Phenotypical and Functional Analysis of Peripheral T Cells in Foxn1 Transgenic Mice: Effects of Aging

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PHENOTYPICAL AND FUNCTIONAL ANALYSIS OF PERIPHERAL T CELLS IN FOXN1 TRANSGENIC MICE:
EFFECTS OF AGING

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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MASTER OF SCIENCE

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY, AND ANATOMY

BY
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LIST OF ABBREVIATIONS AND CELL SURFACE MARKERS

APC  allogycocyanin
APC  antigen presenting cell
APC-AF750  allogycocyanin-alexa-Fluorochrome 750
CFSE  5-(and-6)-carboxyfluorescein succinimidyl ester
DNA  deoxyribonucleic acid
DN  double negative thymocyte
ELISA  enzyme-linked immunosorbonent assay
ETP  early T lineage progenitors
FACS  fluorescence-activated cell sorting
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
Foxn1  Forkhead box N1
GH  growth hormone
IGF-1  Insulin-like growth factor-1
IL-2  Interleukin-2
ITAM  immunoreceptor tyrosine-based activation motif
m  months
MAPK  mitogen-activated protein kinase
MFI  mean fluorescence intensity
MHC  major histocompatibility complex
NF-κB  nuclear factor kappa B
PBS  phosphate buffered saline
PE  phycoerythrin
PECy7  phycoerythrin-Cy7
PerCP-Cy5.5  peridinin chlorophyll protein-Cy5.5
PI3K  phosphatidylinositol 3-kinase
SD  standard deviation
T_{CM}  central memory T cells
T_{EM}  effector memory T cells
Tg  transgenetic
Wt  C57BL/6 wild type mice
| **CCR7** | C-C chemokine receptor 7; also designated CD197; expressed on central memory T cells |
| **CD3** | cell surface marker that associates with T cell receptor |
| **CD4** | co-receptor for helper T cells |
| **CD8** | co-receptor for cytotoxic T cells |
| **CD25** | high affinity α-chain of the IL-2 receptor |
| **CD28** | co-stimulatory molecule required for T cell activation |
| **CD44** | T cell marker, used to define naïve and memory T cells, also expressed on thymocytes |
| **CD45RB** | T cell marker, used to define naïve and memory T cells |
| **CD62L** | L-selectin, adhesion molecule expressed on naïve and central memory T cells |
| **CD80** | B7.1 cell antigen, expressed on antigen presenting cells, binds to CD28 to co-stimulate T cells |
| **CD86** | B7.2 cell antigen, expressed on antigen presenting cells, binds to CD28 to co-stimulate T cells |
| **CD122** | IL-2 receptor β chain |
| **CD132** | common γ-chain receptor |
| **CD127** | IL-7 receptor α chain; T cell marker, expressed on central and effector memory T cells |
| **TCR** | T cell receptor |
ABSTRACT

The thymus is the primary organ for the development and production of TCRαβ naïve T cells. However, with increasing age thymic involution occurs, causing a decline in the output of naïve T cells. The decline in naïve T cell production results in a contraction in the peripheral naïve and expansion of the memory T cell pools. Not only are the production and compositions of peripheral T cells altered with age, T cell functions such as T cell proliferation and production of cytokines required for cell proliferation are also declined. Currently, it is not known if restoring the decline in the production of naïve T cells in aged animals would affect the peripheral T cell pools and their functions. In a mouse model, in which the transcription factor Foxn1 is over expressed under the regulation of the keratin 14 promoter, age-associated decline in the number of thymocytes is attenuated. Using the Foxn1 transgenic mouse model, we hypothesized that attenuation of thymic involution would prevent the age-associated decline in the naïve T pool and T cell functions. We first determined if over expression of Foxn1 in the thymus prevents the age-associated decrease in the number of peripheral naïve T cells. In comparison to aged wild type mice, the number of CD8+ naïve T cells in Foxn1 transgenic mice is not reduced with age and is equivalent to that found in the young wild type and young transgenic mice. In contrast, the number of CD4+ naïve T
cells declines with age in Foxn1 transgenic mice, as seen in wild type mice. However, the number of CD4\(^+\) naïve T cells in aged Foxn1 transgenic mice is 3.5 fold higher as compared to that in aged wild type mice. We next determined if Foxn1 transgenic mice demonstrated an age-related increase in the numbers of CD4\(^+\) and CD8\(^+\) memory T cells. In contrast to the naïve T cell pool, over expression of Foxn1 in aged mice does not prevent age-associated expansion of CD4\(^+\) and CD8\(^+\) memory T cells. While both wild type and Foxn1 transgenic mice show an age-related decrease in the number of total CD4\(^+\) T cells, the number of total CD8\(^+\) T cells does not change with age in Foxn1 transgenic mice as compared to wild type mice. We also determined if over expression of Foxn1 alters the decline in T cell functions with age. As previously shown, wild type mice demonstrated an age-related decrease in IL-2 production and CD4\(^+\) T cell proliferation. Similarly, IL-2 production and CD4\(^+\) T cell proliferation were also reduced with age in Foxn1 transgenic mice. Yet, CD8\(^+\) T cell proliferation did not change with age in wild type or Foxn1 transgenic mice. Surprisingly, T cells from young Foxn1 transgenic mice showed lower IL-2 production and CD4\(^+\) and CD8\(^+\) T cell proliferation upon activation as compared to T cells from young wild type mice. The reduction in T cell functions demonstrated by both aged wild type, young and aged Foxn1 transgenic mice could be due to the decrease in CD3 expression. These data demonstrate that maintaining the production of naïve T cells does not affect age-associated expansion of
the memory T cell pool and suggest that there are factors other than thymic production of naive T cells that alter the peripheral memory T cell compartments and T cell functional responses in aged mice.
CHAPTER 1
LITERATURE REVIEW

Thymic involution

The mammalian thymus is the major site for the development and output of TCRαβ naïve T cells. However, the thymus undergoes thymic involution, an age-related degeneration process, which alters T cell development and the production of naïve T cells. Thymic involution is an evolutionary conserved process that occurs within vertebrates that have a thymus (1). Thymic involution is also one of the major causes of the age-associated decline in the immune response among several vertebrate species, including humans and mice. The process of thymic involution begins at 3 months in mice as the thymus begins to atrophy (2). Adipose tissue infiltrates the thymic parenchymal tissue, causing the shape of the thymus to become distorted (3). With increasing age, the thymus atrophies, resulting in a decrease in the total number of thymocytes (4). Thymic architecture deteriorates, transitioning from a distinct cortico-medullary junction and abundant thymic epithelial cells (TECs) in young mice to undefined compartmentalization in aged mice (5, 6). Thymic epithelial cell volume
decreases as well, which affects thymopoiesis, as TECs are required to interact with and support thymocytes during T cell development (7). Early T cell progenitors (ETP), lineage marker negative, c-kit⁺ CD44hi CD25⁻ CD127⁻ thymocytes, are required to seed the thymus for thymopoiesis, which is highly enriched for T cell progenitors. ETP from aged mice are reduced in both percent and number, proliferate less, and demonstrate a high rate of apoptosis (8). These changes also lead to a continuous decline in both thymocyte number and naïve T cell output, resulting in an age-related contraction of the naïve T cell pool (9). As a result of these age-related changes within the thymus, the number of peripheral naïve T cells declines with age, causing a decreased T cell immune response in the elderly, particularly in response to new antigens (5, 6). Consequently, it is thought that there is a strong association between thymic involution and immunosenescence in the elderly.

