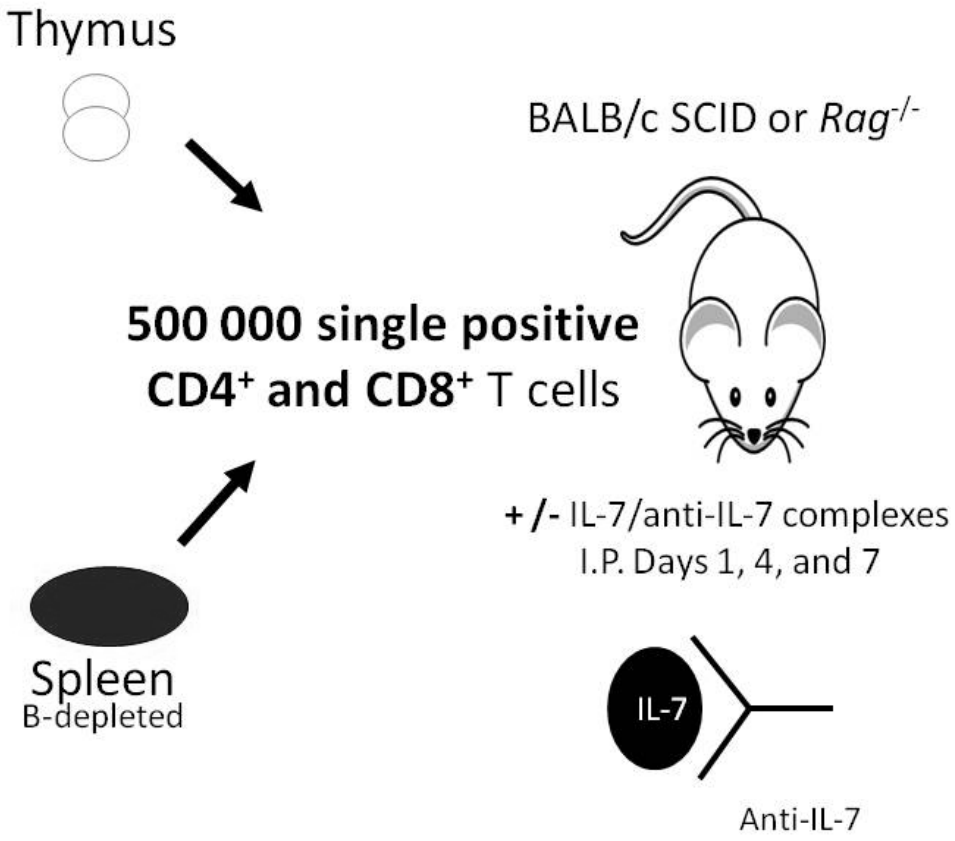


Figure 4.1. Experimental procedure used to compare the transfer of BALB/c wild type thymocytes (thoracic or cervical) or splenocytes into immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mice.
Mice were I.V. injected with whole thymocytes containing $5x10^5$ SP CD4 and CD8 T cells or $5x10^5$ T cells from B cell-depleted splenocytes with or without IL-7/anti-IL-7 complexes given I.P. on days 1, 4 and 7.

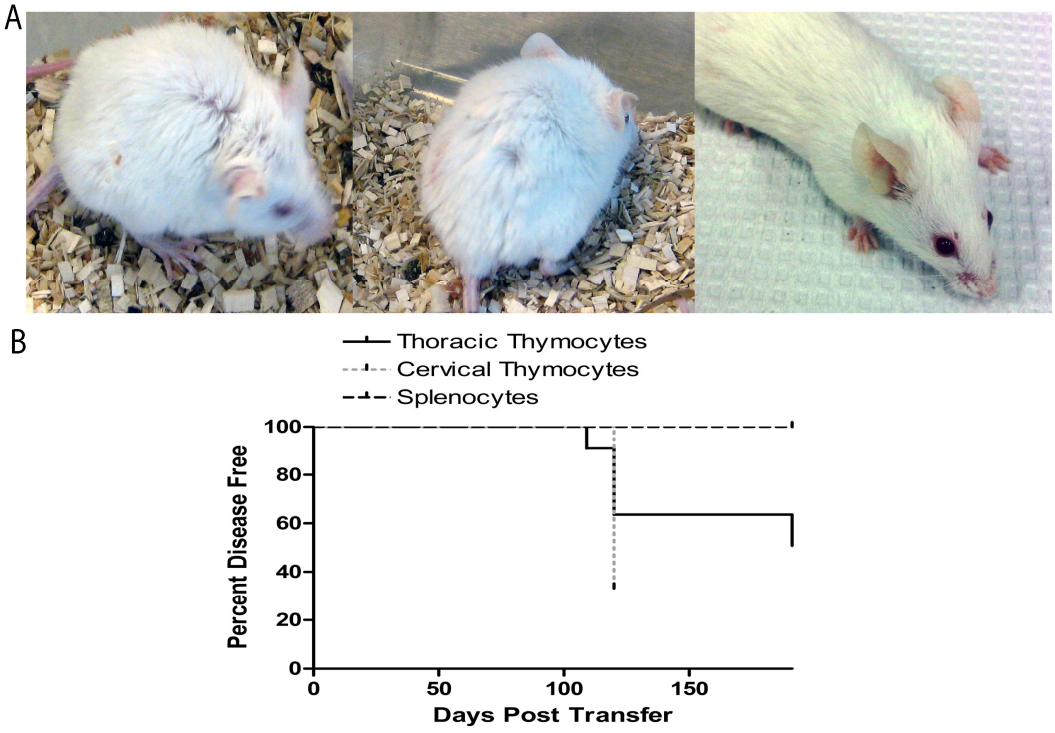


Figure 4.2. Disease induced by thymocyte transfers.
Adoptive transfer of cervical or thoracic thymocytes, but not splenocytes generates disease in immunodeficient, lymphopenic mice. (A) A mouse that received cervical thymocytes (*left panel*), a mouse that received thoracic thymocytes (*middle panel*) and a mouse that received B cell-depleted splenocytes (*right panel*). (B) Disease incidence in mice that received cervical thymocytes (n=3), thoracic thymocytes (n=10), or splenocytes (n=5). Data includes one experiment for cervical thymocyte and splenocyte transfers and two independent experiments for thoracic thymocyte transfers.

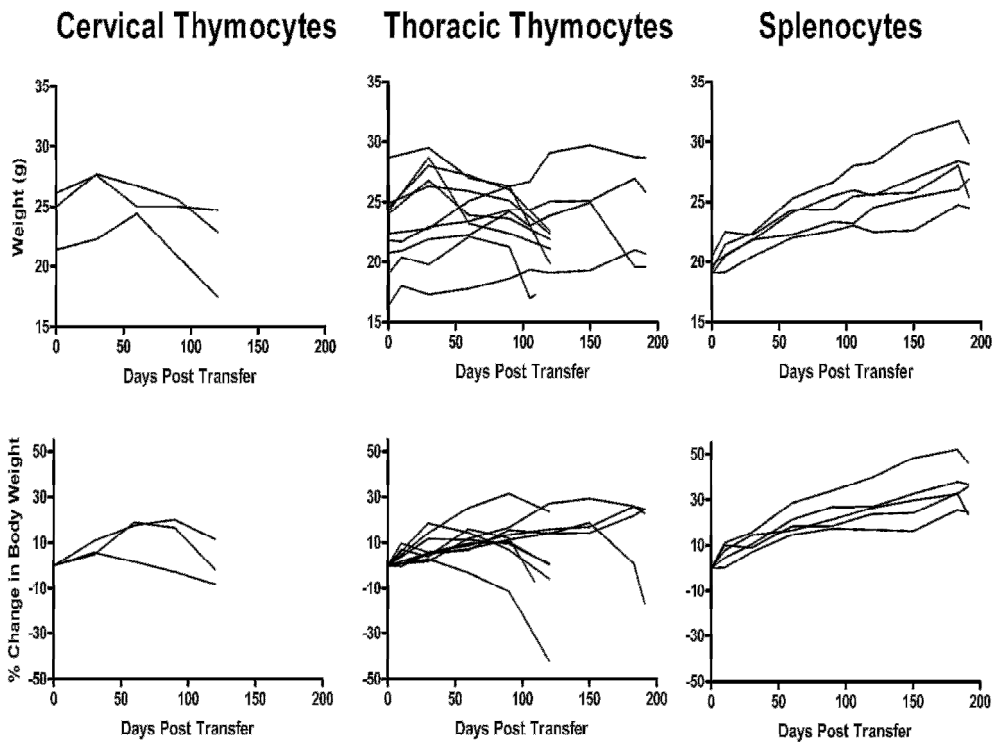


Figure 4.3. Weight loss in thymocyte transfer mice.
Mice that receive cervical or thoracic thymocytes but not splenocytes lose weight with disease. (A) Weight (g) of mice that receive cervical thymocytes (n=3), thoracic thymocytes (n=10) or splenocytes (n=5) after transfer. (B) Percent change in the initial body weight of mice after transfer. Each line is representative of one mouse. Weight measurements cease at the endpoint for that particular mouse determined by disease or use as a control.

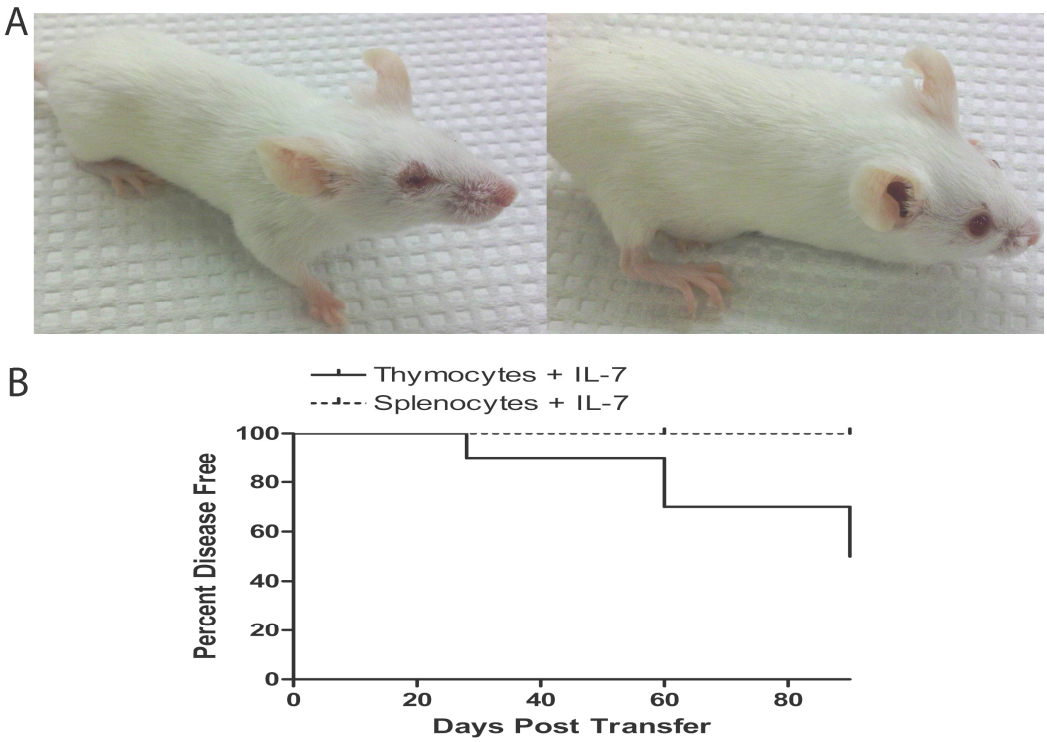


Figure 4.4. Disease augmentation in thymocyte transfer mice with IL-7/anti-IL-7 complexes.
Immunodeficient mice that receive transfer of thoracic thymocytes but not splenocytes in addition to IL-7/anti-IL-7 complexes get enhanced disease. (A) A diseased mouse that received thoracic thymocytes (*left panel*) and a healthy mouse that received splenocytes (*right panel*) in addition to IL-7/anti-IL-7 complexes on days 1, 4, and 7 post transfer. (B) Disease incidence in mice that receive thoracic thymocytes and IL-7/anti-IL-7 complexes (n=9) compared to mice that receive splenocytes and IL-7/anti-IL-7 complexes (n=9). This data includes two independent experiments.

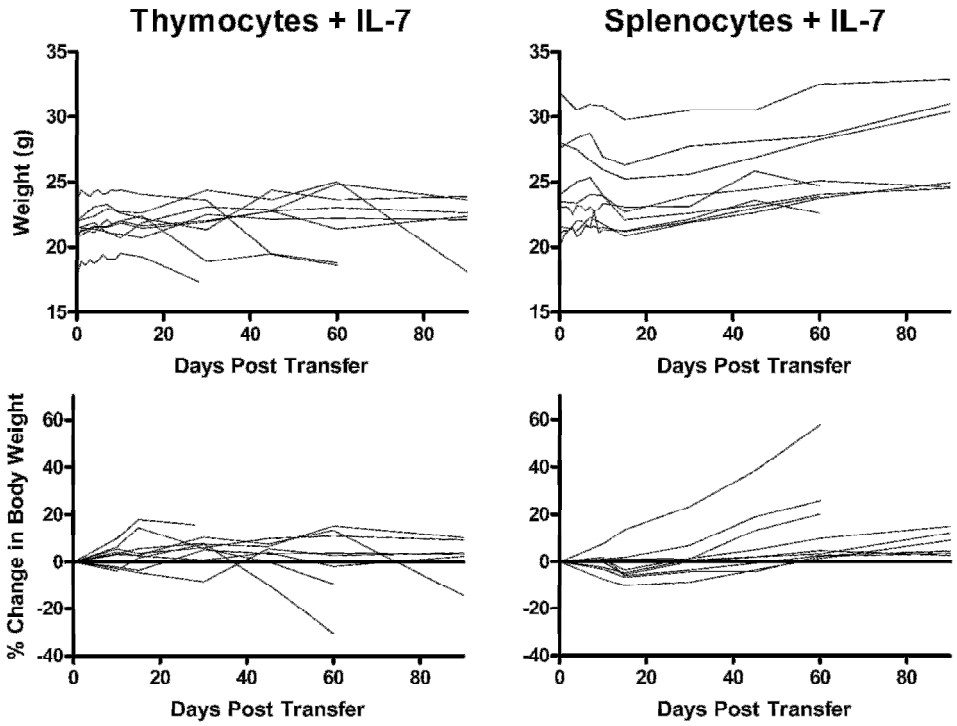


Figure 4.5. Weight loss in thymocyte transfer mice receiving IL-7/anti-IL-7 complexes.

Immunodeficient mice that receive transfer of thoracic thymocytes and IL-7/anti-IL-7 complexes but not splenocytes and IL-7/anti-IL-7 complexes lose weight with disease. Immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mice were given thoracic thymocytes (n=9) or splenocytes (n=9) in addition to IL-7/anti-IL-7 complexes on days 1, 4, and 7 post transfer. (A) Weight (g) of mice post transfer. (B) Weight expressed as percent change in initial body weight of mice post transfer. Each line is representative of one mouse. Cessation of weight measurements represents the endpoint in the experiment for that particular mouse.