**The T cell immune response**

The thymus exports single positive CD4⁺ and CD8⁺ naïve T cells to peripheral secondary lymphoid organs, such as lymph nodes and spleen, and mucosa-associated lymphoid tissue (MALT) (4). Naïve T cells are small lymphocytes in the G₀ phase of the cell cycle that have not encountered antigen and express CD44lo CD45Rabh CD62Lhi CD25⁻ (10). An antigen presenting cell (APC) presents a foreign peptide in the context of MHC I or II to a naïve T cell. The TCR binds to the peptide and induces a naïve T cell to develop into an effector T cell. A naïve T cell requires 2 activation signals for full activation to occur. The first activation signal occurs when the TCR binds to a peptide presented by an APC
in the context of MHC I or II. CD8 or CD4 then binds to and recognizes the MHC complex. This induces T cell activation to occur. The second T cell activation signal is a co-stimulatory signal that occurs when the T cell molecule CD28 binds to B7.1/CD80 or B7.2/CD86 on an APC (11, 12). Engagement of the TCR initiates the T cell immune response, which consists of three phases: 1. Expansion, 2. Contraction, and 3. Memory (Figure 1) (10). During the expansion phase, antigen-specific T cells proliferate in response to antigen and effector T cells are produced. Upon differentiating to effector T cells, activated CD4+ and CD8+ T cells produce IL-2, with the former being the main contributor (13). The IL-2 receptor (IL-2R) is composed of 3 subunits: CD122 (IL-2β), CD132 (the common γ-chain), and CD25 (IL-2Rα) (10, 14). While CD122 and CD132 are constitutively expressed on resting T cells as a low affinity IL-2R, all 3 receptor subunits are required to form the high affinity IL-2R that is expressed on activated T cells (14). IL-2 production acts in an autocrine manner, triggering upregulation of CD25 and clonal expansion, after which CD25 and IL-2 bind. Binding of IL-2 to CD25 induces CD122 and CD132 to associate with CD25 and IL-2 to form a stable quaternary structure, which leads to cytoplasmic signaling through the tails of CD122 and CD132. This induces activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, both of which are important for cell growth and survival (14). Binding of IL-2 to IL-2R induces a T cell to enter the cell cycle and proliferate, producing effector T cells, which are large lymphocytes in the G1 phase that express CD62Llo CD25hi (11, 12).
The last phases in the T cell immune response are contraction and memory, during which the pathogen is cleared. The number of antigen-specific T cells decreases due to apoptosis, with the number of antigen-specific T cells stabilizing as CD4$^+$ and CD8$^+$ memory T cells are produced (13). A lower number of antigen-specific memory T cells remain in circulation for a faster secondary immune response. Memory T cells are long lived cells that display CD44$^{hi}$CD62L$^{-}$CD45RB$^{lo}$CD127$^+$ (6, 12, 15, 16). Memory T cells can be further divided into central (T$_{CM}$) and effector (T$_{EM}$) memory T cell subsets. T$_{CM}$ and T$_{EM}$ differ in terms of location, phenotype, function, and cytokine production. T$_{CM}$ recirculate throughout the spleen, lymph nodes, and blood; whereas, T$_{EM}$ are located in non-lymphoid tissues, such as liver, gut, and lungs, as well as in the spleen, and blood. T$_{CM}$ express CD62L$^{+}$CD127$^{+}$CCR7$^{+}$ and upon antigen stimulation and proliferation rapidly differentiate into effector memory T cells (13, 16). In comparison, T$_{EM}$ are phenotypically as CD62L$^{-}$CD127$^{+}$CCR7$^{-}$ and produce IFN-$\gamma$, IL4, and IL-5 (17).
The T cell immune response

The T cell immune response consists of 3 phases: 1. Expansion, 2. Contraction, and 3. Memory. The expansion phase begins when an APC activates a naïve T cell with foreign antigen. Antigen-specific T cells proliferate in response to antigen, producing effector T cells. T cell contraction occurs once the pathogen is cleared. Apoptosis and cell death occurs, eliminating effector T cells. The memory phase occurs allowing for a population of antigen-specific memory T cells to remain in circulation for a more robust secondary T cell immune response.
Age-associated changes within the peripheral T cell compartment

With age, several changes occur among peripheral T cells that affect both T cell function and composition. The function of CD4⁺ T cells decreases, including their cognate helper activity, which negatively affects both T and B cell responses during infection (18). With increasing age, several changes also occur in the levels of cytokines secreted by peripheral T cells, which affect their ability to function normally. One of the most prominent age-related changes is a decrease in IL-2 production by activated T cells (19, 20). With age, activated CD4⁺ T cells produce less IL-2, IL-3, and increased levels of IL-4, IL-5, and IL-10. Activated CD8⁺ T cells produce elevated levels of IFN-γ and less IL-2 (19, 21, 22). In addition to the dramatic decrease in IL-2 production with age, activated CD4⁺ T cells in aged mice demonstrate lower levels of CD25 expression and T cell proliferation in response to in vitro and in vivo stimulation via antigen (18, 23). Research has also shown that there is decreased nuclear factor κB (NF-κB) DNA binding in nuclear extracts from stimulated aged CD4⁺ effectors compared to stimulated young CD4⁺ effectors (24). Age-related changes also occur among CD8⁺ T cells, demonstrating decreased responsiveness to newly encountered antigens and decreased diversity in CD8⁺ T cell TCR repertoire (25, 26). As a result, vaccinations administered against influenza and other opportunistic pathogens exhibit decreased efficacy in elderly (27).

With increasing age, alterations also occur within the composition of peripheral T cell subsets. In a young host, the thymus exports a large number of naïve T cells to the periphery, resulting in the majority of the peripheral T cell pool being naïve T cells and a small fraction being memory T cells (Figure 2). However, with age, thymic involution
occurs and subsequently, thymic export of naïve T cells to the periphery decreases (4, 5). This results in a reduction of the number and proportion of peripheral naïve T cells, beginning between 1 and 3 months of age in mice (5). Consequently, as age increases memory T cells expand in both number and percent, in order to maintain peripheral T cell homeostasis (Figure 2) (6, 15, 28).

This “space-driven” proliferation of T cells is referred to as homeostatic expansion. Homeostatic expansion of memory T cells occurs in response to lymphopenic conditions, such as in the elderly with a decreasing naïve T cell pool and in cancer patients after chemotherapy or radiation therapy. Requirements for homeostatic expansion include IL-7, TCR recognition of self peptide/MHC ligands, and space or low numbers within the peripheral T cell compartment (29). In contrast, conventional activation of naïve T cells with foreign antigen requires IL-2, TCR engagement with foreign peptide/MHC complex, and costimulation with B7.1/CD80 or B7.2/CD86 molecules. The expansion in the memory T cell pool may also be due to the increased exposure to antigens, environmental pathogens, and immunizations that occur with age (18). Furthermore, clonal expansion also contributes to the increase in memory T cells, particularly in CD8+ T cells, resulting in a decrease in the peripheral TCR repertoire. Clonal expansion in mice and humans may occur in response to irregulated growth of antigenic stimulation, resulting in a decrease in the peripheral TCR repertoire. (30). It has also been shown that $T_{CM}$ and $T_{EM}$ increase with age in humans (31). As a result, memory T cells dominate in aged individuals, in both the splenic and peripheral blood mononuclear cell populations (6).
This shift from a naïve to memory T cell predominance is detrimental as it affects the ability of the host to respond to foreign antigens. The contraction of the peripheral naïve T cell pool in results in homeostatic proliferation of naïve T cells, in an attempt to maintain naïve T cell homeostasis (32). Homeostatic proliferation is cell proliferation that occurs in the absence of antigen stimulation, such as TCR engagement with MHC molecules. Naïve T cell homeostasis is the ability of an organism to maintain a constant number of naïve T cells. Homeostatic proliferation and the subsequent increased lifespan of CD4+ naïve T cells, contribute to DNA damage and decreased T cell function (33). Furthermore, memory T cells generated from aged naïve T cells are often hyporesponsive due to the accumulation of age-related deficiencies, resulting in a reduction of the secondary immune response generated upon re-exposure to foreign antigens (34, 35). Together, the decreased number of naïve T cells and hyporesponsiveness of memory T cells severely compromises the ability of aged populations to respond to new invading antigens.

Thymic atrophy and the decrease in total thymocyte number have been shown to be linked to the age-associated decrease thymic output of naïve T cells, causing a contraction of the naïve T cell pool in both mice and humans (5, 7, 36). The age-related decrease in the naïve T cell pool has several consequences, including a subsequent expansion of the memory T cell pool. Also, with increasing age, IL-2 production by peripheral T cells declines, which is also related to the decrease in naïve T cells (37). Reductions in IL-2 production have been associated with reductions in CD25 expression, T cell activation, and T cell proliferation by activated T cells (18). Therefore, it is
necessary to investigate and further understand the relationship between thymic involution and age-related changes in peripheral T cells.
Decline in Thymic Production of Thymocytes and Naïve T Cells Affects the Composition of Peripheral T Cell Pool

In young mice, thymocyte number is high and the thymus produces a high number of naïve T cells. Consequently, the peripheral T cell pool is mainly comprised of naïve T cells, with a small fraction of memory T cells. Thymic involution results in thymic atrophy and a decrease in the number of thymocytes. As a result, thymic output of naïve T cells decreases, causing a reduction in the naïve T cell pool and a subsequent expansion in the memory T cell pool.

Figure 2. Decline in Thymic Production of Thymocytes and Naïve T Cells Affects the Composition of Peripheral T Cell Pool. In young mice, thymocyte number is high and the thymus produces a high number of naïve T cells. Consequently, the peripheral T cell pool is mainly comprised of naïve T cells, with a small fraction of memory T cells. Thymic involution results in thymic atrophy and a decrease in the number of thymocytes. As a result, thymic output of naïve T cells decreases, causing a reduction in the naïve T cell pool and a subsequent expansion in the memory T cell pool.
**Effect of Foxn1 Over Expression on the Age-Related Changes in Peripheral T Cell Pool**

Figure 3. Effect of Foxn1 Over Expression on the Age-Related Changes in Peripheral T Cell Pool. The purpose of aim 1 was to determine the effect of Foxn1 over expression and attenuation of the age-related decrease of the number of thymocytes on the age-associated changes within the peripheral T cell compartments, particularly within the memory and naïve T cell pools.
Foxn1 and thymic involution

In order to investigate the relationship between thymic involution and aging in the peripheral T cell compartment more thoroughly, we used a mouse model that demonstrated attenuation of thymic involution, with the anticipation that the age-associated decrease in the number of naïve T cells would be prevented. To achieve attenuation of thymic involution, we used C57BL/6 transgenic mouse that over expressed the transcription factor Forkhead box N1 (Foxn1), under the control of the human keratin 14 promoter. This promoter was chosen because keratin 14 is expressed in epithelial cells (P. Le et. al., Manuscript in preparation).