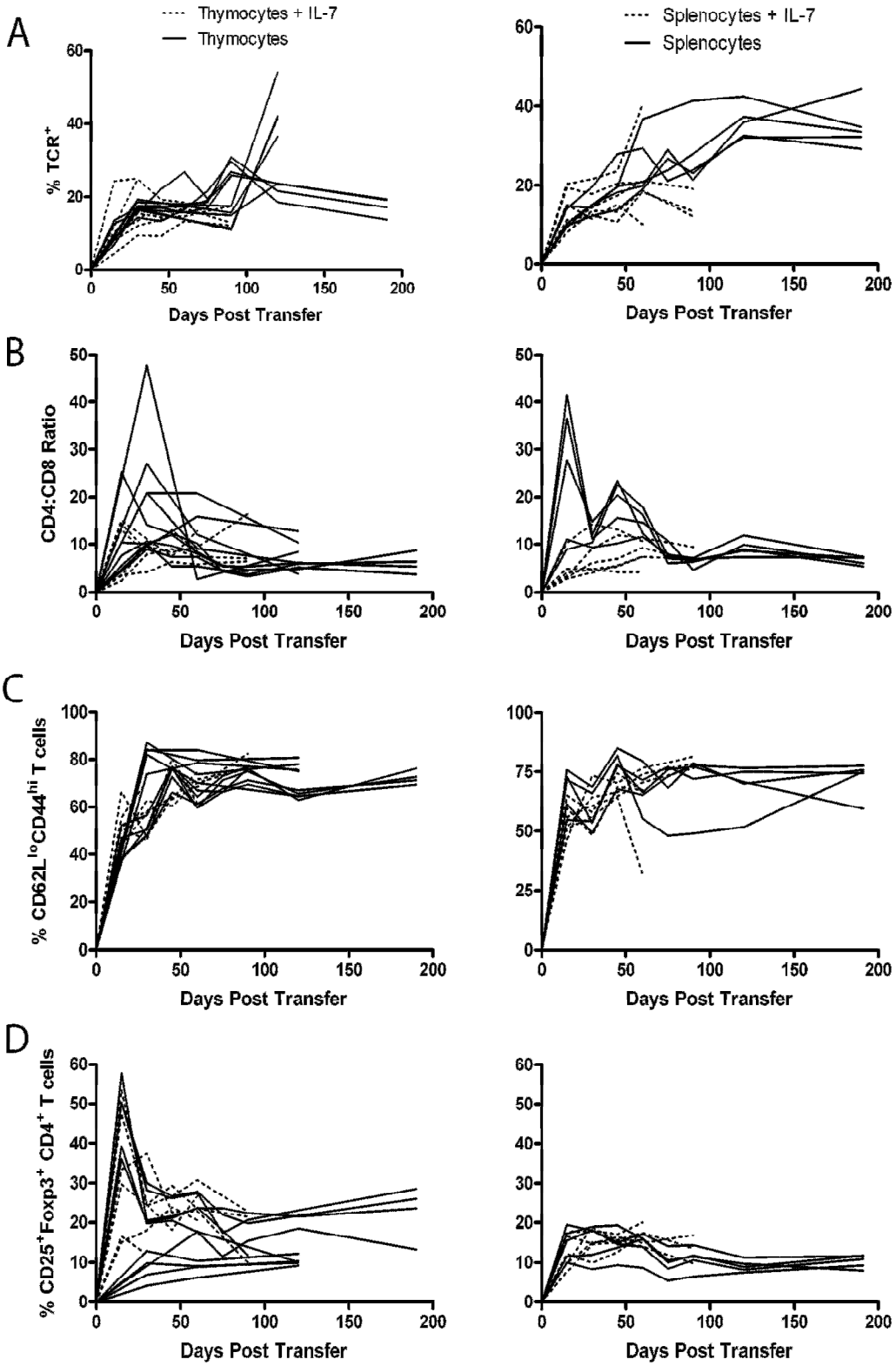

A
Thymocytes + IL-7
Thymocytes
% TCR+
Days Post Transfer
Splenocytes + IL-7
Splenocytes
Days Post Transfer
B
CD4:CD8 Ratio
Days Post Transfer
Days Post Transfer
C
% CD62L loCD44hi T cells
Days Post Transfer
Days Post Transfer
D
% CD25+Foxp3+ CD4+ T cells
Days Post Transfer
Days Post Transfer

Figure 4.6. Similar peripheral blood T cell phenotypes in thymocyte or splenocyte transfer recipients with or without IL-7/anti-IL-7.
Mice that receive thoracic thymocytes or splenocytes with or without IL-7/anti-IL-7 complexes have similar peripheral T cell phenotypes. Mice that receive thoracic thymocytes (*left panel*) or splenocytes (*right panel*) without IL-7/anti-IL-7 complex (*solid line in both panels*) or with IL-7/anti-IL-7 complexes (*dashed line in both panels*). Thoracic thymocytes (n=10), thoracic thymocytes + IL-7/anti-IL-7 (n=6), splenocytes (n=5), splenocytes + IL-7/anti-IL-7 (n=6). All cells were gated on lymphocytes in the periphery. (A) Percentage of lymphocytes that are TCR β^+. (B) CD4:CD8 ratio. (C) Percentage of T cells that are effector memory cells ($CD62L^{lo}CD44^{hi}$). (D) Percentage of $CD4^+$ T cells are regulatory T cells ($CD25^+Foxp3^+$). All cells are gated on lymphocytes in the peripheral blood. Each line is representative of one mouse. Measurement termination represents the endpoint of the experiment for that particular mouse determined by development of disease or use as a control.

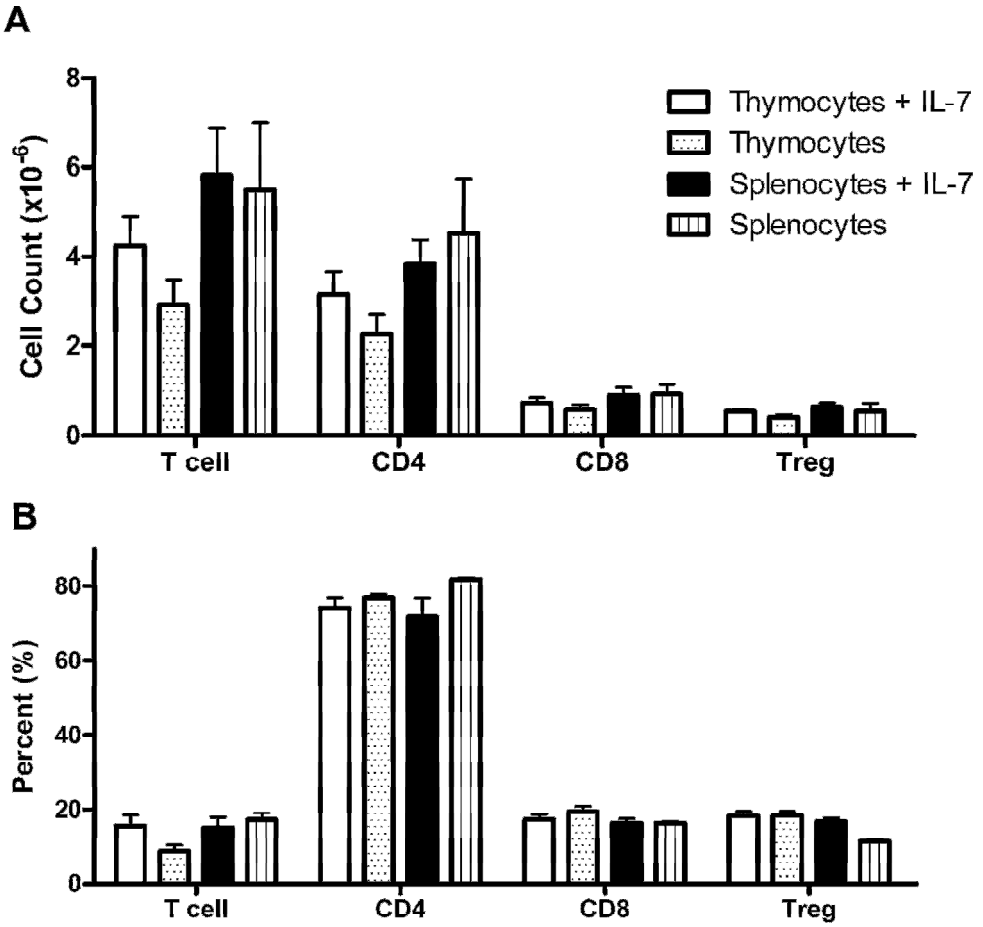


Figure 4.7. Peripheral T cell counts and percentages are similar in thymocyte or splenocyte transfer recipients with or without IL-7/anti-IL-7. There are no significant differences in absolute T cell number or percentage in the spleen to explain the enhancement of disease with IL-7/anti-IL-7 complexes or the differences between thymocytes and splenocytes. (A) Graph (mean + SEM) of T cell, CD4, CD8 and Treg numbers in the spleen. (B) Graph (mean + SEM) of the percentage of T cell, CD4, CD8 and Tregs in the spleen. Tregs were determined by $CD4^+$ T cells that are $CD25^+Foxp3^+$. Mice that received thymocytes and IL-7/anti-IL-7 complexes (n=9), thymocytes (n=9), splenocytes and IL-7/anti-IL-7 complexes (n=9) and splenocytes (n=5). Total cell counts are gated on all live cells and percentages are gated on lymphocytes. T cells are gated on TCR^+ then subsequently gated on $CD4^+$ or $CD8^+$ cells.

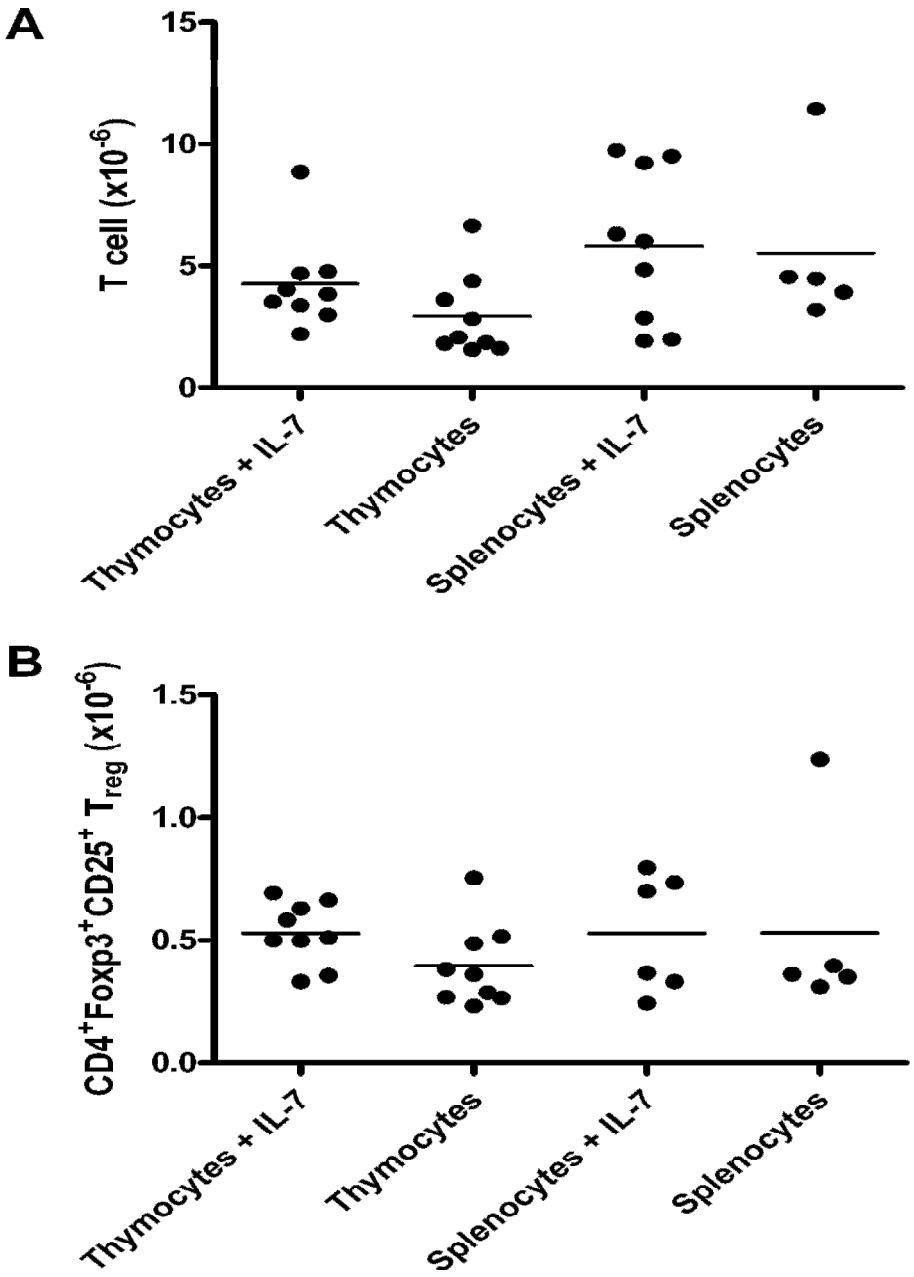


Figure 4.8. Peripheral T cell numbers and Treg numbers are similar in all thymocyte and splenocyte transfer recipients with or without IL-7/anti-IL-7. IL-7 does not significantly decrease the number of T cells or T regulatory cells. (A) T cell numbers in the spleen. (B) The number of $CD4^+$ T cells that are $CD25^+Foxp3^+$. Each dot is representative of one mouse from **Figure 4.7**..

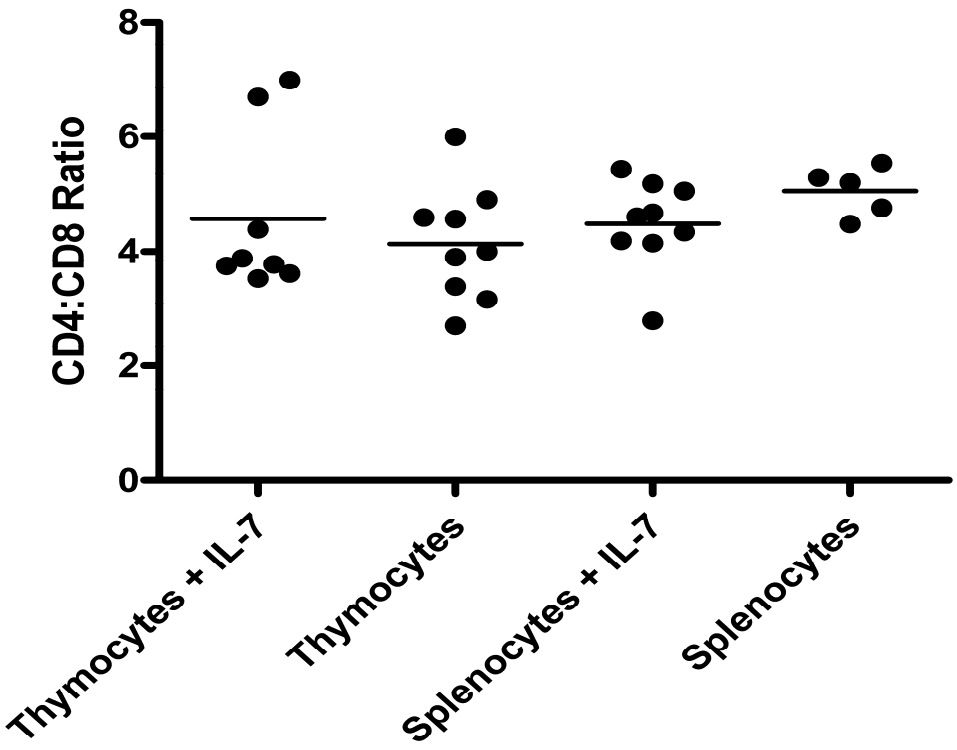


Figure 4.9. Peripheral CD4:CD8 T cell ratios are similar in all thymocyte and splenocyte transfer recipients with or without IL-7/anti-IL-7.
The addition of IL-7 does not alter the CD4:CD8 ratio of T cells in the spleen. The CD4:CD8 ratio was determined by dividing the percentage of $CD4^+$ T cells by the percentage of $CD8^+$ T cells in the spleen. Each dot is representative of one mouse.

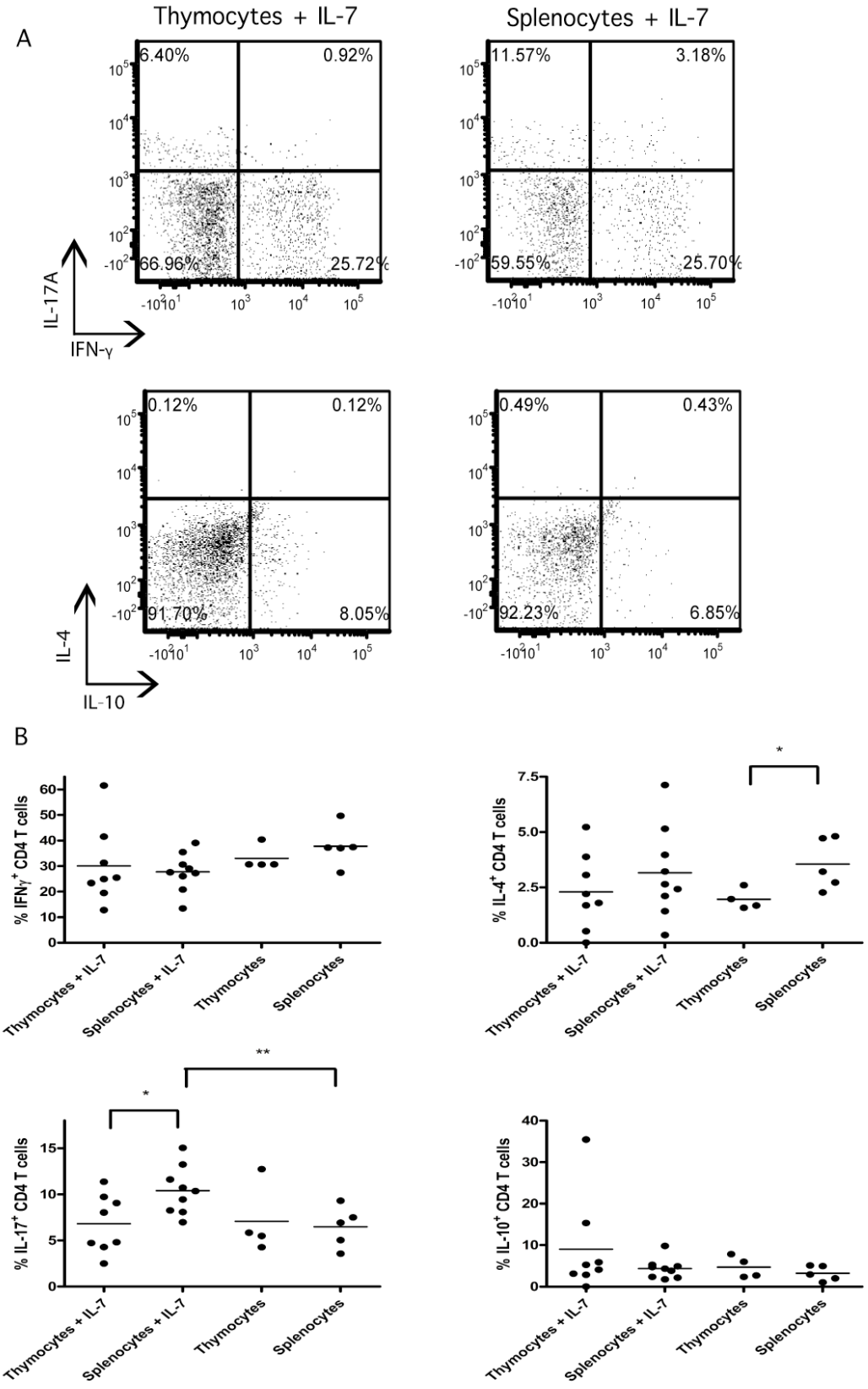

A
Thymocytes + IL-7
Splenocytes + IL-7
6.40%
0.92%
66.96%
25.72%
11.57%
3.18%
59.55%
25.70%
IL-17A
IFN-γ
0.12%
0.12%
91.70%
8.05%
0.49%
0.43%
92.23%
6.85%
IL-4
IL-10
B
% IFNγ+ CD4 T cells
% IL-4+ CD4 T cells
% IL-17+ CD4 T cells
% IL-10+ CD4 T cells
Thymocytes + IL-7
Splenocytes + IL-7
Thymocytes
Splenocytes
*
**

Figure 4.10. Differences in intracellular cytokine profiles of CD4 T cells. Mice that receive thymocytes compared to splenocytes with or without IL-7/anti-IL-7 complexes have differences in their cytokine profiles for $CD4^+$ T cells secreting IFN-γ, IL-4, and IL-17A but not IL-10. Splenocytes were re-stimulated for 5 hours with PMA and ionomycin in the presence of brefeldin A and monensin. (A) Representative flow cytometry dot plots from thoracic thymocyte (n=8) or splenocyte (n=9) with IL-7/anti-IL-7 complexes. (B) Graphs depicting the differences in intracellular cytokines present in $CD4^+$ T cells in mice that receive thymocytes or splenocytes with or without IL-7/anti-IL-7 complexes. *Top left*: IFN-γ (* p=0.36), *top right*: IL-4 (* p=0.038), *bottom left*: IL-17 (* p=0.0207, ** p=0.0105) and *bottom right*: IL-10. Each dot is representative of one mouse.

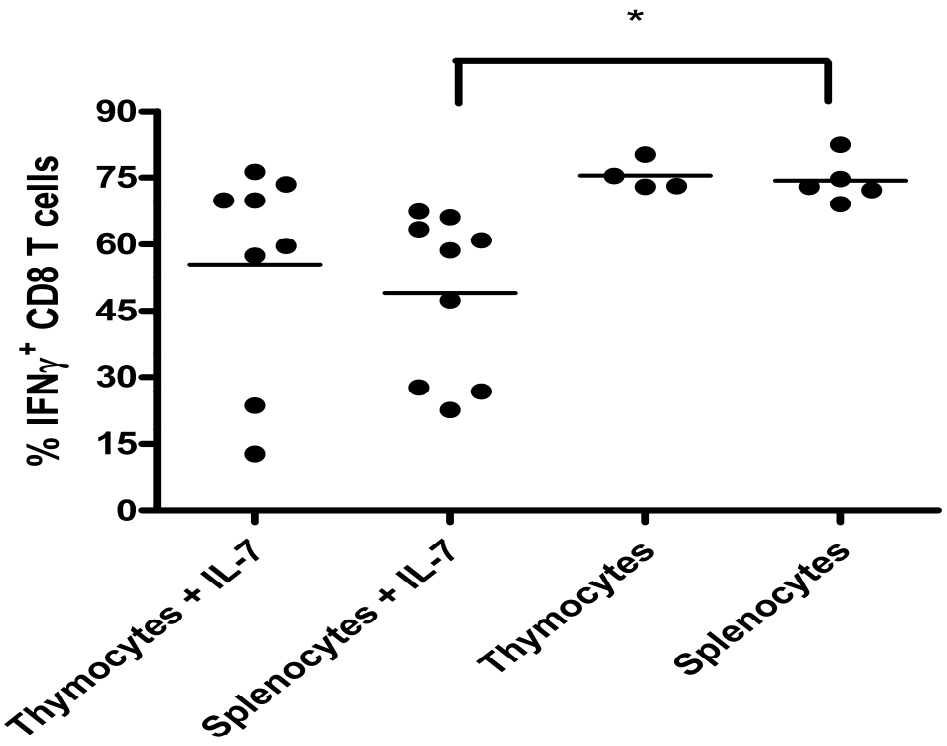


Figure 4.11. Differences in intracellular cytokine profiles of CD8 T cells. Mice that receive IL-7/anti-IL-7 complexes have decreased percentages of $CD8^+$ T cells with intracellular IFN-γ. Splenocytes were re-stimulated for 5 hours with PMA and ionomycin in the presence of brefeldin A and monensin. Graph depicting the differences in intracellular IFN-γ in $CD8^+$ T cells in mice that receive thymocytes or splenocytes with or without IL-7/anti-IL-7 complexes. (* p=0.0119). Each dot is representative of one mouse.

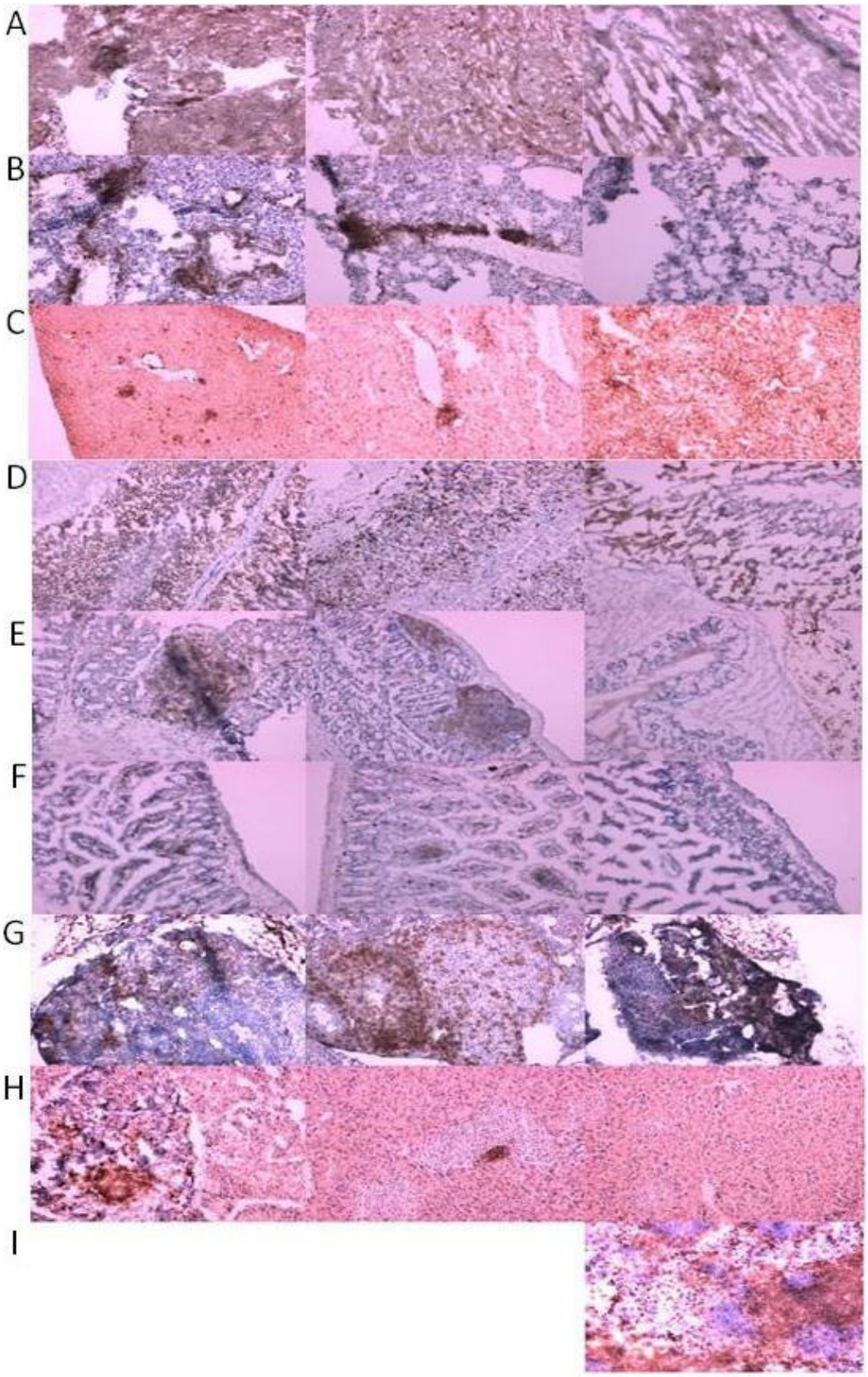

A
B
C
D
E
F
G
H
I

Figure 4.12. CD4 organ infiltration is similar in splenocyte and thymocyte transfer recipients with IL-7/anti-IL-7.
Immunodeficient mice given thoracic thymocytes or splenocytes and IL-7/anti-IL-7 complexes have $CD4^+$ T cells in their lung, liver, small intestine, large intestine, ovary and pancreas. The left column is representative of mice that received thymocytes and IL-7/anti-IL-7 complexes, the middle column is representative of mice that received splenocytes and IL-7/anti-IL-7 and the right column is representative of BALB/c wildtype mice. Each organ is representative of three mice. (A) Heart. (B) Lung. (C) Liver. (D) Stomach. (E) Large intestine. (F) Small intestine. (G) Pancreas. (H) Ovary. (I) Spleen. $CD4^+$ staining was detected on cryosections by immunohistochemistry.

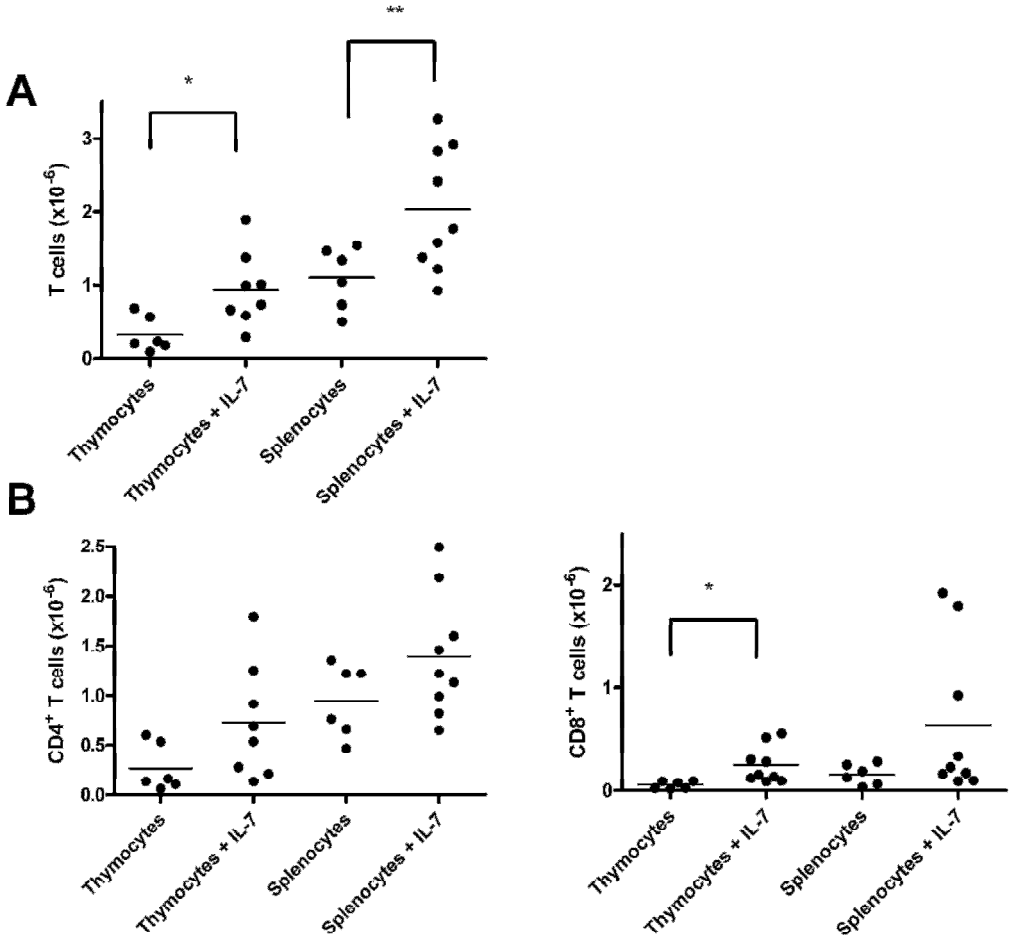


Figure 4.13. Short-term effects of IL-7/anti-IL-7 complexes lead to increased T cell numbers.

IL-7 increases the number of T cells in mice given thoracic thymocytes or splenocytes after 10 days. Immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mice were given whole thymocytes containing 500 000 single positive T cells or 500 000 T cells from B cell-depleted splenocytes with or without IL-7/anti-IL-7 complexes on days 1, 4 and 7. Mice were euthanized on day 10 after transfer and T cells were quantified in the spleen. (A) Total number of T cells in the spleen (* p=0.0174, ** p=0.0276). (B) *Left panel*: total number of CD4$^+$ T cells. *Right panel*: total number of CD8$^+$ T cells (* p=0.022). Data are representative of three independent experiments. Each dot is representative of one mouse.

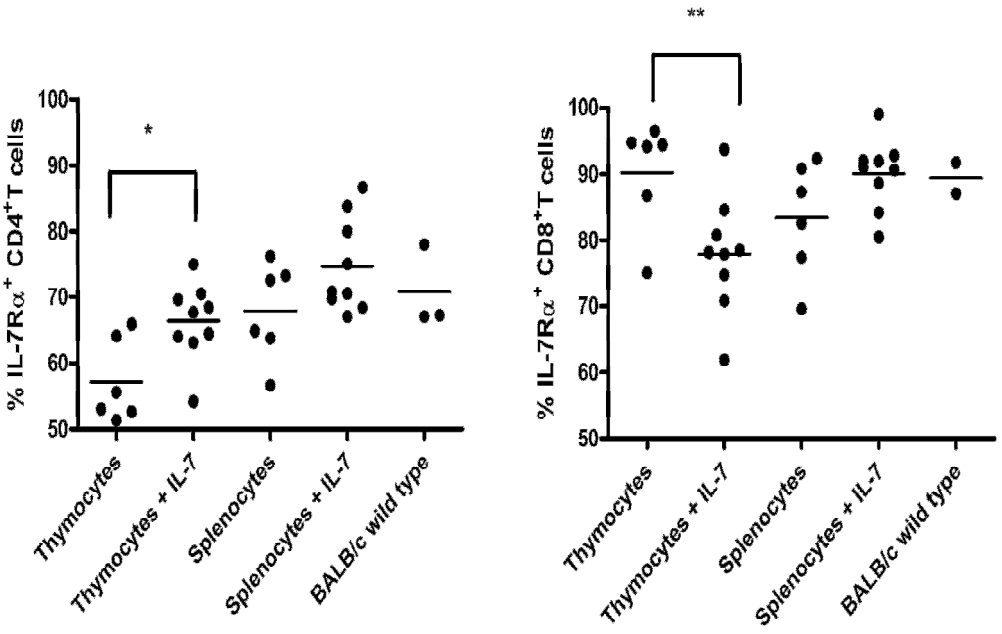


Figure 4.14. Short-term effects of IL-7/anti-IL-7 complexes lead to changes in T cell IL-7 receptor expression.
Mice that receive thoracic thymocytes and IL-7/anti-IL-7 complexes have an increased percentage of $CD4^+$ T cells expressing IL-7Rα and a decreased percentage of $CD8^+$ T cells expressing the IL-7Rα compared to transfer of thoracic thymocytes alone. Immunodeficient BALB/c *Rag*$^{-/-}$ or SCID mice were given whole thymocytes containing 500 000 single positive T or 500 000 T cells from B cell-depleted splenocytes with or without IL-7/anti-IL-7 complexes on days 1, 4 and 7. Mice were euthanized on day 10 after transfer. *Left panel*: percentage $CD4^+$ T cells expressing IL-7Rα (* p=0.0124). *Right panel*: percentage of $CD8^+$ T cells expressing IL-7Rα (** p=0.0172). Data are representative of three independent experiments. Each dot is representative of one mouse.

Table 4.1. Summary of CD4 organ infiltration in transfer mice given IL-7/anti-IL-7 complexes

The *left* column displays the organ examined for the presence of $CD4^+$ by immunohistochemistry of frozen sections. The *middle* and *right* columns summarize the number of mice with CD4 cells present in that organ.

Organ	Thymocytes + IL-7	Splenocytes + IL-7
Heart	1/3	1/3
Lung	3/3	3/3
Liver	3/3	3/3
Stomach	3/3	1/3
Large Intestine	3/3	3/3
Small Intestine	3/3	3/3
Ovary	3/3	3/3
Pancreas	2/3	1/3

CHAPTER 5: DISCUSSION

5.1 T CELLS GENERATED AFTER REMOVAL OF THE THORACIC THYMUS IN LYMPHOPENIA ARE NOT FULLY SELF-TOLERANT

T cells that develop in the absence of the thoracic thymus are not fully self-tolerant in a lymphopenic mouse. Generation of T cells in the absence of the thoracic thymus would likely occur in the cervical thymus, if present, and our data supports this conclusion. There were a number of mice who developed B cells only and no T cells presumably due to the absence of the cervical thymus. If T cells that developed in mice in the absence of the thoracic thymus were due to extra-thymic T cell development we would expect all mice that receive a thoracic thymectomy to generate T cells. Nevertheless, we cannot fully exclude the possibility that T cell development in non-cervical or thoracic thymus tissue exists although there is no evidence to date that non-cervical or thoracic thymus tissue is present in mice. This is because we only directly assessed minimal numbers of mice that developed T cells in the absence of the thoracic thymus for the presence of cervical thymus. However, the lack of ability to detect cervical thymus could be due to technical failure as opposed to the absence of cervical thymus in the mice. Therefore, to re-iterate, the term “cervical thymus” used in this thesis includes both cervical thymus and any other potential non-thoracic thymus tissue that may exist. Moreover, disease in mice that develop T cells in the absence of the thoracic thymus could be due to several reasons. First, an inherent defect in

the cervical thymus or non thoracic thymus tissue could be leading to an increase in the number of autoreactive T cells escaping central deletion. Second, the absence of the thoracic thymus and development of T cells through presumably the cervical thymus in a lymphopenic environment leading to homeostatic proliferation could be acting as a co-factor in disease. For instance, the cervical thymus is much smaller in size compared to the thoracic thymus; therefore, the export of T cells will be largely decreased compared to the thoracic thymus. In order to maintain T cell homeostasis in the lymphopenic environment of a *Rag*$^{-/-}$ or SCID mouse the small numbers of T cells that are being exported could be undergoing lymphopenia-induced homeostatic proliferation which has been known to lead to autoimmunity under certain circumstances[125].