The transcription factor Foxn1, also referred to as winged-helix nude (Whn), is required for both fetal and postnatal regulation of the thymus (38, 39). Postnatally Foxn1 is expressed in TECs (39). While Foxn1 is required for TEC differentiation and proliferation, expression is not required to initiate thymic organogenesis (39, 40). During postnatal development, TECs also express Foxn1 (39). A single base pair deletion in Foxn1 results in a nonsense mutation and the loss of its DNA binding domain. Mice and humans with this mutation have a nude phenotype, lacking TEC differentiation and proliferation, T cells, hair, and are severely immune compromised (40, 41). While Foxn1 is expressed within the thymus and skin, its expression and role in other organs is not well characterized (40, 41). TECs expression of Foxn1 continuously decreases with age, and was shown to be significantly reduced at 3, 12, and 18 months of age (5). Foxn1 downregulation also lead to rapid deterioration of thymic compartments and decreases in
thymocyte number, thymopoiesis, and proliferation of medullary TECs, with the requirement for Foxn1 expression being dose dependent (42).

Over expression of Foxn1 results in attenuation of thymic involution, as demonstrated by the increased number of thymocytes and ETP, compared to age-matched wild type (Wt) mice (P. Le et. al., Manuscript in preparation). Over expression of Foxn1 also ameliorates atrophy of thymic tissue, as seen by the deposition of adipose tissue in old Wt thymus, giving the thymus a more translucent appearance (P. Le et. al., Manuscript in preparation). Also, as shown in H & E staining, in the old Wt thymus, there was a lack of distinction and organization between the cortical and medullary compartments. In comparison, in the old Foxn1 transgenic (Tg) thymus, the organization and distinction between the cortical and medullary compartments was unaltered (P. Le et. al., Manuscript in preparation). For this study, we conducted a series of experiments that focused on testing the functions and composition of peripheral T cells from Foxn1 Tg mice. The focus of our investigation was to characterize and compare the functions and composition of peripheral T cells from young and old Foxn1 Tg and C57BL/6 Wt mice.
CHAPTER 2

SPECIFIC AIMS

It is established that with the age-associated decline of the immune system, peripheral T cell functions are more affected than any other cell types (37). Decreases in T cell functions result in diminished responses within both cell-mediated and humoral immunity. The composition of the peripheral T cell pool also changes with age, with a dramatic contraction of the naïve T cell pool and expansion in the memory T cell pool (12, 15). These changes promote a decrease in T cell functions, humoral response, vaccine efficacy, and the ability of the host to respond to a broad spectrum of infections, resulting in aged individuals mounting weaker immune responses to new foreign antigens and being more vulnerable to various cancers, autoimmune diseases, and infections (27, 33). Projections for 2030 estimate that worldwide population of people 65 and older will have grown to 1 billion – 1 out every 8 people (43). This makes immune insufficiency in the elderly a key process necessary to understand in order to care for the world’s fastest growing population.

One of the main contributing factors to the age-associated decline in the immune response is thymic involution. Thymic atrophy occurs, with thymic tissue structurally deteriorating and adipocyte content increasing (3). Thymic output of naïve T cells decreases, allowing for a reciprocal expansion in memory T cells. Numerous
investigators have also shown that T cell function is also diminished in both CD4$^+$ and CD8$^+$ T cell subsets (30). This causes reduced B cell and cytotoxic T cell activation and decreased clearance of viruses. These changes in peripheral T cell subsets result in the elderly having a reduced T cell response to new invading pathogens.

The overall aim of this study was to determine if the decrease in thymic output of naïve T cells was prevented, would it prevent the age-associated decrease in peripheral T cell functions and the naïve T cell pool. This goal lead to the following hypothesis: Attenuation of thymic involution ameliorates the age associated decrease in the numbers and proportions of peripheral CD4$^+$ and CD8$^+$ naïve T cells and T cell functions. The results from this study provide further insight on the role of thymic involution and the T cell immune response with increasing age. To test this hypothesis, we proposed the following specific aims:

**Aim I:** To determine whether attenuation of thymic involution prevents age-related changes within the peripheral naïve and memory T cell compartments. We predicted that if the age-related decrease in thymic output of naïve T cells was attenuated, then the decline in the number of peripheral naïve T cells would be lessened and consequently the expansion of memory T cells would also be lessened.

*Sub-aim 1.1:* To determine if peripheral T cells from aged Foxn1 Tg mice demonstrate minor age-associated changes within peripheral CD4$^+$, CD8$^+$, and naïve T cell pools.
Sub-aim 1.2: To determine if the age-associated expansions of memory and effector and central memory T cells are prevented in aged Foxn1 Tg mice.

Aim II: To determine if attenuation of thymic involution prevents the age-associated decrease in T cell functions. We predicted that if the age-related decrease in naïve T cells was lessened in Foxn1 Tg mice, then aged Foxn1 Tg mice would demonstrate improved T cell functions as compared to aged Wt mice.

Sub-aim 2.1: To determine if the age-related decrease in IL-2 production by activated peripheral T cells from aged Foxn1 Tg mice is prevented.

Sub-aim 2.2: To determine if the decrease in T cell proliferation by activated T cells from aged Foxn1 Tg mice is prevented.

Sub-aim 2.3: To determine if peripheral T cells from aged Foxn1 Tg mice demonstrate an age-associated decrease in the expression of CD3.
CHAPTER 3
RATIONALE AND EXPERIMENTAL DESIGN

Specific Aim I: To determine whether attenuation of thymic involution prevents age-related changes within the peripheral naïve and memory T cell compartments.

Rationale: Age associated changes occur within peripheral T cell subsets, specifically the concurrent decrease in naïve T cell pool and increase in memory T cell pool, in both the CD4$^+$ and CD8$^+$ T cell subsets (6, 44). These shifts are due to a decrease in thymic output of naïve T cells and to peripheral T cell increasing exposure to environmental pathogens and vaccinations with age (3, 12). CD4$^+$ memory T cells produced from aged naïve CD4$^+$ T cells exhibit hyporesponsive activity upon secondary activation, indicating that memory T cells responses produced late in life function poorly (11, 35). Research also shows that prolonged exposure to latent viruses results in expansion of antigen-specific CD8$^+$ memory T cells and a gradual restriction in binding at the variable region of the TCR-β chain (45). As a result, as age increases, the peripheral T cell pool becomes composed of increasing number of hyporesponsive memory cells and a reduced naïve T cell population. While research shows that the overall size of the peripheral T cell pool remains unchanged, the percent and numbers of CD4$^+$ and CD8$^+$ T cells may change with age. In addition, a decrease in the expression of CD3 on peripheral CD3$^+$ T cells may also be linked to reductions in T cell functions. As a result, these alterations may
compromise the ability of aged animals and patients to respond to new environmental antigens.

**Experimental Design:** This aim has multiple segments. First, we used flow cytometry to determine if overexpression of Foxn1 affects the number and proportion of CD3^+CD4^+ and CD3^+CD8^+ T cells. We next determined if attenuation of thymic involution prevents the age-related contraction of the naïve T cell pool. We also used flow cytometry to determine if the decrease of naïve T cells was prevented, would this ameliorate the age-associated increase in memory T cells. We predicted that if an age-related decrease in the naïve T cell compartment was prevented, then expansion of the memory T cell pool will be lessened as well.

1.1 **To determine if peripheral T cells from aged Foxn1 transgenic mice demonstrate age-associated changes within peripheral CD3^+CD4^+, CD3^+CD8^+, and naïve T cell pools.**

Flow cytometry was used to determine if attenuation of thymic involution prevents the age-associated decrease in the percents and total cell numbers of CD3^+CD4^+ and CD3^+CD8^+ T cells from aged Foxn1 compared to T cells in young and old Wt and young Foxn1 Tg mice.

Secondly, we used flow cytometry to compare the percents and total cell numbers of naïve T cells from young and old Foxn1 Tg and Wt mice to determine if attenuation of thymic involution prevents the age-associated decrease in CD4^+ and CD8^+ naïve T cells. CD4^+ and CD8^+ naïve T cells were defined as CD44^lo within CD3^+CD4^+ and CD3^+CD8^+ T cells (19, 20). For analysis of total CD3^+CD4^+ and CD3^+CD8^+ T cells and naïve T
cells, young Wt and young Foxn1 Tg mice were 2-3 months of age. Aged Wt and aged Foxn1 Tg mice were divided into 2 groups: 20-26 months and 28-35 months of age. For both Wt and Foxn1 Tg mice, only male mice were used for these experiments. For samples with normal distribution, Student’s t-test was used and for samples with non-normal distribution, the Mann-Whitney U test was used. The F-test was used to determine if samples had a normal distribution. Significance for all statistical tests was determined at p≤0.05.

1.2 To determine if the age-associated expansion of memory and effector and central memory T cells is prevented in aged Foxn1 Tg mice.

We also used flow cytometry to determine if the age-related decline in naïve T cells was prevented, would this prevent the age-related increase in memory T cells. To determine this, the percents and total cell numbers of CD4$^+$ and CD8$^+$ memory T cells from young and aged Wt and Foxn1 Tg mice were compared. CD4$^+$ and CD8$^+$ memory T cells were defined as CD44$^{hi}$ within CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells (6, 15). Young Wt and young Foxn1 Tg mice were 2-3 months of age. Aged Wt and aged Foxn1 Tg mice were each divided into 2 separate groups: 20-26 months and 28-35 months of age. All Wt and Foxn1 Tg mice used for these experiments were males.

To determine if an age-related increase in $T_{CM}$ and $T_{EM}$ was prevented, we compared percents and total cell numbers of $T_{CM}$ and $T_{EM}$ populations from young and aged Foxn1 Tg and Wt mice. Young Wt and young Foxn1 Tg mice were 2-3 months of age. Aged Wt and aged Foxn1 Tg mice were 20-26 months of age. All Wt and Foxn1 Tg mice were males. All flow cytometry data was analyzed using fluorescent activated cell
sorting (FACS) on a FACS Canto flow cytometer and FlowJo software version 7.5 (Tree Star, Inc, Ashland, OR). Student’s $t$-test was used for samples with normal distribution to determine significance, with significance at $p \leq 0.05$. For samples with non-normal distribution, the Mann-Whitney U test was used with significance at $p \leq 0.05$. The overall goal of this aim is to determine if slowing the process of thymic involution will prevent age-related changes within peripheral T cell subsets.

**Specific Aim II: To determine if attenuation of thymic involution prevents the age-associated decrease in T cell functions.**

**Rationale:** Age-related decreases in T cell activation, IL-2 production, and T cell proliferation negatively impact cell mediated and humoral immune responses. Addition of exogenous IL-2 to naïve T cell cultures from aged mice rescues the function of aged naive T cells, enabling them to proliferate and produce IL-2 at levels equivalent to levels produced by naive T cells from young mice (46). IL-2 therapy has also been used *in vivo* studies, which showed that intraperitoneal injection of allogeneic tumor cells with exogenous IL-2 in aged mice restored T cell proliferation, cytotoxic T cell function, and IgM and IgG antibody production to levels equivalent to those seen young mice (47). These results suggest that T cells from aged mice have the ability to functionally respond to exogenous IL-2 at levels comparable to those seen in young Wt mice. Therefore, we want to investigate if attenuation of thymic involution in Foxn1 Tg mice prevents the age-associated decrease in peripheral T cell functions.

**Experimental Design:** This aim has three parts. First, we compared levels of IL-2 production by activated T cells from young and aged Wt and Foxn1 Tg mice. We also
compared levels of T cell proliferation by activated T cells from young and old Wt and Foxn1 Tg mice. Lastly, we analyzed peripheral T cells from young and aged Wt and Foxn1 Tg mice to determine if the expression of CD3 in peripheral T cells changed with age. We predicted that if the age-associated decrease in the naïve T cell pool was prevented in Foxn1 Tg mice, then activated T cells from old Foxn1 Tg mice will demonstrate improved T cell functions compared to T cells from old Wt mice.

2.1 To determine if the age-related decrease in IL-2 production by activated peripheral T cells from aged Foxn1 transgenic mice is prevented.

We compared IL-2 production by activated peripheral T cells from young and aged Foxn1 Tg and Wt mice. Peripheral T cells were activated in splenocyte cultures using equivalent concentrations of plate bound anti-CD3 and anti-CD28 monoclonal antibodies. IL-2 concentrations were measured using ELISA assays. Young Wt and young Foxn1 Tg mice were 2-3 months of age. Old Wt and old Foxn1 Tg mice were each divided into 2 groups: 20-26 months and 28-35 months of age. All Wt and Foxn1 Tg mice were males. For samples with normal distribution, Student’s t-test was used, with significance at p≤0.05. For samples with non-normal distribution, the Mann-Whitney U test was used, with significance at p≤0.05. The F-test was used to determine if samples had a normal distribution.

2.2 To determine if activated peripheral T cells from aged Foxn1 transgenic mice demonstrate an age-related decrease in T cell proliferation.

We also used flow cytometry to compare T cell proliferation by stimulated peripheral T cells from young and aged Foxn1 Tg and Wt mice. To induce T cell
proliferation, splenic T cells were activated using soluble Concanavalin A (ConA) in splenocyte cultures. T cell proliferation was measured using flow cytometry and CFSE, an intracellular dye that divides equally between two daughter cells with each cell division (48, 49). Young Wt mice and young Foxn1 Tg mice were 2-3 months of age. Aged Wt and aged Foxn1 Tg mice were 20-26 months of age. For samples with normal distribution, Student’s t-test was used, with significance at p≤0.05. For samples with non-normal distribution, the Mann-Whitney U test was used, with significance at p≤0.05.

2.3 To determine if peripheral T cells from aged Foxn1 transgenic mice demonstrate an age-associated decrease in the expression of CD3.

Lastly, the expression of CD3 on peripheral CD3+ T cells from young and old Wt and Foxn1 Tg mice was analyzed, with the anticipation that the age-associated decrease in T cell functions may be related to a reduction in the cell surface expression of CD3 on peripheral CD3+ T cells. An age-associated decrease in the expression of CD3 in peripheral T cells has been shown within the elderly, but it is undetermined if it also occurs in mice (50). Young Wt and young Foxn1 Tg mice were 2-3 months of age. Old Wt and old Foxn1 Tg mice were divided into 2 groups: 20-26 months and 28-35 months of age. For samples with normal distribution, Student’s t-test was used, with significance at p≤0.05. For samples with non-normal distribution, the Mann-Whitney U test was used, with significance at p≤0.05. The F-test was used to determine if samples had a normal distribution. The overall goal of this aim was to determine if attenuating thymic involution would prevent the age-associated decline in T cell functions.
CHAPTER 4
RESULTS

Aim I

*Attenuation of thymic involution ameliorates the age-associated decrease in the naïve T cell pool and but does not prevent expansion of the memory T cell pool.*

*Comparison of Total CD4⁺ and CD8⁺ T Cells in Wt and Foxn1 Tg mice*

With increasing age changes occur in the distribution of peripheral T cell subsets in both humans and mice that affect the ability of the host to respond to new antigens (27, 33). It has been shown that the ratio of peripheral CD4⁺ to CD8⁺ T cells decreases with age due to the expansion of CD8⁺ memory T cells (28). This lead us to test if over expression of Foxn1 would affect the distribution of total CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in Foxn1 Tg mice with age. As shown in Figure 4A, CD3⁺ T cells were selected for, which were further analyzed for either CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells. In Wt mice, no change was found in the percent of CD3⁺CD4⁺ T cells between 2-3 months and 20-26 months of age (Figure 4B). However, the percent of CD3⁺CD4⁺ T cells declined in Wt mice between 20-26 months to 28-35 months of age (p=0.007, Figure 4B). In Foxn1 Tg mice, the percent of CD3⁺CD4⁺ T cells decreased between 2-3 months and 28-35 months (p=0.02, Figure 4B). No difference was found in the percent of CD3⁺CD4⁺ T cells in young Wt and young Foxn1 Tg mice (Figure 4B). The proportions of CD3⁺CD4⁺
T cells in old Wt and old Foxn1 Tg mice 20-26 months and 28-35 months were similar (Figure 4B). While the change was not significant, the total cell number of CD3^+CD4^+ T cells in Wt mice decreased by 13% between 2-3 months and 20-26 months (Figure 4B). The total cell number of CD3^+CD4^+ T cells in Wt mice decreased by 13% between 2-3 months and 20-26 months (Figure 4B). In Foxn1 Tg mice, there was no change in the total cell number of CD3^+CD4^+ T cells between 2-3 months and 20-26 months or 20-26 months and 28-35 months (Figure 4B). However, in Foxn1 Tg mice there was an overall 46% decline in the number of CD3^+CD4^+ T cells between 2-3 months and 28-35 months (p=0.007, Figure 4B). No change was found in the number of CD3^+CD4^+ T cells in young Wt and young Foxn1 Tg mice (Figure 4B). Also, no differences were found in the number of CD3^+CD4^+ T cells from between old Wt and old Foxn1 Tg mice 20-26 months or 28-35 months (Figure 4B).