We hypothesize that the incidence of disease in the absence of a thoracic thymus may be due to newly generated T cells/recent thymic emigrants (RTEs) exiting the cervical thymus (or non thoracic thymus tissue) at a lower thymic output in a lymphopenic environment allowing for the homeostatic proliferation and generation of autoimmunity. Autoimmunity may be generated when RTEs enter into the periphery in a lymphopenic environment leading to homeostatic proliferation of self-reactive T cells that would normally undergo peripheral tolerance. Proliferation of self-reactive T cells via homeostatic proliferation would increase the number and frequency of clones of these self-reactive T cells to disproportionate numbers. Overrepresentation of these self-reactive T cell numbers may be too extreme for the regulation that would normally occur in the periphery by Tregs or the overall decrease in T cell numbers could decrease the

Treg:APC ratio. In accordance with this, Rocha *et al*. (1989) have shown that $CD4^+$ T cells transferred into a lymphopenic environment can divide upwards of 54 times in vivo[286]. Furthermore, recent evidence has shown that RTEs are both functionally and phenotypically different compared to a "mature" naïve T cell[152,262,263]. Consequently, these RTEs must undergo continued maturation in the periphery before they can become incorporated into the naïve T cell pool[152,264]. As well, RTEs undergo increased homeostatic proliferation and out-compete "mature" naïve T cells in a lymphopenic environment[264]. Therefore, these potentially "non-tolerized" RTEs may increase in frequency if a decreased output of T cells from the cervical thymus leads to increased homeostatic proliferation of T cells.

We wanted to determine if there was an increased proportion of recent thymic emigrants in mice that receive thoracic Tx + FL compared to sham Tx + FL or BALB/c WT mice. CD24 has traditionally been used as a marker of recent thymic emigrants[152]. It has been shown to be important in homeostatic proliferation of T cells[266] and polymorphisms in CD24 represent a risk factor in multiple sclerosis patients[292]. We found that there were an higher percentage of CD4 and CD8 T cells that expressed CD24 in mice that developed disease after thoracic Tx + FL. This suggests that in the absence of the thoracic thymus there is a higher proportion of RTEs even 200^+ days post transfer. Unfortunately, CD24 is not a definitive marker of RTEs; therefore, we cannot unequivocally conclude that these cells are RTEs. Mice that receive thoracic Tx + FL also have lower numbers of splenic T cells; therefore, this could suggest that the cervical thymus is not

fully capable of supporting a complete T cell repertoire like the thoracic thymus. In conclusion, the cervical thymus may be continually exporting T cells whereas the thoracic thymus may decrease export once homeostasis is reached. However, we do not know the overall contribution of thymic export compared to lymphopenia-driven proliferation in this model.

In addition, we speculated that the cervical thymus may play a role in day 3 neonatal thymectomy-induced autoimmunity or that our model may represent an adult version of d3Tx-induced autoimmunity. When mice undergo neonatal thoracic thymectomy there is the potential for cervical thymus to remain present in the mouse and to continue to export T cells into the periphery. The incidence of cervical thymus in BALB/c mice is anywhere from 50-90% and they are highly susceptible to d3Tx-induced autoimmunity (but not 100% incidence) whereas the incidence of cervical thymus in C57BL/6 mice is under 50% which could explain why this strain is generally considered d3Tx-induced autoimmune resistant[191,279,280].

Since a defect in Treg numbers was long speculated as the reason for disease in d3Tx-induced autoimmunity[180,211] we wanted to determine if a defect in Treg numbers was the reason for disease in our thoracic Tx + FL mice. However, we found that there was in increased frequency of CD4 T cells that were $CD25^{+}Foxp3^{+}$ Tregs in the spleens of mice that got disease. In addition, there was no significant difference in absolute number of Tregs in the spleen. Considering there was a significantly lower number of overall T cells in the spleens of mice that had undergone thoracic Tx + FL there was actually an increased number of

Tregs in terms of frequency. However, we did not assess the functionality of Tregs from thoracic Tx + FL mice. So, while there is an increased frequency of CD4 T cells that are Tregs in the spleens of mice that develop disease, they may not be fully functional or capable of suppression. However, d3Tx papers have shown that disease-specific Tregs exist in the periphery and are actively trying to suppress disease[214] which may be the similar in our model. Alternatively, the decreased number of T cells in mice that underwent thoracic Tx + FL could decrease the Treg:APC ratio and decrease the effectiveness of suppression. An increase in intracellular IL-10 in CD4 T cells was detected in thoracic Tx + FL mice, which has been shown to be important in Treg suppression. These IL-10^{+} CD4 T cells may represent a population of Tregs actively trying to suppress disease. Determining the phenotype of the CD4 subsets with these cytokines being produced will help determine the role and effect of these cytokines on disease. Therefore, disease occurs even in the presence of CD4 T cells with a regulatory T cell phenotype.

Interleukin-7 is an important cytokine in homeostatic proliferation of T cells[104,119]. Therefore, we analyzed the IL-7Rα expression on T cells to begin to determine if excess amounts of IL-7 in a lymphopenic environment are a contributing factor to disease in thoracic Tx + FL mice. We did not find any differences between IL-7Rα expression on CD4 T cells in either thoracic Tx + FL, sham Tx + FL or BALB/c WT mice. However, IL-7Rα expression was lower in CD8 T cells in mice that developed disease. Downregulation of the IL-7R suggests that IL-7 has signalled through the receptor; therefore, CD8 T cells may

be more responsive to the cytokine or CD8 T cells may be mediating disease[149]. Nevertheless, evidence that IL-7R signalling leads to downregulation was only shown in CD8 T cells. In addition, d3Tx-induced autoimmunity and CD4 T cell transfer experiments evidence has pointed towards CD4 T cells rather than CD8 T cells being important in and mediating autoimmunity[189].

Next, we analyzed serum immunoglobulin isotypes and intracellular cytokines in T cells to determine if there was any Th-skewing in mice that develop disease when T cells are generated in the absence of the thoracic thymus. We found an increase in serum IgG1 levels which occurs in the presence of IL-4 suggesting that disease may be Th2-mediated[14,15]. However, there was also an increase in IgG2b and an small increase in IgA (although not statistically significant) which occurs in the presence of TGF-β and Tregs[14,16] suggesting again that these cells are present and actively trying to suppress disease. However, we found an increase in intracellular IL-17A and IFN-γ as opposed to IL-4 in CD4 T cells. IL-17A and IFN-γ are important in tissue damage and destruction and are traditionally Th17 or Th1 cytokines, respectively[293]. Therefore, disease may involve an unbalanced T helper cell response.

The organs affected by disease have remained unclear in thoracic Tx + FL experiments. However, preliminary studies of intracellular cytokine staining suggest that there are differences in the presence of CD4 T cells expressing IL-17A depending on the location of the LNs. Pooled axillary, mesenteric and periaortic LNs had CD4 T cells expressing IL-17A whereas cervical LNs did not have a population of CD4 T cells expressing IL-17A (**Appendix Figure A2.1**). In

addition, there were no differences in IL-4, IL-10 or IFN-γ between the two types of LNs. This data suggests that IL-17A is important in disease and that disease is likely occurring in the stomach similar to d3Tx-induced autoimmunity[191]. However, we cannot exclude the possibility that disease is occurring in an organ that drains into the axillary, inguinal or periaortic LNs. More experiments are required to determine which LN has increased numbers of IL-17A^{+}CD4 T cells and if the increase can be replicated (n=1). Detection of autoantibodies by western blotting has suggested that autoantibodies to the stomach, liver and pancreas are present in diseased mice that undergo thoracic Tx + FL (n=3) but not in sham Tx + FL mice (n=1)(**Appendix Figure A2.2**) but further experiments need to be done to confirm this finding. The data from these western blots indicates that disease is autoimmune in nature, but more experiments and determining the presence of autoreactive T cells are necessary to fully conclude this. We also found CD4 staining and infiltration in the lung of one mouse tested that received thoracic Tx + FL and got ill but not in a mouse that received sham thoracic + Tx (**Appendix Figure A2.3**). Altogether, further experiments are required to be able to definitively determine which organs are being affected by disease and if it is in fact autoimmunity. Preliminary data also suggests that splenocytes from thoracic Tx + FL mice that get disease can transfer disease to a new immunodeficient recipient although ineffectively (>150 days post transfer; **Appendix Figure A2.4**).

Initial experiments were done to try to determine whether T cell development in the absence of the thoracic thymus itself is pathogenic or whether

a decrease in any thymic tissue is a factor in disease. Therefore, a group of mice had half of their bi-lobed thoracic thymus removed and were given fetal liver cells (0.5Tx + FL). These mice had similar numbers of T cells in their spleen (n=3) compared to thoracic Tx + FL mice (**Appendix Figure A2.5**). In addition, the pattern of intracellular CD4 cytokines detection in 0.5Tx + FL was similar to thoracic Tx + FL for IFN-γ and IL-10 (**Appendix Figure A2.6**) and CD8 T cells with IFN-γ (**Appendix Figure A2.7**). However, while there was an increase in intracellular IL-17A in CD4 T cells from thoracic Tx + FL mice, there was only a slight increase in 0.5Tx + FL. Instead there was an increase in CD4 T cells with intracellular IL-4 in 0.5Tx + FL. This data suggests that there may be slight differences in T cell development in a lymphopenic environment between thoracic and non-thoracic thymus. Removing half a lobe of thoracic thymus still leaves thymic tissue that is much larger in size compared to the LN-sized cervical thymus. So, any differences between the two groups could simply be attributed to that. In conclusion, more experiments need to be done to validate these observations and these 0.5Tx + FL mice need to be monitored for incidence of disease.

5.2 DISEASE GENERATED BY THYMOCYTES TRANSFERRED INTO A LYMPHOPENIC ENVIRONMENT IS AUGMENTED BY IL-7/ANTI-IL-7 COMPLEXES

It has previously been shown that transfer of thymocytes but not splenocytes into an immunodeficient, lymphopenic recipient leads to disease[189]. However, it remains unknown whether or not there are differences in the T cell populations which lead to disease. One potential reason why thymocyte transfers may generate disease could be due to the transfer of T cells that have not fully undergone central tolerance. This may, therefore, lead to transfer of a population containing T cells that would have normally been deleted prior to entering the periphery. Alternatively, the increased number of RTEs in the thymus that have not undergone maturation in the periphery and are potentially autoreactive could contribute to disease. Furthermore, RTEs preferentially move into the LNs in the periphery as opposed to the spleen[140]; therefore, the spleen has a very low proportion of RTEs compared to "mature" naïve T cells whereas the thymus has a large proportion of RTEs. Therefore, when thymocytes or splenocytes are transferred into a lymphopenic environment the RTEs will preferentially undergo homeostatic proliferation to maintain homeostasis. Since the thymus has a larger number of RTEs these T cells will homeostatically proliferate and potentially increase the proportion of autoreactive T cells in the periphery compared to a population of splenocytes. In addition, previous transfer experiments where done with whole splenocytes. In our experiments we depleted the splenocytes of B

cells; therefore, B cells within the spleen do not prevent autoimmunity in the splenocyte population.

We initially wanted to determine if disease generated in mice that receive a thoracic Tx + FL was due to autoreactive T cells escaping negative selection in the cervical thymus or if increased amounts of homeostatic proliferation due to decreased T cell export from the cervical thymus was a probable cause of disease. Therefore, we transferred whole thymocytes from either pooled cervical thymi or thoracic thymus into an immunodeficient, lymphopenic host. Surprisingly, both populations of thymocytes generated disease in these mice suggesting that disease was due to homeostatic proliferation and lymphopenia-driven autoimmunity. We later used T cell transfers from B cell-depleted splenocyte as a control for no disease and for assessing the effect of IL-7/anti-IL-7 complexes on disease since splenocyte transfer mice had been shown to be incapable of generating disease during lymphopenia[189]. Mice that receive either splenocytes or thymocytes have an increased percentage of effector T cells in the periphery suggesting that both populations of cells are undergoing homeostatic proliferation. Therefore, homeostatic proliferation is likely only a co-factor in generation of disease.

Interleukin-7 is an important cytokine in homeostatic proliferation so, we wanted to determine if IL-7 enhanced disease in transfer mice since treatment with IL-7 is becoming a widely accepted treatment method for restoring T cell numbers in patients after lymphopenia induced by viral infection or cancer treatment[149]. In addition, clinical trials are currently underway for assessing the benefits of IL-7 treatment in HIV infected individuals who are

lymphopenic[137,149,159,160,269,294]. Therefore, we gave thymocyte or splenocyte transfer recipients IL-7/anti-IL-7 complexes short term to determine if they had an effect on disease. These complexes accelerated the time course of disease, but did not increase the severity of the disease generated. This may be due to the fact that after 10 days of transfer the addition of the IL-7/anti-IL-7 complexes led to almost double the number of T cells in the spleens of thymocyte or splenocyte recipients. Therefore, the IL-7/anti-IL-7 complexes may just be increasing the number of autoreactive T cell clones at a faster rate.

Interleukin-7 has been shown to have several effects on T cells themselves. For instance, the addition of IL-7 to T cells causes them to become refractory to inhibition by Tregs but does not affect the Treg's ability to suppress T cells[155]. Therefore, IL-7/anti-IL-7 complexes could be making autoreactive T cells less susceptible to suppression by Tregs. Furthermore, the addition of IL-7 has been suggested to selectively deplete Tregs since they have low levels of IL-7R expression and may proliferate less in response to it[138]. However, we did not find any significant differences in the Treg frequencies, and the Treg numbers were slightly increased with the addition of IL-7/anti-IL-7 complexes in thymocyte recipients. Additionally, RTEs have been shown to be more responsive to homeostatic proliferation in lymphopenic conditions yet they too express low levels of the IL-7R[264].

CD4 T cells have been shown to be important in disease in thymocyte transfer experiments, and can generate disease even in the absence of CD8 T cells[189]. However, CD8 T cells are more responsive to IL-7[149], so it remains

unknown whether the IL-7/anti-IL-7 complexes are affecting CD4 T cells, CD8 T cells or both. IL-7 can alter the CD4:CD8 ratio[146], but in our model the CD4:CD8 ratio remains the same in thymocyte or splenocyte recipients with or without IL-7/anti-IL-7 complexes. In addition, we assessed the expression of the IL-7Rα on CD4 and CD8 T cells 10 days post transfer since activation through the IL-7R can lead to its downregulation. We found that there was no difference in CD4 T cell expression of the IL-7Rα in thymocyte recipients with or without IL-7/anti-IL-7 complexes. However, there was a decrease in the IL-7Rα on CD8 T cells in thymocyte recipients given IL-7/anti-IL-7 complexes suggesting that the receptor is getting activated and downregulated by the availability of IL-7. In addition, there were no apparent differences in CD4 and CD8 cytokine expression that could explain enhanced disease with IL-7/anti-IL-7 complexes. However, preliminary data suggests that there are increased amounts of serum IL-17A, IL-1α and IL-1β in mice that receive thymocytes and IL-7/anti-IL-7 complexes compared to mice that receive splenocytes and IL-7/anti-IL-7 complexes (**Appendix Figure A3.1**). It remains to be determined if there are any differences in serum cytokines between thymocytes alone or thymocytes in addition to IL-7/anti-IL-7 complexes.

In conclusion, caution should be taken when using IL-7 to increase recovery of T cell numbers in patients after becoming lymphopenic. IL-7 promotes disease in lymphopenic mice that receive thymocyte transfers and could potentially cause autoimmunity in patients as a side effect. If RTEs are important in disease there is a chance that the risk of autoimmunity could decrease with age

due to age-dependent atrophy of the thymus which decreases the number of T cells being exported into the periphery[258]. However, since RTEs are preferentially incorporated into the T cell repertoire in a lymphopenic environment[264] it could still a pose a risk for autoimmunity.

5.3 FUTURE DIRECTIONS

In these experiments we cannot directly conclude that T cells are being generated by the cervical thymus after removal of the thoracic thymus. Therefore, it will be important to assess the presence of cervical thymus in these mice in the future. However, to date there is no evidence that non thoracic thymic tissue in addition to the cervical thymus exists in mice. Further experiments are required to characterize disease in mice that generate T cells in the absence of the thoracic thymus. First, the organ(s) affected by disease need to be identified and to determine if this disease is in fact autoimmunity. This can be done through pathology or indirectly by assessing the presence of autoantibodies towards different organs using immunohistochemistry or a western blot with different tissues. In addition, immunohistochemistry can be used to determine organs which have CD4 or CD8 T cell infiltration then further characterize the types of cells infiltrating. For instance, are the infiltrating T cells Treg, Th1, Th2 or Th17 cells? In addition, looking at serum cytokines may provide insights into which cytokines are important in the disease induced after thoracic Tx + FL.

Determining which cytokines are important can be done using neutralizing antibodies to cytokines such as IFN-γ (commercially available). Experiments can be done to determine if the cytokine is important in the beginning development of disease or if blocking the cytokine once disease has begun to progress can prevent or delay disease. However, these experiments may be hindered by the long time course of disease. The presence of Tregs was found in mice that developed disease; however, it will be important to determine if these Tregs are capable of suppressing. Suppression assays can be done in vitro using anti-CD3/anti-CD28 stimulation of T cells co-cultured with Tregs, or can be assessed by purifying Tregs from mice that generate disease and adoptively transferring them into a new host to determine if they can prevent disease. Alternatively, Tregs could be depleted in vivo using an anti-CD25 depleting antibody. If depletion of Tregs causes enhanced disease then it is likely that Tregs are actively trying to suppress disease but less efficiently in mice that receive thoracic Tx + FL.

Further experiments can be done to characterize disease and determine the role of IL-7 in immunodeficient, lymphopenic mice that receive thymocytes and IL-7/anti-IL-7 complexes. For instance, we can determine if the T cells in mice can be suppressed by Tregs or if the Tregs present in these mice are capable of suppression. Also, experiments can be done to assess the effect of IL-7 on transferring SP CD4 or CD8 T cells from thymocytes to determine which cells are important and which cells are being affected by the addition of IL-7/anti-IL-7 complexes. As well, we found that there were similar amounts of CD4 T cells infiltrating certain organs with the addition of IL-7/anti-IL-7 complexes

independent of which population of cells are transferred. However, we did not assess the phenotype or Ag-specificity of these CD4 T cells or whether there was a difference in numbers or presence of CD8 T cells in these organs. Furthermore, we did not assess CD4 infiltration in thymocytes and splenocytes without IL-7/anti-IL-7 complexes, so it will be interesting to determine if these mice have similar amounts of CD4 T cells in certain organs as well.

The importance of recent thymic emigrants and whether they represent a population of T cells that have more potential to become autoreactive needs to be addressed. Recent thymic emigrants can be sorted from the thymus or spleen based on CD24 expression and transferred into a lymphopenic host to determine if splenocytes are then capable of generating disease. In addition, the presence or absence of autoreactive T cells can be determined in the initial and end population of T cells (assuming disease is similar to d3Tx-induced autoimmunity). In d3Tx-induced autoimmunity the H+/K+ ATPase in the stomach is the main target of autoimmunity in BALB/c mice. Tetramers are commercially available (NIH tetramer core facility, Atlanta, GA) to detect reactive T cells to the H+/K+ ATPase which would allow us to determine the frequency of initial autoreactive T cells in thymocytes versus splenocytes.

5.4 SUMMARY OF CONCLUSIONS

1. The cervical thymus can function fully independently from the thoracic thymus.

2. T cells generated in the absence of the thoracic thymus do not generate a repertoire of T cells that are fully self-tolerant in a lymphopenic environment.
3. Disease in mice that received a thoracic thymectomy and fetal liver cells occurs in the presence of T cells with a regulatory T cell phenotype.
4. There are increased numbers of CD4 T cells expressing IL-17A, IFN-γ and IL-10 and CD8 T cells expressing IFN-γ in the spleen and pooled axillary, periaortic and mesenteric lymph nodes in mice that receive thoracic Tx + FL.
5. IL-7/anti-IL-7 complexes accelerate the time course of disease in lymphopenic mice that are given thymocytes but still do not lead to disease in mice given splenocytes.
6. IL-7/anti-IL-7 complexes significantly increase the number of T cells in thymocyte and splenocyte transfer recipients at 10 days post transfer.

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APPENDIX:

A1: REAGENTS AND PROTOCOLS

Anaesthesia

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

Cell Culture Medium

Per one 500 mL bottle of Dulbecco's Modified Eagle's Medium (DMEM)

1. Add 50 mL of fetal bovine serum (FBS), 5 mL of penicillin/streptomycin (10 000 units/mL of penicillin G sodium and 10 000 µg/mL of streptomycin sulphate), 5 mL of 200 mM L-glutamine and $5x10^{-3}$ M 2-mercaptoethanol.
2. Mix, filter sterilize and store at 4 °C.

Flow Cytometry

a) Antibody Diluent

Per 50 mL

1. Add 5 mL of 10% dialyzed (w/v) bovine serum albumin (BSA) to 45 mL of 1x PBS.
2. Filter, sterilize and store at 4 °C.

b) Fc receptor block

Per 10 mL

1. Mix together 3.33 mL each of mouse, hamster and rat serum.
2. Add 300 µg of anti-CD16/32 antibody (2.4G2).
3. Mix, filter sterilize and store at 4 °C.

Intracellular Cytokine Staining

a) FACS Buffer (0.2% BSA in PBS)

a) Dissolve 1 g BSA (Sigma-Aldrich) in 499 mL PBS

b) Saponin (10x)

1. Dissolve 1.5 g of saponin (Sigma-Aldrich, Oakville, ON) in 13.4 mL FACS Buffer

Permeabilization Buffer (0.1% saponin in FACS Buffer)

1. Add 500 µl of 10x saponin to 49.5 mL FACS Buffer

Phosphate Buffered Saline (PBS; 10x)

Per 1 L

1. Dissolve 2 g of potassium chloride, 2 g of potassium dihydrogen phosphate, 80 g of sodium chloride and 9.2 g of disodium hydrogen phosphate to 800 mL of ddH_2O.
2. Adjust pH to 7.2-7.4.
3. Bring volume up to 1 L, filter sterilize at store at RT.

Red blood cell lysis buffer (1x)

Per 1 L

1. Mix 8.29 g of ammonium chloride, 1 g of potassium bicarbonate and 37.2 mg of disodium ethylenediaminetetraacetic acid to 800 mL of ddH_2O.
2. Adjust pH to 7.2-7.4.
3. Bring volume up to 1 L, filter sterilize and store at 4 °C.

Tissue Culture Media

Per one 500 mL bottle of 1x high glucose Dulbecco's Modified Eagle's Medium (DMEM):

1. Add 50 mL of fetal bovine serum (FBS), 5 mL of penicillin/streptomycin (10,000 units/mL of penicillin G sodium and 10,000 µg/mL of streptomycin sulphate), 5 mL of 200 mM L-glutamine, 5 mL NEAA (non essential amino acids), 1 mM sodium pyruvate and 500 µL of 5×10^{-3} M 2-mercaptoethanol.

2. Mix, filter sterilize and store at 4°C

Western Blot

a) 30% Acrylamide/0.8% Bisacrylamide:

Per 100 mL

1. Add 30 g acrylamide, and 0.8 g N, N'-methylenebisacrylamide to 100 mL ddH_2O. Filter and store at 4°C in the dark.

b) 4X Tris.Cl/SDS, pH 8.8:

Per 100 mL

1. Dissolve 18.2 g Tris base in 75 mL ddH_2O. Adjust pH to 8.8. Add 0.4 g SDS and bring volume up to 100 mL with ddH_2O. Filter and store at 4°C.

c) 4X Tris.Cl/SDS, pH 6.9:

Per 50 mL

1. Dissolve 3.03 g Tris base in 40 mL ddH_2O. Adjust pH to 6.8. Add 0.2 g SDS and bring volume up to 50 mL with ddH_2O. Filter and store at 4°C.

d) 10% Ammonium Persulfate (APS):

Per 10 mL

1. Add 1 g APS to 10 mL ddH_2O. Store at 20°C.

e) 1% Bromophenol Blue:

Per 5 mL

1. Add 0.05 g bromophenol blue to 5 mL ddH_2O.

f) 2M Tris, pH 7.6:

Per 2.5 mL

1. Dissolve 0.6057 g Tris base in 1 mL ddH_2O. Adjust pH to 7.6 then bring volume up to 2.5 mL with ddH_2O.

g) Lysis Buffer for Tissue:

1. Add 0.018g NaCl, 20 µl Triton-X, 100 µl 1M Tris, pH 8.0, and 20 µl protease inhibitor cocktail to 1.86 mL ddH_2O.

h) 2X Loading (Laemmli) Buffer:

Per 10 mL

1. Add 2.5 mL 4X Tris.Cl/SDS, pH 6.8, 2 mL glycerol, 1 mL β 2-mercaptoethanol, 0.4 g SDS and 0.2 mL 1% bromophenol blue to 10 mL ddH_2O. Store at -80°C.

i) 10X Running Buffer:

Per 500 mL

1. Dissolve 72.08 g glycine, 15.15 g Tris base and 5 g SDS in 100 mL ddH_2O. Bring volume up to 500 mL with ddH_2O.

j) Wash Buffer:

Per 1 L

1. Dissolve 5 mL Tween 20, 5 mL 2M Tris, pH 7.6, and 5.84 g NaCl in 1 L ddH_2O. Store at 4°C.

k) Transfer Buffer:

Per 1 L

1. Add 200 mL methanol, 3.03 g Tris base, 14.43 g glycine and 0.5 g SDS to 800 mL ddH_2O.

l) Blocking Buffer:

Per 100 mL

1. Add 5 g non-fat milk powder to 100 mL of wash buffer

m) 10% Resolving Gel:

Per 15 mL (2 gels)

1. Add 5 mL 30% polyacrylamide, 3.75 mL 4X Tris.Cl/SDS, pH 8.8, and 10 µl TEMED to 6.25 mL ddH_2O.
2. Add 50 µl 10% APS and pour gel.

n) 4% Stacking Gel:

Per 5 mL (2 gels)

1. Add 0.65 mL 30% polyacrylamide, 1.25 mL 4X Tris.Cl/SDS, pH 6.8, and 5 µl TEMED to 3.05 mL ddH_2O.
2. Add 25 µl 10% APS and pour stacking gel.

ANIMALS

B6-RAG B6.129S7-*Rag1*^{tm1Mom} (B6 $Rag^{-/-}$) were obtained from Jackson Laboratories. TCR transgenic Rag2-/- Marilyn (originally obtained from the NIAID Exchange Program)[286], and TCR transgenic Rag2-/- Marilyn x C57BL/6-*Pdcd1-/-* (PD-1-/-; originally generated by T. Honjo[287]). All three stains were bred on-site at the University of Alberta under viral and specific pathogen-free conditions.

WESTERN BLOT FOR AUTOANTIBODIES

Various tissues were dissected from BALB/c *Rag*$^{-/-}$ mice and placed in ice cold lysis buffer. Tissues were homogenized in lysis buffer for at least 15 min on ice. Samples were then centrifuged at 12 000 g for 10 min at 4°C. The supernatant was removed and the pellet discarded. The concentration of protein in the supernatant was determined using a Bradford Assay. A standard curve was prepared by diluting 1.47 mg/ml BSA in ddH_2O to make a stock solution. This stock solution was further diluted to 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml and ddH_2O was used for 0 µg/ml. Samples were diluted 1:40 and 1:100 and 950 µl of Bradford reagent was added to 5 µl of sample. All samples were read at 595 nm using a spectrophotometer. Supernatant that was not used immediately was stored at -80°C.

Samples were prepared by adding 30 µg of protein at a 1:1 dilution to Laemmli buffer on ice. Protein samples were then denatured by boiling at 100°C for 5 min and loaded into the gel assembly prepared with a 10% resolving gel and 4% stacking gel. Amersham ECL Plex™ Fluorescent Rainbow Markers (GE Healthcare, Fairfield, CT) was used as a molecular weight standard. Samples were run in 1x Running Buffer for 35 min at 200V in a Mini Trans-Blot® Cell (Bio-Rad, Hercules, CA). The protein gels were then transferred onto a nitrocellulose membrane in 1x transfer buffer at 4°C for 1 hr at 100V and 350 mA. Upon completion membranes were blocked for 1 hr at RT in blocking buffer on a plate shaker. The primary antibody (mouse serum) was diluted 1:480 in blocking buffer and membranes were incubated at 4°C overnight on a plate shaker. Membranes

were then washed in 1x wash buffer, 3x at 5 min each on a plate shaker to remove residual primary antibody. To detect primary antibody binding, anti-mouse IgG-HRP (eBioscience) was diluted at 1:1000 in blocking buffer and membranes were incubated for 1 hr in the dark at RT on a plate shaker. Membranes were then washed 3x for 5 min each. To detect and image the antibody binding, blots were incubated for 5 min with 2 mL of ECL^{+} (GE Healthcare) in the dark. Blots were then imaged using a Typhoon Trio^{TM} (GE Healthcare).

HISTOLOGY

a. Fixation for Paraffin Sections

Organs were fixed for 24-48 hours in buffered zinc formalin fixative (Z-Fix; Anatech Ltd, Battle Creek, MI) before processing. Organs were then stored in 70% ethanol until processing. Organs were paraffin-embedded and tissues were sectioned at 3 μM and placed on histobond slides (Marienfield, Germany).

b. Hematoxylin and Eosin Staining

Protocol adapted from the Alberta Diabetes Institute Histology Core

1. Dehydrate paraffin sections:
    a. Histoclear, 3x (5 min per).
    b. 100% EtOH, 3x (2 min per).
    c. 95% EtOH, 1x (1 min).
    d. 70% EtOH, 1x (1 min).
    e. ddH_2O (5 min).

2. Harris' Hematoxylin (1 min 30 sec). Rinse under tap water until water clears.
3. 0.25% acid alcohol (3 sec). Agitate slides in water (5 sec).
4. Lithium carbonate (30 sec). Rinse in water (30 sec).
5. 90% EtOH (30 sec).
6. Alcoholic Eosin Y (35 sec).
7. Dehydrate slides:
    a. 95% EtOH (30 sec).
    b. 100% EtOH (4x 30 sec).
    c. Xylene (3x 5 min).
8. Coverslip and mount in Enellan mounting media (Electron Microscopy Sciences, Hattlefield, PA).

c. Counterstain protocol for Harris' hematoxylin.

    a. Rinse in water (1 min).

    b. Hematoxylin (1 min). Rinse in water (2 min).

    c. 0.25% acid alcohol (0.03 min). Rinse in water (1 min).

    d. Lithium carbonate (0.03 min). Rinse in water (1 min).

    e. Dehydrate slides

        i. 70% EtOH (1 min)

        ii. 95% EtOH (1 min)

        iii. 100% EtOH (3x 2 min)

        iv. Xylene (2x 5 min)

f. Coverslip and mount slides in Permount (Electron Microscopy Sciences)

SERUM CYTOKINES

At the time of sacrifice, mice were anaesthetized by inhalation of isofluorane. They were exsanguinated using a 3 mL syringe and 23-gauge needle and blood was transferred into a 1.5 mL microcentrifuge tube and allowed to clot at RT for at least 20 min. Blood was then centrifuged for 20 min at >2000 x g for serum recovery. Serum was stored at -80°C until analysis was performed. Serum cytokine was performed using a mouse cytokine bead assay (Millipore, Billerica, MA) for IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, and TNF-α according to the manufacturer's instructions. Data was acquired using a Luminex 100TM System (Applied Cytometry Systems, Sheffield, UK) and analyzed using STarStation 2.0 software.

T CELL PURIFICATION

T cells were purified from the spleen or thymus using a StemCell EasySep® (Vancouver, BC, Canada) negative selection T cell kit according to the manufacturer's instructions. The population of T cells from thymocytes was split in half and one population was subjected to CD4 positive selection and the other to CD8 positive selection using StemCell EasySep® kits according to the

manufacturer's instructions. The negatively selected fractions were then mixed together and adoptively transferred by I.V. into BALB/c SCID mice.

VIOLET PROLIFERATION DYE TRANSFER EXPERIMENTS

Spleens and thymi were removed from PD-$1^{-/-}$ Marilyn mice. Tissues were homogenized between two frosted glass slides and filtered through a 70 µm nylon cell strainer. Thymocytes and splenocytes were then stained using a CellTrace™ Violet Proliferation Dye Kit (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. Cells were assessed for viability and verified for staining using flow cytometry. Then 200 000 unsorted SP thymocytes or T cells from splenocytes were adoptively transferred by I.V. injection into immunodeficient B6 $Rag^{-/-}$ males or females. Mice were sacrificed on day 7 post transfer and the spleens and inguinal LNs were removed for analysis of T cell proliferation using flow cytometry.