As with CD3^+CD4^+ T cells, a similar age-related decline was found in total CD3^+CD8^+ T cells in Wt mice. No changes were found in the percent of CD3^+CD8^+ T cells in Wt mice with increasing age (Figure 4C). While no difference was found in the proportion of CD3^+CD8^+ T cells in Foxn1 Tg mice from 2-3 months and 20-26 months, there was an overall increase in proportion between 2-3 and 28-35 months (p=0.008, Figure 4C). Also, young Wt mice had a higher percent of CD3^+CD8^+ T cells than young Foxn1 Tg mice (p=0.005, Figure 4C). However, despite the difference in the proportions of CD3^+CD8^+ T cells in young mice, no differences were found between old Wt and old Foxn1 Tg mice 20-26 months or 28-35 months (Figure 4C). Significant changes were
also found in the total cell numbers of CD3⁺CD8⁺ T cells in Wt mice with age. The total cell number of CD3⁺CD8⁺ T cells in young Wt 2-3 months decreased 24% from an average of 7.9 ± 2.0 x10⁶ cells to 6.0 ± 1.5 x10⁶ cells in aged Wt 20-26 months (p=0.02, Figure 4C). While the reduction was not significant, in Wt mice the total cell number of CD3⁺CD8⁺ T cells declined another 42% between 20-26 months and 28-35 months (Figure 4C). In comparison, in Foxn1 Tg mice no difference was found in the total cell number of CD3⁺CD8⁺ T cells with age (Figure 4C). The number of CD3⁺CD8⁺ T cells in young Wt was greater than in young Foxn1 Tg (p=0.02, Figure 4C). There was no change in number of CD3⁺CD8⁺ T cells in old Wt and old Foxn1 Tg mice 20-26 months or 28-35 months (Figure 4C). While the data was not included here, no changes were found in the proportion or number of total peripheral CD3⁺ T cells in young and old Wt and Foxn1 Tg mice. In summary these results show that the number of CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells decreased with age in Wt mice. However, in Foxn1 Tg mice only the number of CD3⁺CD4⁺ T cells decreased with age, whereas the number of CD3⁺CD8⁺ T cells remained unchanged, suggesting that over expression of Foxn1 may favor survival of peripheral CD3⁺CD8⁺ T cells over CD3⁺CD4⁺ T cells.
A. Identification of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells

![Diagram showing CD3 and CD4](image1)

B. CD3⁺CD4⁺ T Cells

![Graph showing total cell number and percentage of CD3⁺CD4⁺ T cells](image2)

C. CD3⁺CD8⁺ T Cells

![Graph showing total cell number and percentage of CD3⁺CD8⁺ T cells](image3)

Figure 4. Total number of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells peripheral T cells in young and old Wt and Foxn1 Tg mice. A) Identification of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells. Lymphocytes from the spleen of Wt and Foxn1 mice were isolated and stained for CD4⁺ and CD8⁺ T cells within the CD3⁺ T cell population. CD3⁺ T cells were selected for within the lymphocyte gate. CD4⁺ and CD8⁺ T cells were then selected for. Data shown are from a representative experiment of 12 experiments. B and C) CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells. Data are presented as the total cell number and percent of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells. Numbers in parentheses indicate the number of animals in each group of mice. Data are presented as means ± standard deviation (SD).
Comparison of CD4⁺ and CD8⁺ Naïve T Cells in Wt and Foxn1 Tg Mice

We next determined whether attenuation of the decline of the total number of thymocytes would prevent the age-associated decrease in peripheral naïve T cells. It has been established that thymic output of naïve T cells severely decreases with age in both humans and mice (5, 15, 20, 51). Flow cytometry was used to determine the total cell numbers and percents of CD4⁺ and CD8⁺ naïve T cells from young and aged Wt and Foxn1 Tg mice. The CD4⁺ and CD8⁺ naïve T cells were defined as the CD44lo subset within CD3⁺CD4⁺ and CD3⁺CD8⁺ T cell populations (Figure 5A). The percent of CD4⁺ naïve T cells in Wt mice declined from 47.2 ± 9% (2-3 months) to 31.5 ± 5% (20-26 months) (Figure 5B). The percent of CD4⁺ naïve T cells in Wt mice continued to decline to 14 ± 4% at 28-35 months of age (p=0.0001, Figure 5B). In comparison, there was no change in the percents of CD4⁺ naïve T cells in Foxn1 Tg mice with increasing age (Figure 5B). Also, as expected, the proportions of CD4⁺ naïve T cells in young Wt and young Foxn1 Tg mice were similar (Figure 5B). Percents of CD4⁺ naïve T cells in old Foxn1 Tg mice 20-26 months and 28-35 months age groups were significantly greater than in old Wt mice of similar age groups (p=0.007, 0.0007, Figure 5B).

The age-related decrease in CD4⁺ naïve T cells in Wt mice was confirmed by data shown in total cell numbers. The number of CD4⁺ naïve T cells in Wt mice decreased by 45% between 2-3 months and 20-26 months (p=0.01, Figure 5B). Between 20-26 months and 28-35 months, the number of CD4⁺ naïve T cells decreased another 81% from an average of 4.2 x 10⁶ cells at 20-26 months to 0.78 ± 0.70 x 10⁶ cells at 28-35 months (p=0.0002, Figure 5B). There was no change in the number of CD4⁺ naïve T cells from
2-3 months to 20-26 months in Foxn1 Tg mice (p=0.93, Figure 5B). However, there was still a 41% decrease in the total number of CD4$^+$ naïve T cells in Foxn1 Tg mice between 2-3 months to 28-35 months (p=0.03, Figure 5B). However, the number of CD4$^+$ naïve T cells in old Foxn1 Tg mice 28-35 months was greater than in old Wt mice 28-35 months (p=0.03, Figure 5B). The CD4$^+$ naïve T cell data shown in total cell number in correlate with the data shown in proportions, confirming that the age-related decrease in CD4$^+$ naïve T cells in Foxn1 Tg mice was lessened in Foxn1 Tg mice compared to old Wt mice.

Similar to CD4$^+$ naïve T cells, CD8$^+$ naïve T cells in Wt mice also decreased with age, in both percent and total cell number. Percents of CD8$^+$ naïve T cells in Wt mice declined from 23 ± 7% at 2-3 months to 14 ± 6% at 20-26 months (p=0.006, Figure 5C). In Wt mice there was no significant change in the percent of CD8$^+$ naïve T cells between 20-26 months and 28-35 months of age (Figure 5C). In Foxn1 Tg mice the percent of CD8$^+$ naïve T cells fluctuated with age, decreasing between 2-3 months and 20-26 months (p=0.002, Figure 5C). However, overall the percent of CD8$^+$ naïve T cells did not change between 2-3 months and 28-35 months in Foxn1 Tg mice (Figure 5C). Interestingly, the percent of CD8$^+$ naïve T cells in young Foxn1 Tg mice was 1.9 fold higher compared to the percent of CD8$^+$ naïve T cells in young Wt mice (p<0.0001, Figure 5C). No differences were found in the proportion of CD8$^+$ naïve T cells in old Wt and old Foxn1 mice 20-26 months or 28-35 months (Figure 5C). However, the percent CD8$^+$ naïve T cells in old Foxn1 Tg mice 28-35 months was similar to that in young Wt mice (p=0.712, Figure 5C).
The total cell number of CD8\(^+\) naïve T cells in Wt mice declined with age, decreasing approximately 55% from a mean of 1.9 ± 0.98 \(\times 10^6\) at 2-3 months to 0.86 ± 0.38 \(\times 10^6\) at 20-26 months (\(p=0.005\), Figure 5C). The total cell number of CD8\(^+\) naïve T cells in Wt mice decreased further, with a 73% reduction to 0.23 ± 0.17 \(\times 10^6\) at 28-35 months of age (\(p=0.04\), Figure 5C). In comparison, no changes were found in total cell number of CD8\(^+\) naïve T cells from Foxn1 Tg mice with age (Figure 5C). While there was no difference in the number of CD8\(^+\) naïve T cells from old Foxn1 Tg and old Wt mice 20-26 months, the total cell number of CD8\(^+\) naïve T cells from old Tg mice 28-35 months was significantly greater than cells from old Wt mice 28-35 months (\(p=0.048\), Figure 5C). The number of CD8\(^+\) naïve T cells in old Foxn1 Tg 28-35 months was similar to levels in young Wt mice (\(p=0.9512\), Figure 5C). In summary, these results support our hypothesis, indicating that attenuation of thymic involution not only prevented the age-associated decrease in CD8\(^+\) naïve T cells, but also maintained naïve T cells at a level equivalent to those in young Wt and young Foxn1 Tg mice.
A. Identification of CD4+ and CD8+ naïve T cells

red = young Wt
blue = old Wt

CD4+ T cells

CD8+ T cells
**Figure 5.** Comparison of CD4⁺ and CD8⁺ naïve T cells in young and aged Wt and Foxn1 Tg mice. 

A) Identification of CD4⁺ and CD8⁺ naïve T cells. Splenocytes were isolated from Wt and Foxn1 Tg mice. CD4⁺ and CD8⁺ naïve T cells were defined as CD44lo within CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells. Data shown are from a representative experiment of 12 experiments. 

B) CD4⁺ naïve T cells. Data are presented as the total cell number and percent of CD4⁺ naïve T cells. *p=0.03 old Wt 28-35 m vs. old Tg 28-35 m; #p=0.007 old Wt 20-26 m vs. old Tg 20-26 m; **p=0.0007 old Wt 28-35 m vs. old Tg 28-35 m. 

C) CD8⁺ naïve T cells. Data are presented as the total cell number and percent of CD8⁺ naïve T cells. *p=0.048 old Wt vs. 28-35 m vs. old Tg 28-35 m; Numbers in parentheses represent the number of animals per group. Error bars are values of ±SD.
Comparison of CD4+ and CD8+ memory T Cells in Wt and Foxn1 Tg Mice

We next wanted to determine if maintaining the naïve T cell pool would prevent the age-associated increase in CD4+ and CD8+ memory T cell compartments. It has been established the memory T cell pool expands in both percent and total cell number with increasing age in both mice and humans (15, 20, 51). As shown in Figure 6A, the CD4+ and CD8+ memory T cells were defined as CD44hi within CD3−CD4+ or CD3−CD8+ T cells.