A2: PRELIMINARY DATA FOR MICE DEVELOPING DISEASE AFTER RECEIVING THORACIC THYMECTOMY AND FETAL LIVER

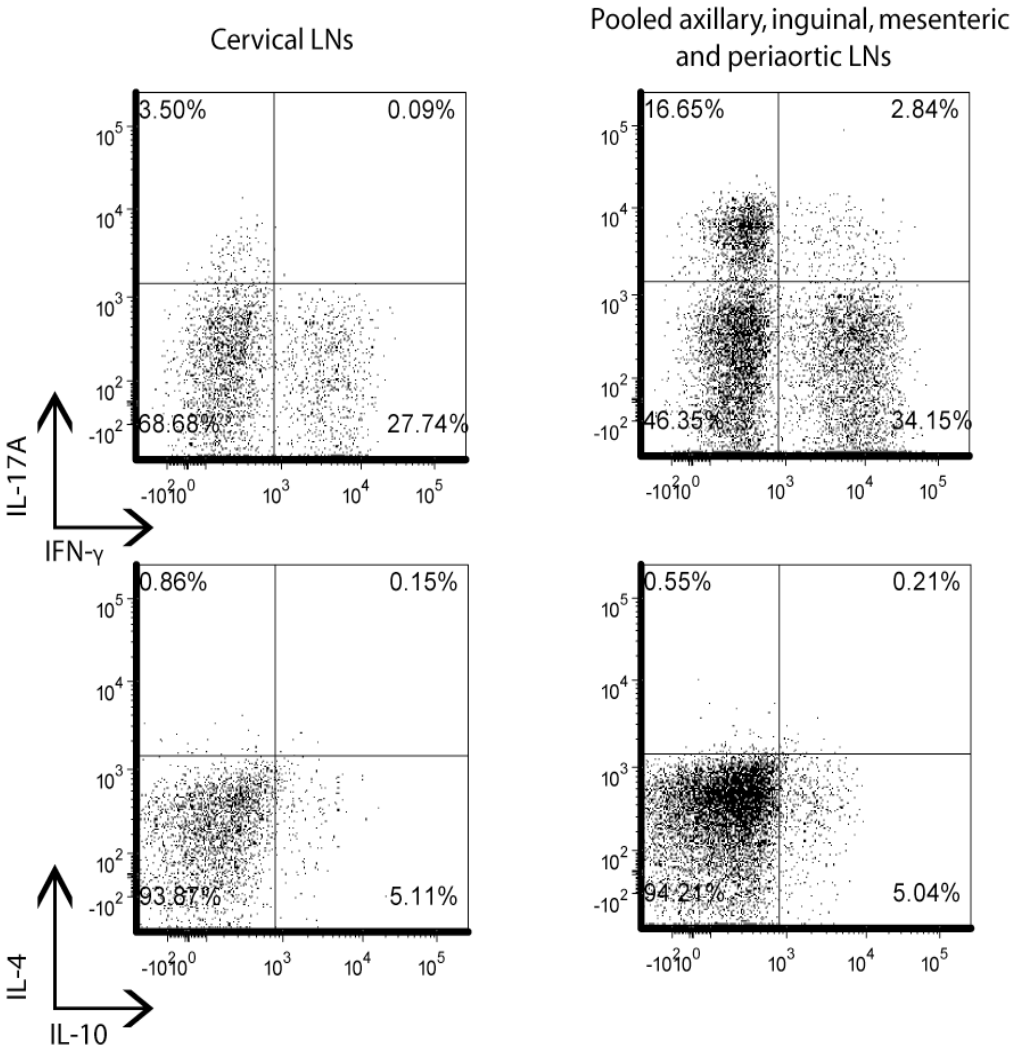

Figure A2.1. Increased intracellular IL-17A is detected in pooled axillary, mesenteric and periaortic lymph nodes but not cervical lymph nodes.
The cervical lymph nodes or pooled axillary, mesenteric and renal lymph nodes from a thoracic Tx + FL mouse that got disease (n=1) were re-stimulated for 5 hours in the with PMA and ionomycin in the presence of brefeldin A and monensin.

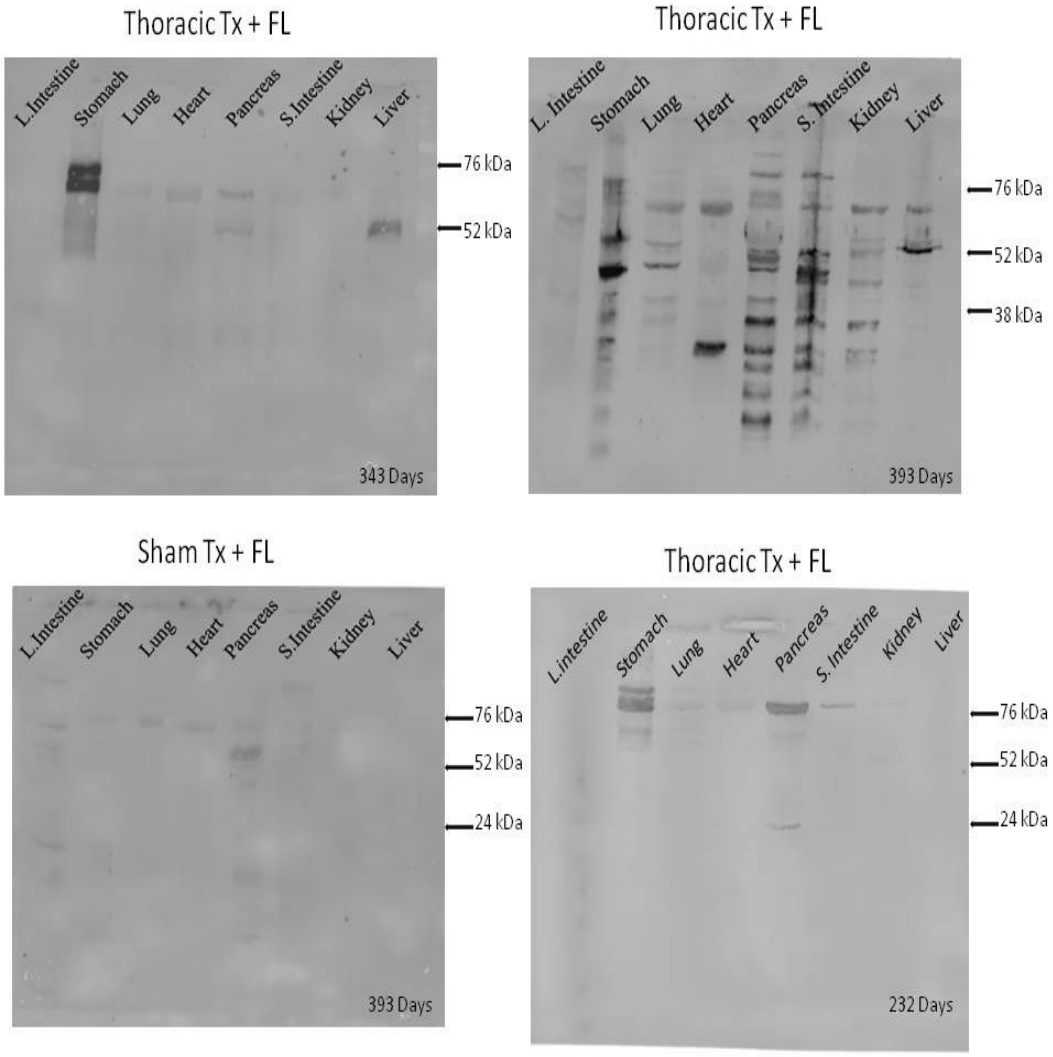

Figure A2.2. Detection of tissue-specific autoantibodies in serum.
Serum antibody binding was detected in the stomach and pancreatic tissue in mice that develop disease after thoracic thymectomy and fetal liver cells. Various tissues from BALB/c *Rag*$^{-/-}$ mice were run on an SDS-Page gel. Proteins were then transferred onto a nitrocellulose membrane and incubated with serum overnight from thoracic Tx + FL mice (n=3) or Sham Tx + FL mice (n=1). Binding of serum antibody was detected using anti-mouse IgG.

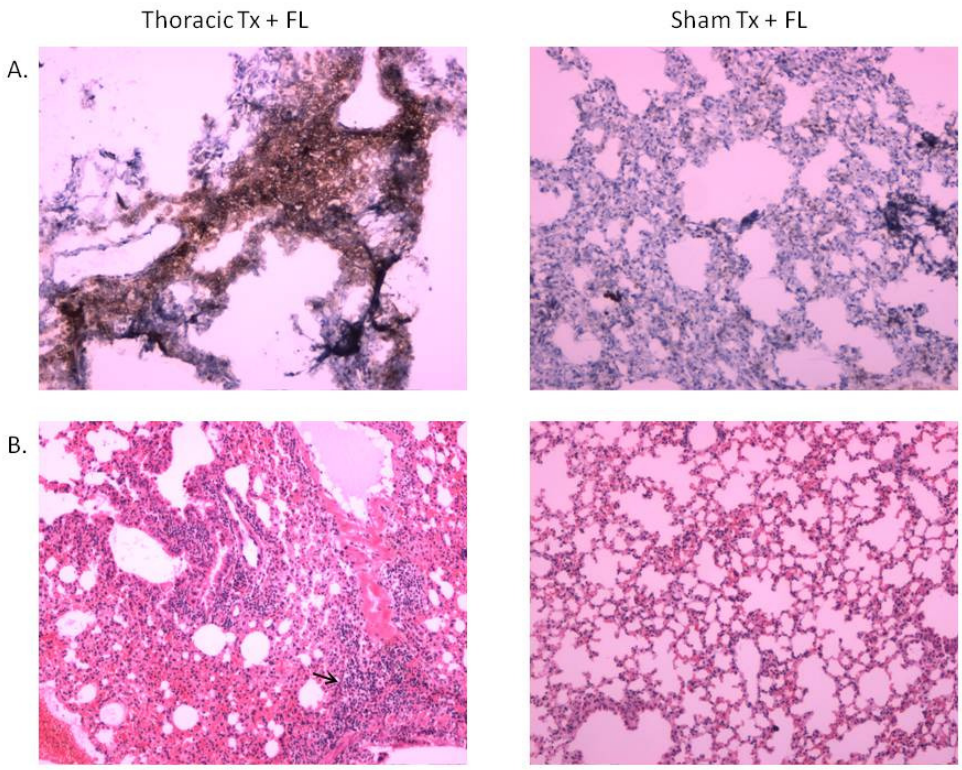

Figure A2.3. CD4 T cell staining and leukocyte infiltration in the lung of a thoracic Tx + FL.
Thoracic Tx + FL (n=1) or sham Tx + FL (n=1) mice were analyzed for CD4 staining and infiltration in the lung. (A) Detection of CD4 cells by immunohistochemistry. 100x magnification. (B) H&E staining in the lung of thoracic Tx + FL (*left panel*) and sham Tx + FL (*right panel*). 100x magnification.

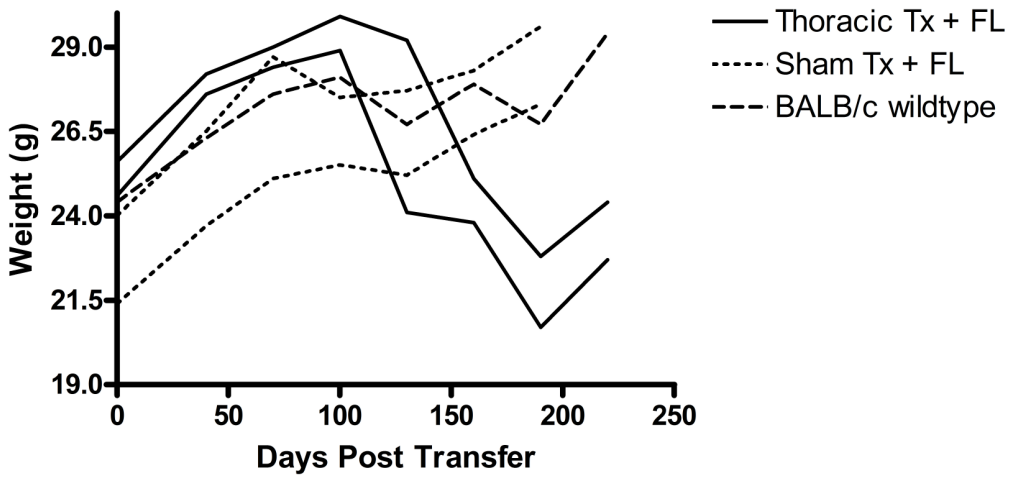

Figure A2.4. Disease can be transferred from thoracic Tx + FL mice but not efficiently.
Splenocytes containing $10x10^6$ T cells from thoracic Tx + FL (n=1; diseased), sham Tx + FL (n=1) or BALB/c WT (n=1) were transferred into immunodeficient BALB/c $Rag^{-/-}$ mice and monitored for disease. The above graph depicts the weight (g) of transfer recipients. Each line represents one mouse and weight measurements end at the endpoint of the experiment for that particular mouse.

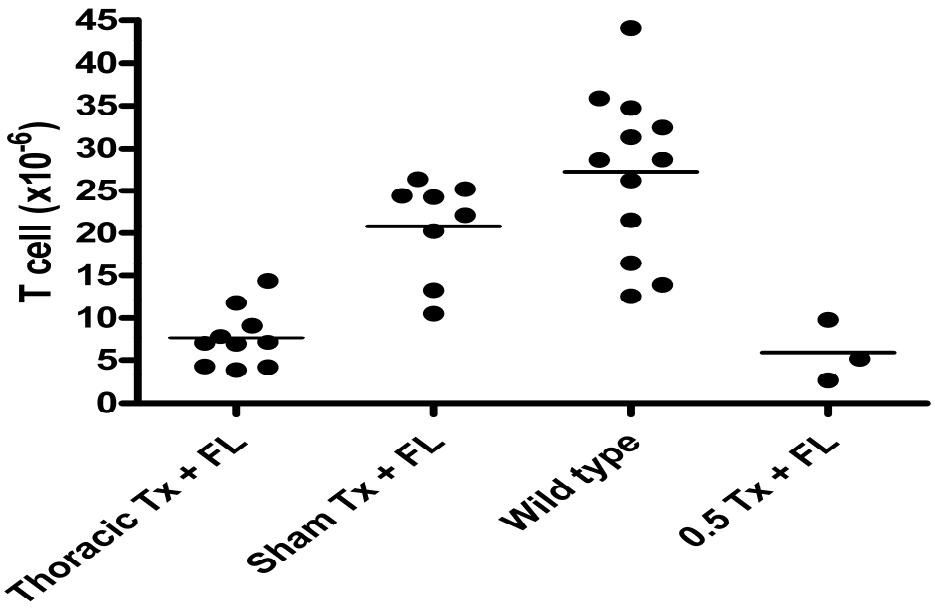

Figure A2.5. Mice that receive half a thoracic thymectomy have lower number of T cells compared to mice with a whole thoracic thymus.
T cell numbers in the spleen were determined from mice that received thoracic Tx + FL, sham Tx + FL, BALB/c WT or half of the thoracic thymus removed (0.5Tx + FL). Each dot is representative of one mouse.

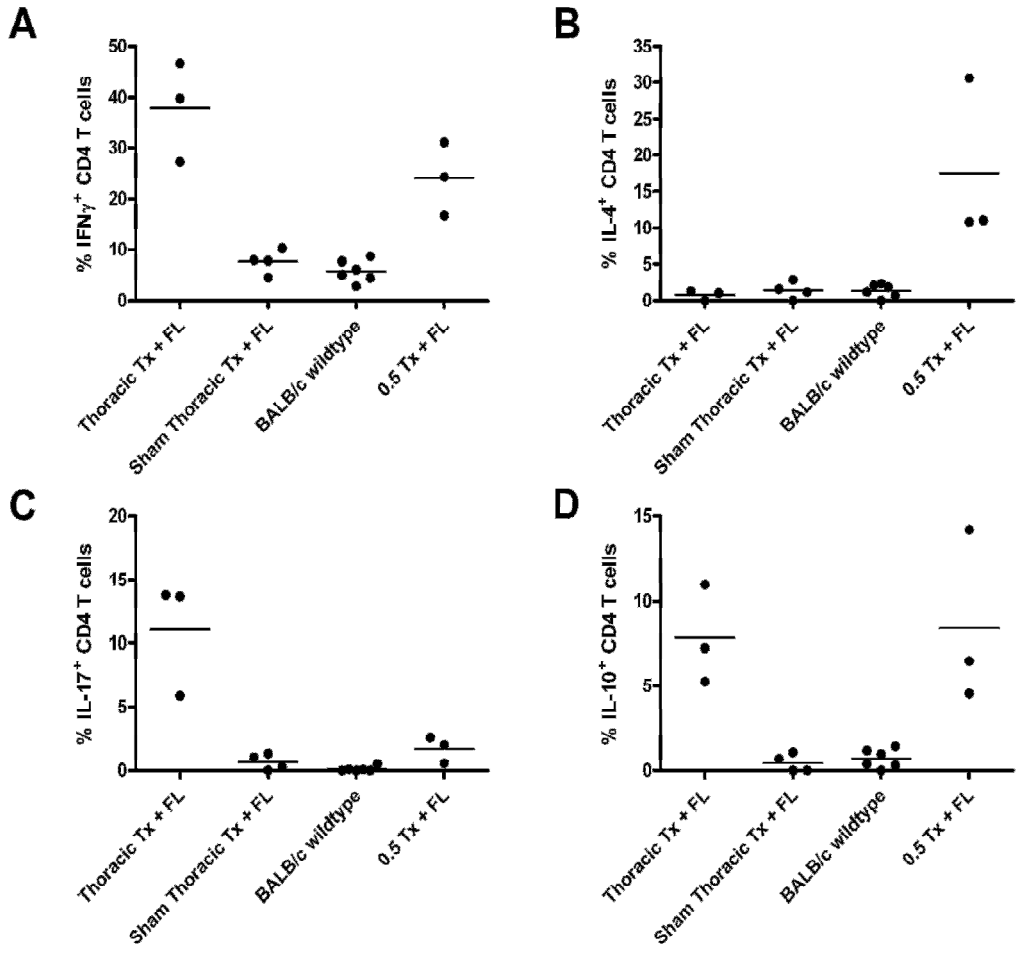

Figure A2.6. Intracellular cytokine profiles of CD4 T cells after 0.5Tx + FL. Mice that receive have a half a thoracic thymectomy + FL mimic thoracic Tx + FL as opposed to sham Tx + FL or WT in terms of CD4 IFN-γ and IL-10 intracellular cytokine percentages, but have decreased IL-17A and increased IL-4. Splenocytes from Thoracic Tx + FL, sham Tx + FL, BALB/c WT or 0.5Tx + FL were re-stimulated for 5 hours with PMA and ionomycin in the presence of brefeldin A and monensin. Cells were then stained intracellularily and assessed for percentages of CD4 T cells with the following cytokines: (A) IFN-γ. (B) IL-4. (C) IL-17A. (D) IL-10. Each dot is representative of one mouse.