The percent of CD4+ memory T cells from Wt mice increased between 2-3 months and 20-26 months (p<0.0001, Figure 6B). However, no change was found in percent of CD4+ memory T cells in old Wt 20-26 months and 28-35 months (Figure 6B). Results also show that in old Foxn1 Tg 20-26 and 28-35 months the percent of CD4+ memory T cells was greater than in young Tg mice (p<0.0001, p<0.0001, Figure 6B). Similar to Wt mice, the percent of CD4+ memory T cells was similar between old Foxn1 Tg mice 20-26 months and 28-35 months (Figure 6B). As expected the proportion of CD4+ memory T cells in young Wt and young Foxn1 Tg mice were also similar (Figure 6B). While there was no change in the percent of CD4+ memory T cells between old Wt and old Foxn1 Tg 20-26 months, the percent of CD4+ memory T cells in old Wt 28-35 months was greater than in old Foxn1 Tg mice 28-35 months (p=0.03, Figure 6B).

The data shown in the total cell number of CD4+ memory T cells reaffirms the trends shown in percents, that CD4+ memory T cells increased with age in both Wt and Foxn1 Tg mice. As shown in Figure 6B, the total cell number of CD4+ memory T cells increased in Wt mice increased as expected, from a mean of 2.6 ± 1.1 x 10^6 cells at 2-3
months to 6.2 ± 2.0 x 10^6 cells at 20-26 months (p<0.0001, Figure 6B). Surprisingly, in Wt mice the number of CD4^+ T cells decreased after 28 months (Figure 6B). While these results seemed unusual, given that the number of memory T cells increases with age, research in mice this old is not common. Therefore, the number of animals in the Wt and Foxn1 28-35 month age group needs to be increased. Results also show that the numbers of CD4^+ memory T cells in old Foxn1 Tg mice 20-26 and 28-35 months were greater than in young Tg mice (p=0.04, p=0.04, Figure 6B). The number of CD4^+ memory T cells in young Wt and young Foxn1 Tg mice were similar (Figure 6B). Also, no changes were found in CD4^+ memory T cell total cell number between old Wt and old Foxn1 Tg mice 20-26 months or 28-35 months (Figure 6B).

The percent of CD8^+ memory T cells in old Wt mice was significantly increased compared to young Wt mice (p<0.0001, Figure 6C). The percent of CD8^+ memory T cells in Wt mice did not change between 20-26 months and 28-35 months (Figure 6C). Similar to Wt mice, the proportion of CD8^+ memory T cells in Foxn1 Tg mice increased between 2-3 months and 20-26 months (p<0.0001, Figure 6C). Also, the proportions of CD8^+ memory T cells in Foxn1 Tg mice 20-26 months and 28-35 months were similar (Figure 6C). No differences were found in the percent of CD8^+ memory T cells in young Wt and young Foxn1 Tg mice (Figure 6C). The percent of CD8^+ memory T cells from old Foxn1 Tg mice 20-26 months was significantly higher than in old Wt mice 20-26 months (p=0.005, Figure 6C). However, no change was found in the percent of CD8^+ memory T cells between old Wt and old Foxn1 Tg mice 28-35 months (Figure 6C).
Similar results were found with the total cell number of CD8\(^+\) memory T cells in Wt mice. As expected, CD8\(^+\) memory T cells in Wt mice expanded in total cell number, from an average of 1.1 ± 0.42 x 10\(^6\) cells at 2-3 months to 2.7 ± 0.65 x 10\(^6\) cells at 20-26 months (p<0.0001, Figure 6C). Similar to the results in CD4\(^+\) memory T cells, the number of CD8\(^+\) memory T cells in Wt mice decreased after 28 months (Figure 6C). In contrast to Wt mice, in Foxn1 Tg mice the number of CD8\(^+\) memory T cells did not decline after 28 months (Figure 6C). The number of CD8\(^+\) memory T cells in old Foxn1 Tg mice 20-26 month and 28-35 month mice was increased compared to young Tg mice (p=0.001, 0.001, Figure 6C). The number of CD8\(^+\) memory T cells in young Wt and young Tg mice were similar (Figure 6C). No changes were found between old Wt and old Foxn1 Tg 20-26 months or 28-35 months in CD8\(^+\) T cell memory total cell number (Figure 6C). In summary, these results do not support our hypothesis, but rather indicate that lessening the decline in the CD4\(^+\) naïve T cell pool in Foxn1 Tg mice did not prevent an expansion of the CD4\(^+\) memory T cell compartment. These results also indicate that preventing the decrease in the number of CD8\(^+\) naïve T cells did not prevent an increase in the number of CD8\(^+\) memory T cells. This suggests that age-related changes within the peripheral environment, such as clonal expansion, heavily influence memory T cell expansion.
A. Identification of CD4+ and CD8+ memory T cells

CD4+ T cells

CD8+ T cells

red = young Wt
blue = old Wt
Figure 6. Comparison of CD4⁺ and CD8⁺ memory T cells in young and aged Wt and Foxn1 Tg mice. A) Identification of CD4⁺ and CD8⁺ memory T cells. Splenocytes were isolated from Wt and Foxn1 Tg mice. CD4⁺ and CD8⁺ memory T cells were defined as CD44⁺ within CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells. The dotted line indicates the division between CD44⁻ and CD44⁺ cells. Data shown are from a representative experiment out of 12 experiments. B and C) CD4⁺ and CD8⁺ memory T cells. Data are presented as the total cell number and percent of CD4⁺ and CD8⁺ memory T cells. Numbers in parentheses represent the number of animals in each group. Data are presented as the mean ± SD for each mouse group.
Comparison of Central and Effector Memory T Cells in Wt and Foxn1 Tg Mice

We sought to further investigate the effect of attenuation of thymic involution on memory T cell subpopulations in Foxn1 Tg mice, specifically central (T_{CM}) and effector (T_{EM}) memory T cells. Central memory T cells are defined as CD44^{hi}CD127^{+}CD62L^{+} (Figure 6A); these cells produce IL-2 and proliferate in response to IL-2 stimulation. In contrast, T_{EM} express CD44^{hi}CD127^{+}CD62L^{-} (Figure 6A) and produce IFN-γ, IL-4, and IL-5; these cells exhibit immediate effector functions (16, 17, 52). Both T_{CM} and T_{EM} are located in the spleen.

In both young and old Wt and Foxn1 Tg mice, low levels of CD4^{+} central memory T cells were found. Surprisingly, no difference was found in the percent of CD4^{+} central memory T cells in young and old Wt mice (Figure 7A). Similar to Wt mice, no change was found in the percents of CD4^{+} central memory T cells in young and old Foxn1 Tg mice (Figure 7A). Also, the percent of CD4^{+} central memory T cells in young Wt mice was greater than in young Foxn1 Tg mice (p=0.0006, Figure 7A). No difference was found between in the percent of CD4^{+} central memory T cells in old Wt and old Foxn1 Tg mice (Figure 7A). In both Wt and Foxn1 Tg mice the number of CD4^{+} central memory T cells did not change with age (Figure 7A). Similar to the results in percents, the number CD4^{+} central memory T cells in young Wt mice was greater than in young Foxn1 Tg mice (p=0.01, Figure 7A). However, the number of CD4^{+} central memory in the old Foxn1 Tg was greater than in old Wt mice (p=0.01, Figure 7A).

The results show that for CD4^{+} memory T cells in young and old Wt and Foxn1 Tg mice, effector memory T cells were the predominate population. The percent of CD4^{+}
effector memory T cells in old Wt mice was significantly higher cells than in young Wt mice \( (p=0.0002, \text{Figure 7B}) \). Similarly, the percent of CD4\(^+\) effector memory T cells in old Foxn1 Tg mice was higher than in young Tg mice \( (p<0.0001, \text{Figure 7B}) \). The proportion of CD4\(^+\) effector memory T cells in young Wt and young Tg mice was similar (Figure 7B). No difference was found in the percent of CD4\(^+\) effector memory T cells in old Wt and old Foxn1 Tg mice (Figure 7B). The numbers of CD4\(^+\) effector memory T cells indicate that there was an age-related increase in both Wt and Foxn1 Tg mice \( (p=0.0007, 0.004, \text{Figure 7B}) \). The numbers of CD4\(^+\) effector memory T cells in young Wt and young Tg mice were similar (Figure 7B). Also, no difference was found in the number of CD4\(^+\) effector memory T cells in old Wt and old Foxn1 Tg mice (Figure 7B). In summary, CD4\(^+\) effector memory T cells increased with age in both percent and number in both Wt and Foxn1 Tg mice, whereas CD4\(^+\) central memory T cells did not.