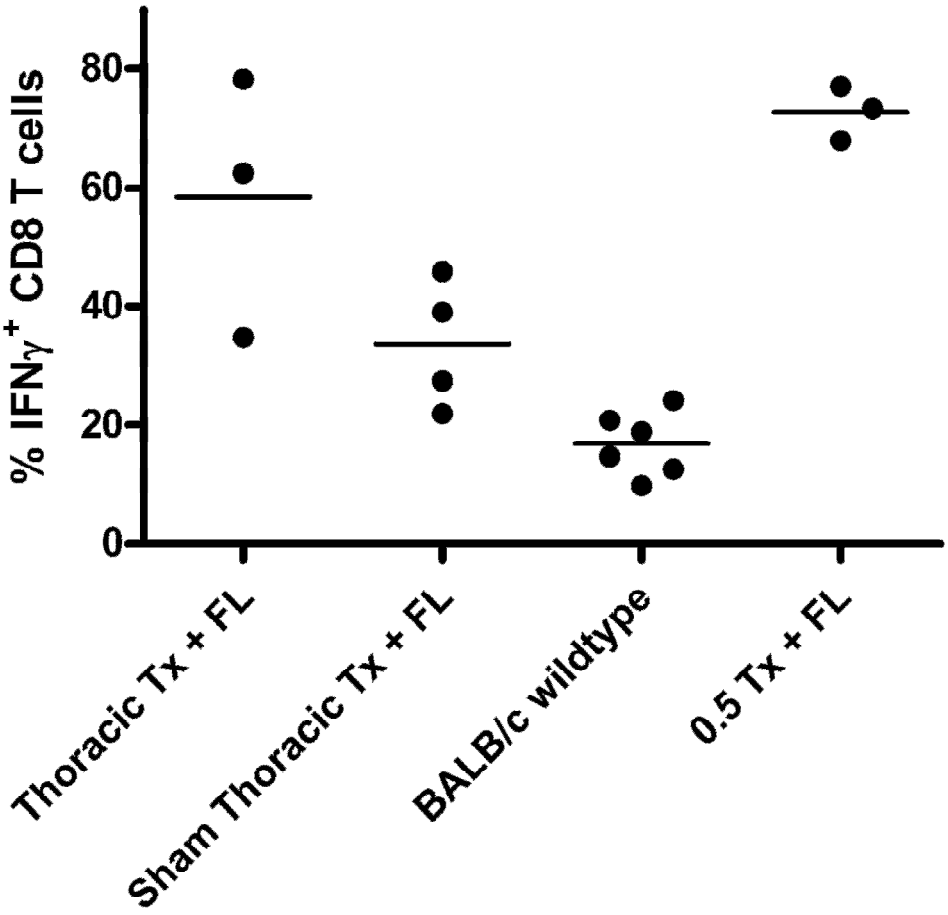

Figure A2.7. Intracellular cytokine profiles of CD8 T cells after 0.5Tx + FL. Mice that receive have a half a thoracic thymectomy + FL mimic thoracic Tx + FL CD8 IFN-γ intracellular cytokine percentages. Splenocytes from Thoracic Tx + FL, sham Tx + FL, BALB/c WT or 0.5Tx + FL were re-stimulated for 5 hours with PMA and ionomycin in the presence of brefeldin A and monensin. Cells were then stained intracellularily and assessed for percentages of CD8 T cells with IFN-γ. Each dot is representative of one mouse.

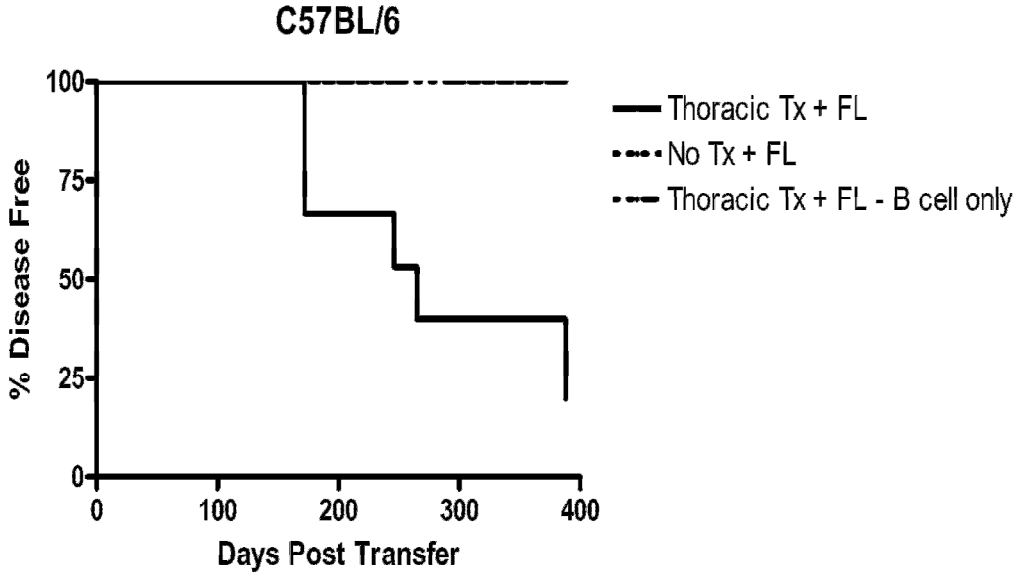

Figure A2.8 C57BL/6 mice given thoracic thymectomy and fetal liver are susceptible to disease.
C57BL/6 *Rag*$^{-/-}$ mice received a thoracic Tx + FL and developed T and B cells (n=8), thoracic Tx + FL and developed B cells only (n=5) or given no thoracic Tx + FL (n=4). Data are representative of two independent experiments. Fetal liver cells were from C57BL/6 WT time pregnant mice at day 14 of gestation.

A3: PRELIMINARY DATA FOR MICE RECEIVING IL-7/ANTI-IL-7 COMPLEXES AFTER THYMOCYTE OR SPLENOCYTE TRANSFER

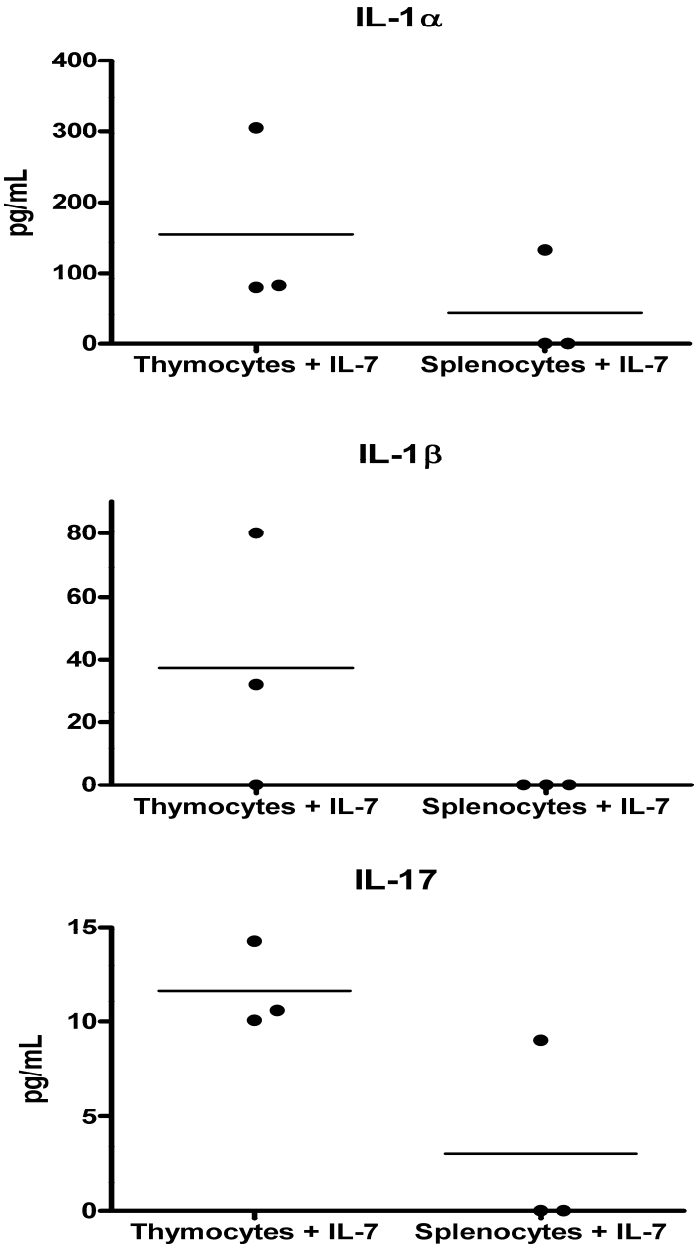

Figure A3.1. Serum cytokines in transfer mice that receive IL-7/anti-IL-7 complexes.
Mice that receive thymocytes and IL-7/anti-IL-7 complexes have a potential increase in serum IL-1α, IL-1βand IL-17. Serum cytokines were quantified using a cytokine multiplex bead assay. Each dot is representative of one mouse

A4: LYMPHOPENIC MICE GIVEN PURIFIED T CELLS FROM SPLENOCYTES HAVE INCREASED WEIGHT GAIN IN A LYMPHOPENIC RECIPIENT

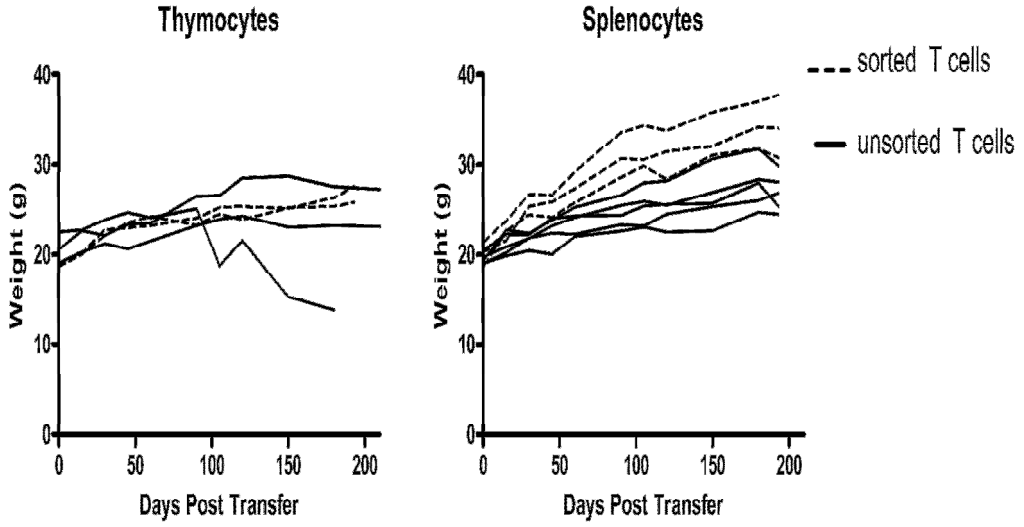

Figure A4.1. Transfer of purified T cells from splenocytes leads to increased weight gain in recipients.
Single positive T cells from thymocytes or T cells from splenocytes from BALB/c WT were purified and $1.5x10^5$ T cells (dotted line) or whole thymocytes or splenocytes from BALB/c WT containing $5x10^5$ single positive T cells (solid line) were transferred into immunodeficient BALB/c SCID mice. Weight (g) was monitored over time. Each line indicates one mouse.

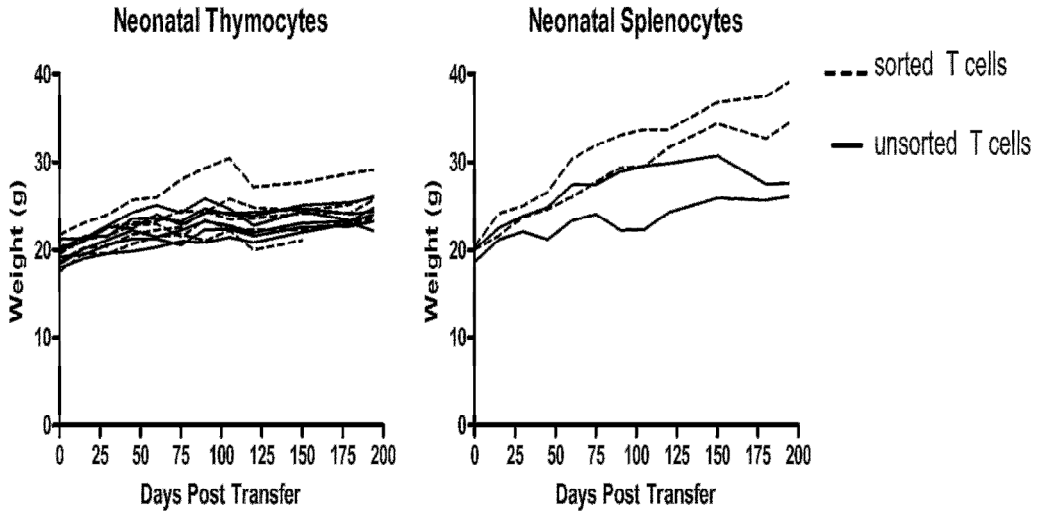

Figure A4.2. Transfer of purified T cells from neonatal splenocytes leads to increased weight gain in recipients.
Single positive T cells from thymocytes or T cells from splenocytes from day 4 neonatal mice were purified and $1.5x10^5$ T cells (dotted line) or whole thymocytes or splenocytes from day 4 neonatal mice containing $5x10^5$ single positive T cells (solid line) were transferred into immunodeficient BALB/c SCID mice. Weight (g) was monitored over time. Each line indicates one mouse.

A5: CD4 T CELLS FROM MARILYN MICE HAVE INCREASED PROLIFERATION CAPACITIES IN THE ABSENCE OF PD-1

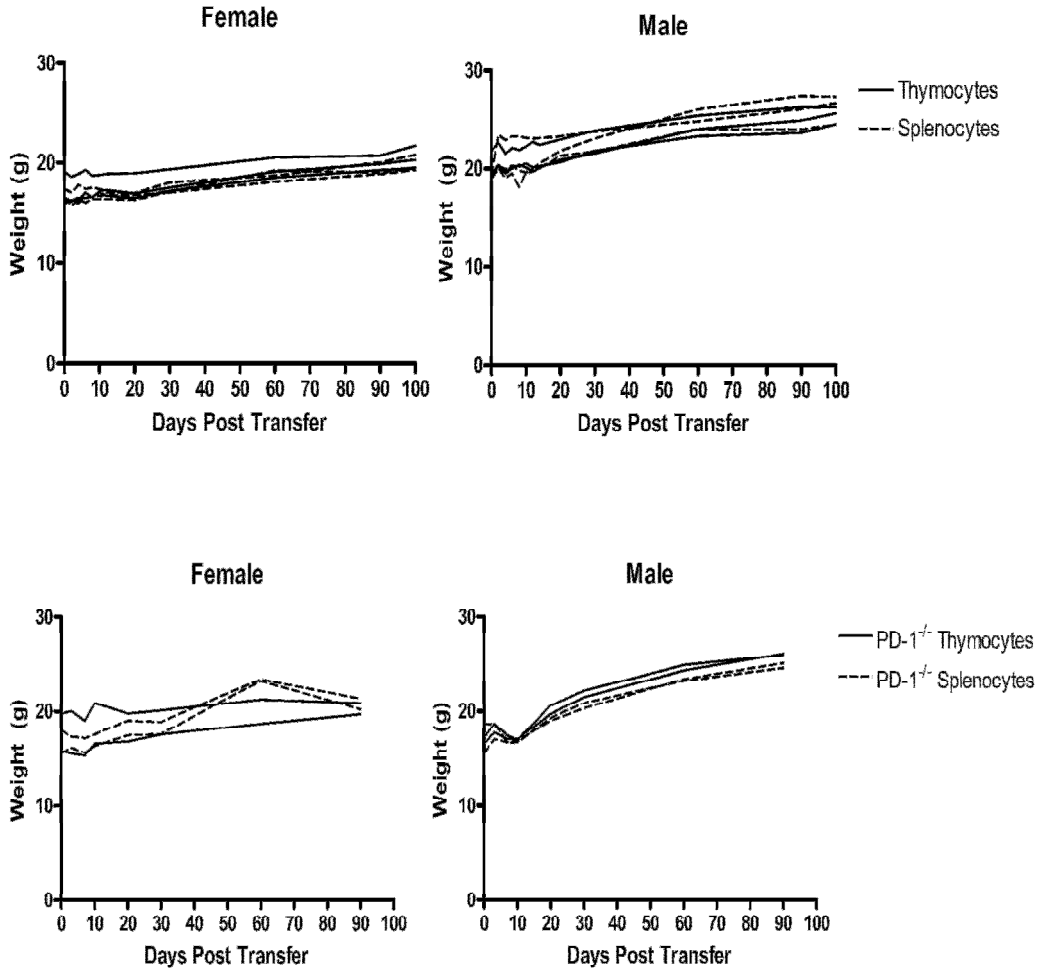

Figure A5.1. Transfer of transgenic PD-1$^{-/-}$ Marilyn thymocytes or splenocytes into an immunodeficient male causes transient weight loss. Whole thymocytes (black line) or splenocytes (blue line) containing 2x10^5 SP T cells from CD4 Marilyn (*top panel*) or PD-1$^{-/-}$ Marilyn (*bottom panel*) female mice were transferred into either male or female C57BL/6 Rag$^{-/-}$ mice. Weight (g) was monitored. Each line is representative of one mouse.

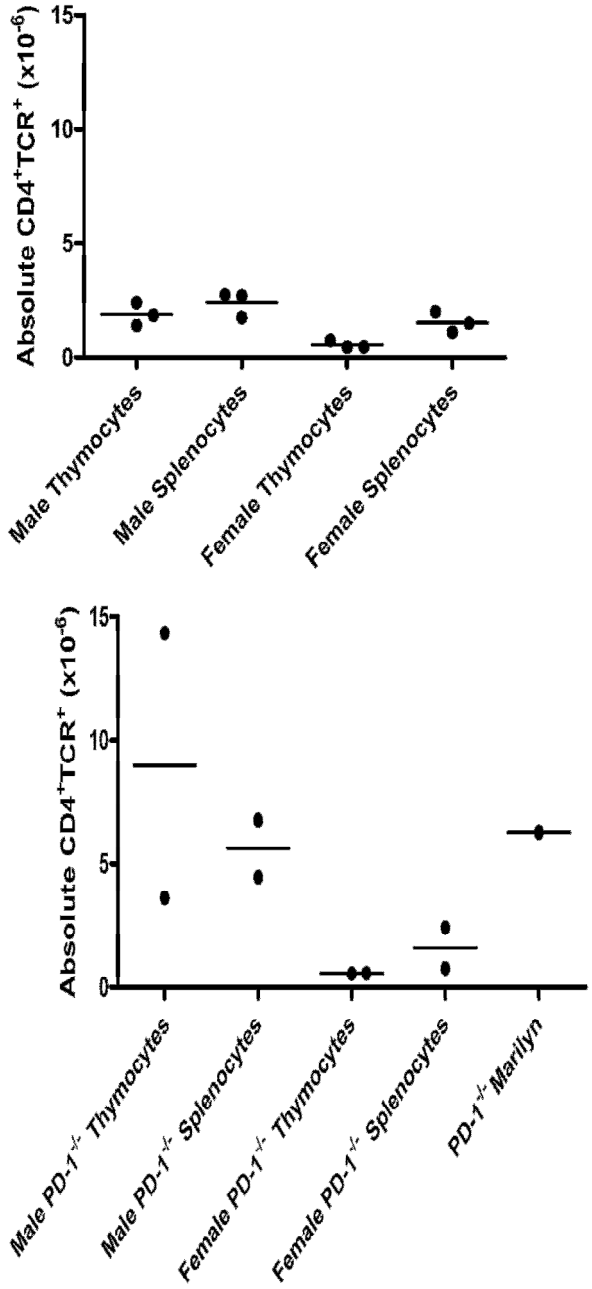

Figure A5.2. Marilyn T cells proliferate at low levels in an immunodeficient male mouse, but lack of PD-1 leads to increased proliferation.
T cells numbers from the spleens of B6 $Rag^{-/-}$ mice receiving either Marilyn or PD-$1^{-/-}$ Marilyn T cells from thymocytes or splenocytes were assessed 90 days post transfer.

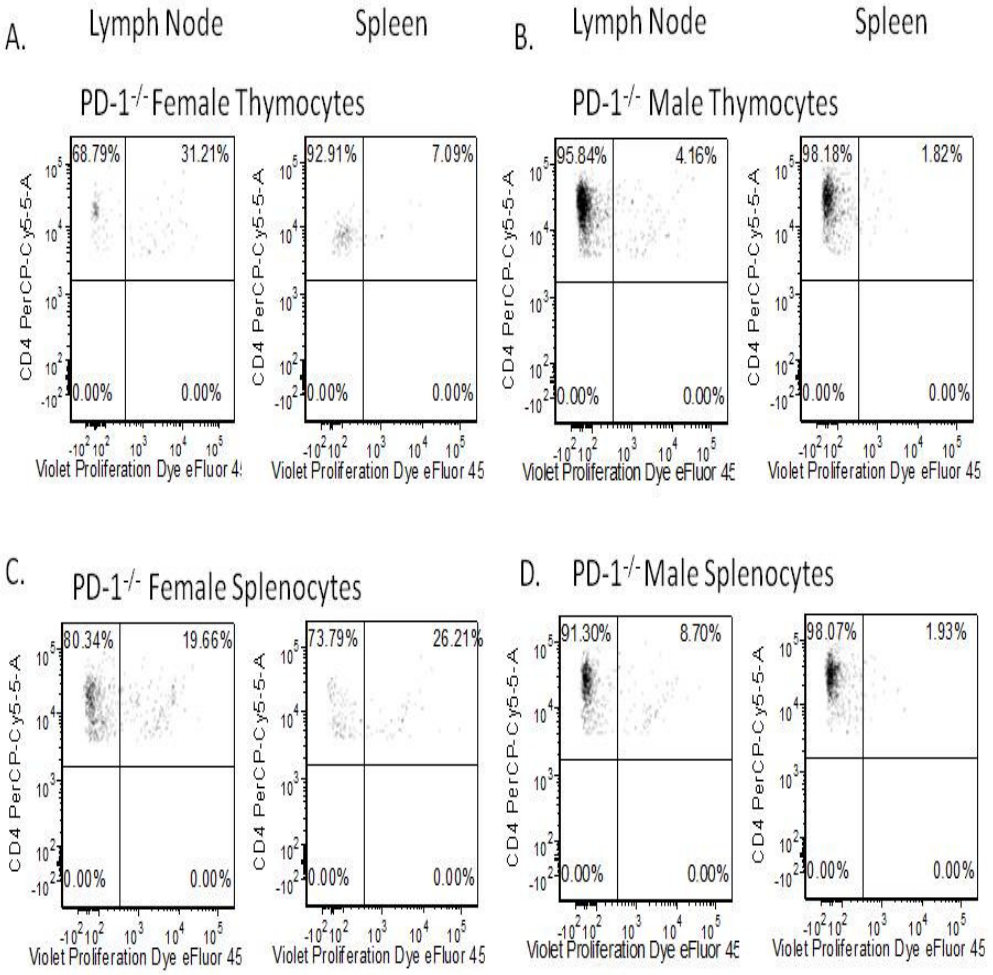

Figure A5.3. PD-1$^{-/-}$ Marilyn T cells from splenocytes and thymocytes undergo increased rates of proliferation in a lymphopenic male compared to female B6 Rag$^{-/-}$ mouse.

Whole thymocytes or splenocytes containing 2x10^5 SP T cells were labelled with Violet Proliferation Dye and transferred into a B6 Rag$^{-/-}$ male or female. The spleens (*left*) and inguinal lymph (*right*) nodes were removed from each mouse 7 days post transferred and assessed for levels of CD4 T cell proliferation. (A) PD-1$^{-/-}$ Marilyn thymocytes transferred into a female B6 Rag$^{-/-}$. (B) PD-1$^{-/-}$ Marilyn thymocytes transferred into a male B6 Rag$^{-/-}$. (C) PD-1$^{-/-}$ Marilyn splenocytes transferred into a female B6 Rag$^{-/-}$. (D) PD-1$^{-/-}$ Marilyn splenocytes transferred into a male B6 Rag$^{-/-}$. (n=1 for each group).

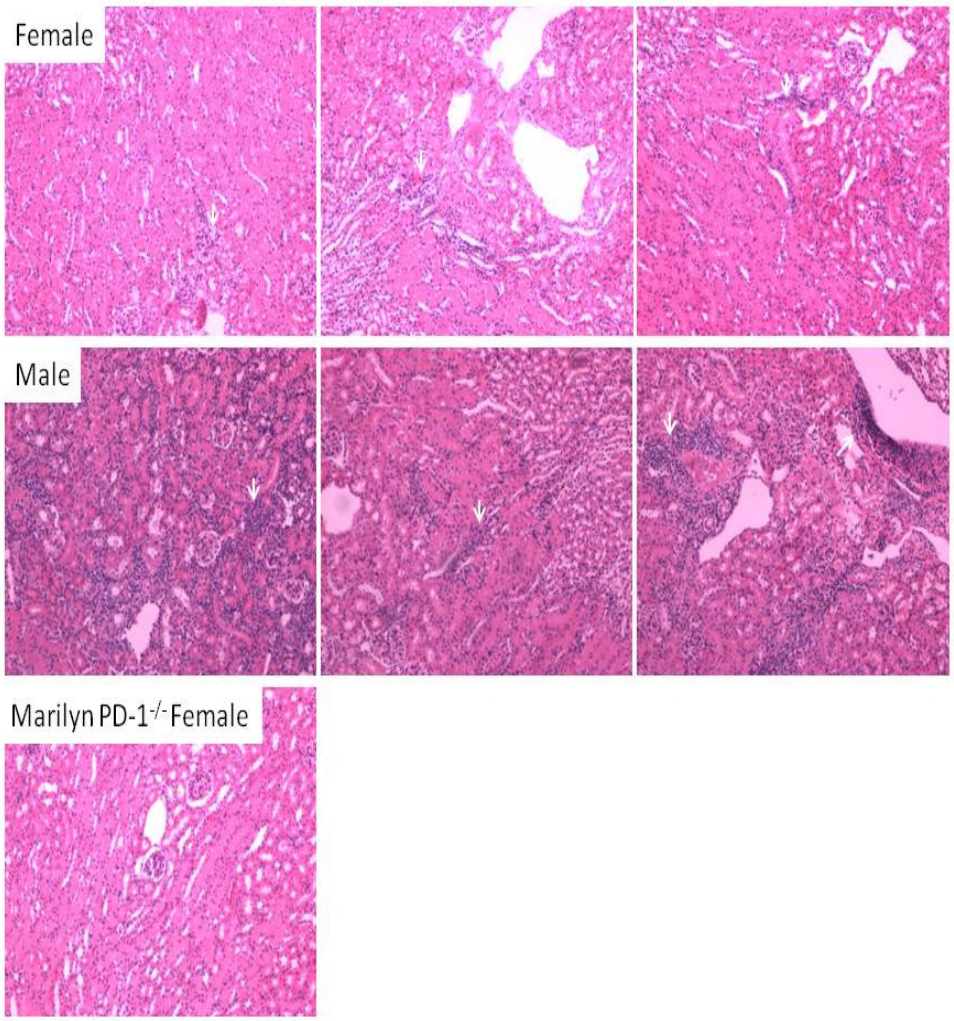

Figure A5.4. Transfer of PD-1$^{-/-}$ Marilyn T cells into a male or female mouse causes infiltration in the kidney but no apparent disease.
PD-1$^{-/-}$ Marilyn whole thymocytes or splenocytes containing 200 000 SP T cells were transferred into a B6 Rag$^{-/-}$ female (*top row*) or male (*middle row*). The kidney of a PD-1$^{-/-}$ Marilyn female is shown for comparison (*bottom row*). H&E staining of the kidney 90 days post transfer. 100x magnification. Data are representative of one experiment.

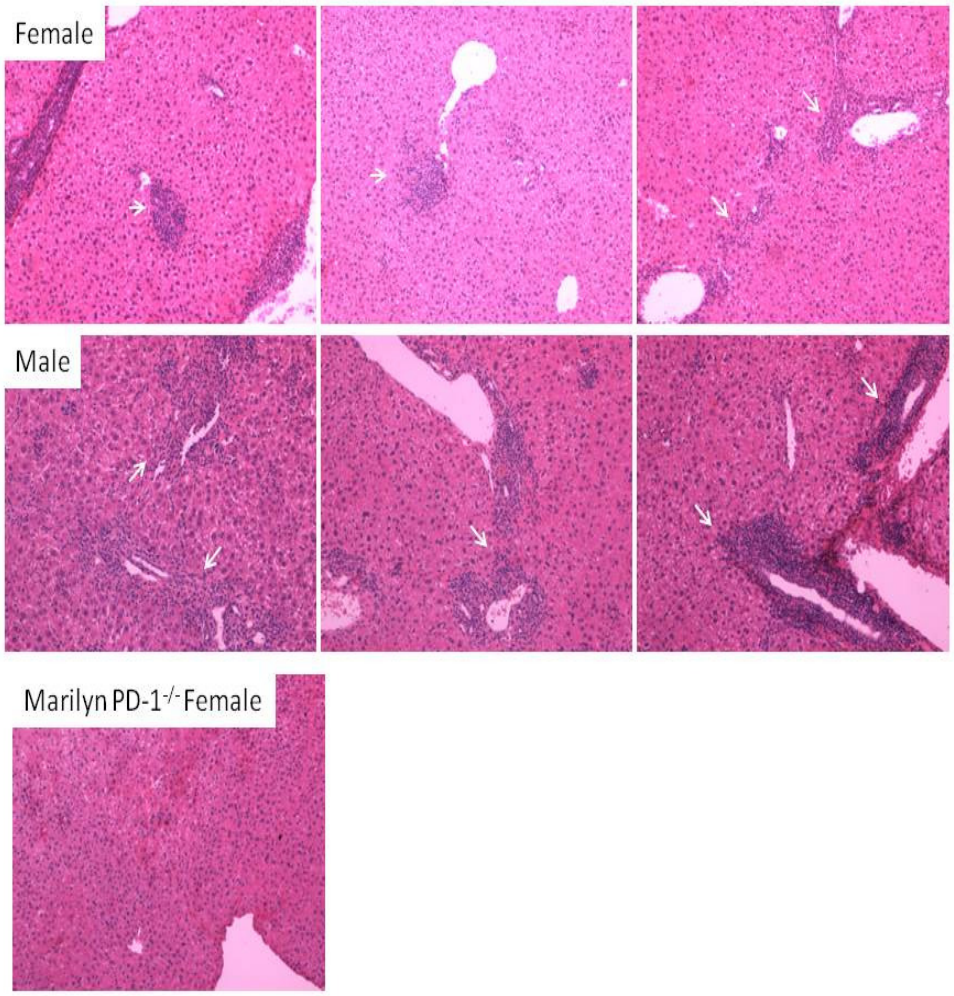

Figure A5.5. Transfer of PD-1$^{-/-}$ Marilyn T cells into a male or female mouse causes infiltration in the liver but no apparent disease.
PD-1$^{-/-}$ Marilyn whole thymocytes or splenocytes containing 200 000 SP T cells were transferred into a B6 Rag$^{-/-}$ female (*top row*) or male (*middle row*). The liver of a PD-1$^{-/-}$ Marilyn female is shown for comparison (*bottom row*). H&E staining of the liver 90 days post transfer. 100x magnification. Data are representative of one experiment.

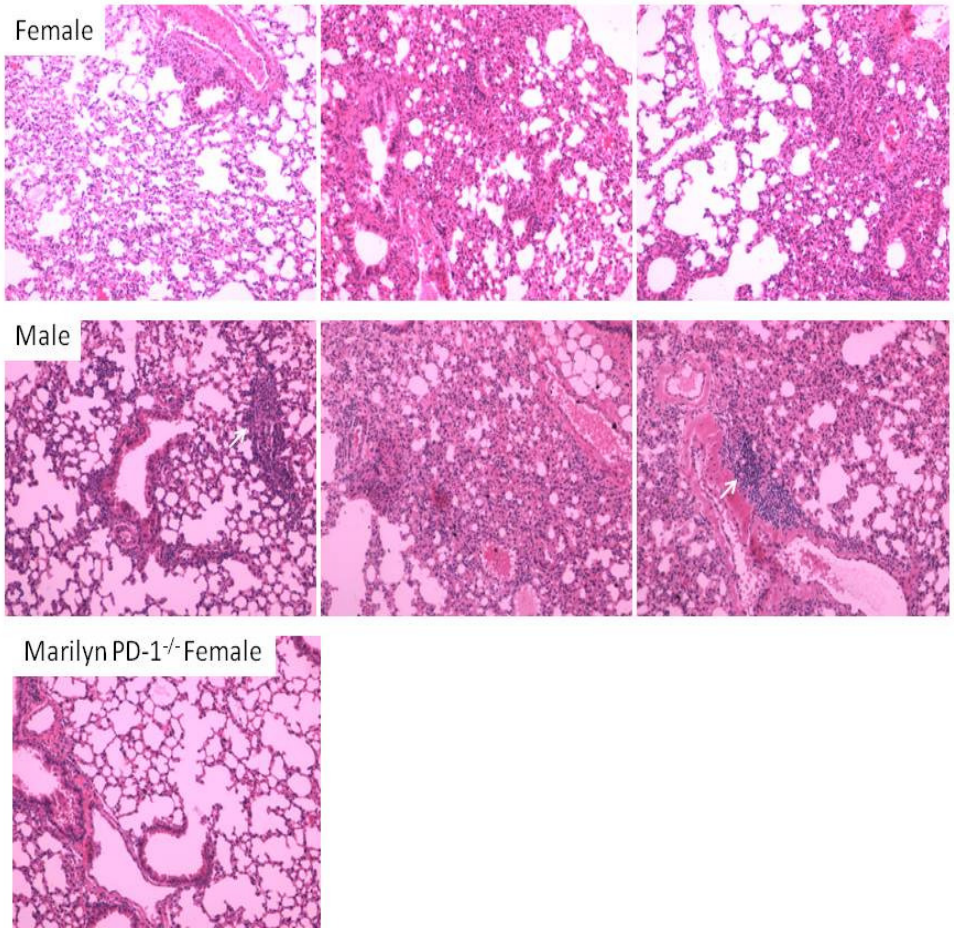

Figure A5.6. Transfer of PD-1$^{-/-}$ Marilyn T cells into a male mouse causes infiltration in the lung but no apparent disease.
PD-1$^{-/-}$ Marilyn whole thymocytes or splenocytes containing 200 000 SP T cells were transferred into a B6 Rag$^{-/-}$ female (*top row*) or male (*middle row*). The lung of a PD-1$^{-/-}$ Marilyn female is shown for comparison (*bottom row*). H&E staining of the lung 90 days post transfer. 100X magnification. Data are representative of one experiment.