For the central memory T cells, CD8\(^+\) T cells were the main population. As expected, the percent of CD8\(^+\) central memory T cells in young Wt was less than in old Wt mice \( (p=0.003, \text{Figure 7C}) \). The percent of CD8\(^+\) central memory T cells from old Foxn1 Tg was significantly higher than in young Tg mice \( (p=0.0002, \text{Figure 7C}) \). The proportion of CD8\(^+\) central memory T cells in young Wt mice was greater than in young Foxn1 Tg mice (Figure 7C). No differences were found in the percent of CD8\(^+\) central memory T cells between old Wt and old Foxn1 Tg mice (Figure 7C). The number of CD8\(^+\) central memory T cells in Wt mice increased 2-fold from \( 0.65 \pm 0.17 \times 10^6 \) in young Wt to \( 1.3 \pm 0.62 \times 10^6 \) in old Wt mice \( (p=0.02, \text{Figure 7C}) \). In comparison, in Foxn1 Tg mice, the number of CD8\(^+\) central memory T cells expanded 8-fold from a
mean of $0.26 \pm 0.29 \times 10^6$ cells in young Tg mice to $2.1 \pm 1.5 \times 10^6$ cells in old Tg mice (p=0.003, Figure 7C). Similar to the results in percents, the number of CD8$^+$ central memory T cells in young Wt was greater than in young Foxn1 Tg mice (p=0.008, Figure 7C). The numbers of CD8$^+$ central memory T cells in old Wt and old Foxn1 Tg mice were similar (Figure 7C).

An age-related increase was also found in CD8$^+$ effector memory T cells, as indicated by the difference in percents in young and old Wt mice (p=0.0001, Figure 7D). Similarly, CD8$^+$ effector memory T cells in old Foxn1 Tg were significantly higher than in young Tg in percent (p<0.0001, Figure 7D). The percents of CD8$^+$ effector memory T cells in young Wt and young Foxn1 Tg mice were similar (Figure 7D). Also, no difference was found in the percents of CD8$^+$ effector memory T cells in old Wt and old Foxn1 Tg mice (Figure 7D). The number of CD8$^+$ effector memory T cells in old Wt was greater than in young Wt mice (p=0.0007, Figure 7D). Similarly, the number of CD8$^+$ effector memory T cells in old Foxn1 Tg was greater than in young Tg mice (p=0.004, Figure 7D). No change was found in the number of CD8$^+$ effector memory T cells between young Wt and young Foxn1 Tg mice (Figure 7D). Also, no change was found in the number of CD8$^+$ effector memory T cells in old Wt and old Foxn1 Tg mice (Figure 7D). In summary, the percent and number of both CD8$^+$ central and effector memory T cells increased with age in Wt and Foxn1 Tg mice. Furthermore, attenuation of thymic involution ameliorates the age-associated decrease in CD4$^+$ and CD8$^+$ naive T cells, but does not reduce expansion of the memory T cell compartment, as seen in CD4$^+$ and CD8$^+$ memory T cells and central and effector memory T cells.
A. **CD4^+ CD44^{hi} CD62L^{+} central memory T cells**

B. **CD4^+ CD44^{hi} CD62L^{-} effector memory T cells**
Figure 7. Comparison of CD4+ and CD8+ central and effector memory T cells in Wt and Foxn1 Tg mice. A) CD4+ central memory T cells. CD4+ central memory T cells were defined as CD44hiCD62L+CD127+ within CD3+CD4+ T cells as shown in Figure 6. B) CD4+ effector memory T cells. CD4+ effector memory T cells were defined as CD44hiCD62L−CD127+ within CD3+CD4+ T cells. Data are presented as the total cell number or percent of CD4+ central or effector memory T cells. C) CD8+ central memory T cells. CD8+ central memory T cells were defined as CD44hiCD62L+CD127+ within CD3+CD8+ T cells. D) CD8+ effector memory T cells. CD8+ effector memory T cells were defined as CD44hiCD62L−CD127+ within CD3+CD8+ T cells. Data are presented as the total cell number or percent of CD8+ central or effector memory T cells. Numbers in parentheses represent the number of animals in each group of mice. Data are presented as mean ± SD for each mouse group.
Over expression of Foxn1 maintains the naïve T cell pool, but does not prevent the age-related expansion of the memory T cell pool

Figure 8. Over expression of Foxn1 maintains the naïve T cell pool, but does not prevent the age-related expansion of the memory T cell pool. In young mice, the majority of peripheral T cell pool consists of naïve T cells, with a small fraction of memory T cells. With age, the number of naïve T cells decreases, with a subsequent increase in memory T cells. Over expression of Foxn1 results in attenuation of the number of thymocytes, resulting in maintenance of the naïve T cell pool, but does not prevent an increase in the memory T cell pool. The percents for each cell subset were calculated by dividing the total cell number of a cell subset by the total cell number of CD3⁺ CD44⁺ T cells. These percents were then used to make a pie chart.
**Aim II**

*Attenuation of thymic involution does not prevent a decrease in IL-2 production, T cell proliferation, or the expression of CD3. Young Foxn1 Tg mice demonstrate lower T cell functions and CD3 expression compared to young Wt mice.*

**Comparison of IL-2 Production by Activated T Cells from Wt and Foxn1 Tg Mice**

Our findings from aim 1 demonstrate that attenuation of thymic involution through Foxn1 over expression lessens the age-related decrease in the number of CD4$^+$ and CD8$^+$ naïve T cells, but does not prevent the expansion of the CD4$^+$ and CD8$^+$ memory T cell compartments. These results suggest that attenuation of thymic involution and number of thymocytes counteracts the age-related decline of naïve T cells. These results also suggest that age-associated changes within the peripheral environment heavily influence memory T cell expansion.

Given the results of aim 1, we further investigated if attenuation of thymic involution would prevent age-associated decrease in T cell functions. First, we compared IL-2 production by stimulating splenic T cells from aged Foxn1 Tg and aged Wt mice. As shown in Figure 9, there is a decline in IL-2 produced by activated T cells from Wt mice, decreasing by 84% from a mean of 8.5 ± 1.9 ng/ml (2-3 months) to 1.4 ± 0.84 ng/ml in (20-26 months) (p<0.0001, Figure 9). IL-2 produced by activated T cells from Wt mice decreased another 79% to 0.30 ± 0.30 ng/ml in 28-35 month mice (p=0.0424, Figure 9). In Foxn1 Tg mice, IL-2 production decreased by 70% from 2-3 months to 20-26 months (p=0.0007, Figure 9). However, in contrast to Wt mice, IL-2 production by stimulated T cells from old Foxn1 Tg mice remained unchanged between 20-26 months and 28-35
months of age (Figure 9). Surprisingly, activated T cells from young Foxn1 Tg mice produced less IL-2 than young Wt mice (p=0.0006, Figure 9). No difference was found in IL-2 production by old Wt and old Foxn1 Tg mice 20-26 months or 28-35 months (Figure 9). IL-2 was not detectable in any of the unstimulated samples that were cultured without anti-CD3 and anti-CD28 antibodies. These results indicate that attenuation of thymic involution did not prevent the age-associated decrease in IL-2 production. However, these results should be repeated with larger sample sizes for old Wt and old Foxn1 Tg mice 28-35 months. In summary, IL-2 production by activated T cells from both Wt and Foxn1 Tg mice declined with age. Also, IL-2 production by activated T cells from young Foxn1 Tg mice was lower compared to T cells from young Wt mice.
IL-2 production by activated T cells from young and aged Wt and Foxn1 Tg mice

Figure 9. Comparison of IL-2 production by activated T cells from young and aged Wt and Foxn1 Tg Mice. Splenocytes were isolated from Wt and Foxn1 Tg mice. T cell activation was induced using monoclonal, plate bound anti-CD3 and anti-CD28 antibodies at a final concentration of 5 µg/ml. 500,000 splenocytes were cultured per well for 24 hr at 37°C, 5% CO₂. IL-2 concentrations were measured using ELISA. Numbers in parentheses represent the number of animals per group. Data shown are the mean ± SD for each group.
Comparison of CD4\(^+\) and CD8\(^+\) T cell Proliferation by Activated T Cells from Wt and Foxn1 Tg Mice

As a result of the age-associated decrease in T cell production of IL-2, there is a corresponding decrease in IL-2 driven T cell proliferation. Therefore, we next sought to compare levels of CD4\(^+\) and CD8\(^+\) T cell proliferation by activated T cells from young and aged Wt and Foxn1 Tg mice to test if attenuating thymic involution would prevent the age-associated decrease in T cell proliferation. As expected, CD4\(^+\) T cells from young Wt proliferated significantly more than T cells from aged Wt mice (p=0.0007, Figure 10B). Similarly, CD4\(^+\) T cells from young Foxn1 Tg mice proliferated more than T cells from old Tg mice (p=0.04, Figure 10B). Similar to the IL-2 production results, stimulated CD4\(^+\) T cells from young Wt proliferated more than T cells from young Tg mice (p=0.0003, Figure 10B). Being that CD4\(^+\) T cells are the main source of IL-2 production, these results support one another (19). Also, CD4\(^+\) T cells from old Wt divided significantly more than T cells from old Foxn1 Tg (p=0.003, Figure 10B).

Surprisingly, CD8\(^+\) T cell proliferation did not decrease with age in Wt mice (Figure 10B). While no difference was found between CD8\(^+\) T cell proliferation in young and old Foxn1 Tg, there was a large standard deviation in the percent of divided CD8\(^+\) T cells from old Tg mice (Figure 10B). Also, CD8\(^+\) T cells from young Wt mice divided more than T cells from young Foxn1 Tg mice (p<0.0001, Figure 10B). Similar to the results of proliferation of CD4\(^+\) T cells, CD8\(^+\) T cells from old Wt mice divided more than in old Foxn1 Tg mice (p=0.03, Figure 10B). In summary, no age-related
difference was found in CD8\(^+\) T cell proliferation in Wt and Foxn1 Tg mice. These results also show that attenuation of thymic involution does not prevent the age-related decline in CD4\(^+\) T cell proliferation. These results also indicate that CD4\(^+\) and CD8\(^+\) T cells from young Wt mice proliferated more than T cells from young Foxn1 Tg mice.
A. **Identification of divided CD4$^+$ and CD8$^+$ T cells**

B. **Percent of divided CD4$^+$ T cells**

B. **Percent of divided CD8$^+$ T cells**

**Figure 10. Comparison of CD4$^+$ and CD8$^+$ T cell proliferation by activated T cells from Wt and Foxn1 Tg mice.** *A*) Identification of divided CD4$^+$ and CD8$^+$ T cells. Lymphocytes were isolated from the spleen of Wt and Foxn1 Tg mice, labeled with CFSE, activated using ConA. Lymphocytes were stained for CD4$^+$ and CD8$^+$ T cells after 5 days of culture. Histograms show cell proliferation of CD4$^+$ and CD8$^+$ T cells. The percent of divided T cells was determined by comparing CFSE histograms from unstimulated cells (black) with stimulated cells (red). Data shown are from a representative experiment of 7 experiments. *B*) Percent of divided CD4$^+$ and CD8$^+$ T cells. Data is presented as the percent of divided CD4$^+$ or CD8$^+$ T cells. Error bars are values of ±SD. Numbers in parentheses indicate the number of animals per group.
Comparison of CD3 expression in peripheral T cells in Wt and Foxn1 Tg mice

We next used flow cytometry to analyze peripheral T cells for the expression of CD3. The anticipation was that the age-related decrease in IL-2 production and T cell proliferation may be related to a decrease in the expression of CD3. Lymphocytes were selected for and CD3$^+$ T cells were gated on. The MFI of CD3$^+$ T cells was calculated using FlowJo software version 7.5 (Tree Star, Inc, Ashland, OR). The results show that in Wt mice there was a 50% decrease in the expression CD3 between 2-3 months and 20-26 months (p<0.0001, Figure 11B). No change was found in the expression of CD3 in Wt mice between 20-26 months and 28-35 months (Figure 11B). In Foxn1 Tg mice, CD3 expression in CD3$^+$ T cells decreased 34% between 2-3 months and 20-26 months (p=0.0464, Figure 11B). Similar to the results in Wt mice, the expression of CD3 in Foxn1 Tg mice remained unchanged between 20-26 months and 28-35 months (Figure 11B). Surprisingly, CD3 expression in young Foxn1 Tg was 34% lower than in young Wt mice (p=0.02, Figure 11B). No differences were found in CD3 expression in old Wt and old Foxn1 Tg mice 20-26 months or 28-35 months (Figure 11B). These data indicate that the expression of CD3 decreases with age in Wt and Foxn1 Tg mice. These data also indicate that CD3$^+$ T cells in young Foxn1 Tg mice express less CD3 than in young Wt mice.
Figure 11. Comparison of CD3 Expression on CD3⁺ T Cells in young and aged Wt and Foxn1 Tg Mice. 

A) Age-associated decrease in the cell surface expression of CD3 on lymphocytes. Lymphocytes were isolated from the spleen of Wt and Foxn1 Tg mice of different ages and stained for CD3⁺ T cells. CD3⁺ T cells were selected for within lymphocytes. Histograms show reduced expression of CD3 in aged Wt and young and aged Foxn1 Tg mice. Data presented are a representative experiment of 3-7 experiments.

B) Expression of CD3 on CD3⁺ T cells. The mean fluorescence intensity of CD3 was calculated using flow cytometry. Data are presented as the mean expression of CD3 within CD3⁺ T cells. Error bars are values of ±SD.
It is well established that with increasing age T cell functions decline, resulting in a reduced T cell response to newly encountered antigens, vaccine efficacy, and the overall immune response (33, 53-55). For example, in the elderly population of 65 years and older, it is estimated that the annual influenza vaccine has a 40 to 60% efficiency rate (27). The decrease in the T cell immune response is partially due to the contraction of the peripheral naïve T cell pool that results from a decrease in the thymic output of naïve T cells that occurs with age and an expansion of the memory T cell compartment (5, 15, 20). The decrease in the number of naïve T cells results in an overall reduction of IL-2 production by peripheral T cells, particularly by CD4+ T cells, causing a subsequent reduction in T cell proliferation (19, 22). Consequently, it is necessary to investigate the relationship between thymic involution and age-related changes among peripheral T cells, particularly the contraction of the naïve T cell pool.

The thymus functions to produce and export TCRαβ naïve T cells to the peripheral T cell pool. However, with age the thymus deteriorates in both structure and function, resulting in aberrations in T cell development and naïve T cell production. The thymic microenvironment is also altered with an age-associated stepwise decrease in the
expression of Foxn1 (45). Foxn1 is a Forkhead box transcription factor required for TEC differentiation during thymic organogenesis (39, 40). In order to study the relationship between thymic involution and the age-associated contraction of the naïve T cell pool, we used a C57BL/6 mouse model that demonstrates attenuation of thymic involution through over expression of the Foxn1 gene. Aged Foxn1 Tg mice show a higher number of thymocytes and ETP compared to age-matched Wt mice (P. Le et. al., Manuscript in preparation). Aged Foxn1 Tg mice also show minor changes in thymic architecture, with little thymic atrophy and infiltration of adipose tissue, compared to old Wt mice.

Also, H & E staining shows that the organization of the medullary and cortical compartments is well preserved in old Foxn1 Tg mice compared to old Wt mice (P. Le et. al., Manuscript in preparation).

Given this information, we tested if attenuation of the decline in the number of thymocytes would prevent age-related changes in peripheral T cell subsets. In Wt mice the numbers and percents of CD4^+ and CD8^+ naïve T cells declined with age, confirming what others have previously shown (Figure 5B and C) (20, 44). The contraction of the naïve T cell pool is due to the reduction in the number of thymocytes and thymic output of naïve T cells. As expected the total cell number and percent of CD4^+ and CD8^+ memory T cells in Wt mice increased with age (Figure 6B and C). We predicted that if the age-related decrease in the naïve T cell pool was prevented in Foxn1 Tg mice, the age-related expansion in the memory T cell pool would be lessened as well. In Foxn1 Tg mice, the number of CD8^+ naïve T cells did not decrease with age but was comparable to that of young Tg and young Wt mice (Figure 5C). In comparison, while the number of
CD4$^+$ naïve T cells in old Foxn1 Tg mice (28-35 months) was lower compared to young Tg mice, the number is 3.5-fold greater than in old Wt mice (28-35 months) (Figure 5C). However, despite the lessening of the decline of naïve T cells, the numbers of both CD4$^+$ and CD8$^+$ memory T cells increased with age in Foxn1 Tg mice (Figure 6B and C). Figure 8 summarizes these results, demonstrating the compensatory changes in the fractions of CD4$^+$ and CD8$^+$ naïve and memory T cells comprising the peripheral T cell pool in young and old Wt and Foxn1 Tg mice. As shown in Figure 8, in Wt mice, the fraction of CD4$^+$ naïve T cells contracts with age, with a reciprocal increase in CD4$^+$ memory T cells. Interestingly, in Wt mice despite the continuous decline in CD4$^+$ naïve T cells between 2-3 months to 28-35 months, the increase in the fraction of CD4$^+$ memory T cells plateaued at 20-26 months (Figure 8). This suggests that the age-related increase in memory T cells was due to reasons besides homeostatic expansion. In Wt mice the fraction of CD8$^+$ naïve T cells was reduced between 2-3 months and 28-35 months, with a reciprocal increase in the fraction of CD8$^+$ memory T cells (Figure 8). Contrary to expected results, lessening the decline in the fractions of CD4$^+$ and CD8$^+$ naïve T cells did not prevent the age-related increase in the CD4$^+$ and CD8$^+$ memory T cell pools (Figure 8). A potential reason for the contradictory results between naïve and memory T cells in Foxn1 Tg mice is that the age-related contraction in the naïve T cell pool is a result of thymic involution and the subsequent decrease in thymic function, total thymocyte number, and thymic output of naïve T cells (18). However, while the age-related increase in memory T cells occurs in response to the decrease in the naïve T cell pool, memory T cell expansion also related to the peripheral environment. The age-
related expansion of the memory T cell pool, particularly CD8$^+$ memory T cells, in Foxn1 Tg mice may be due to age-associated changes within the peripheral environment, that are independent of the size of the naïve T cell pool, given that the CD8$^+$ naïve T cell pool is maintained. Therefore, the expansion of memory T cells in Foxn1 Tg mice is most likely a result of a combination of several contributing factors. For example, clonal expansion, as opposed to homeostatic expansion, may drive the increase of the memory T cell compartment. Clonal expansion of memory T cells in mice and humans are believed to originate from dysregulated growth of T cell clones during chronic infections (30). Also, clonal expansions of memory T cells can also arise from conventionally antigen-activated CD8$^+$ memory T cells (56). The increase in the memory T cell pool in mice that have not been immunized may be the consequence of both antigen-dependent and independent events, as evidence has been found in support of both sides of this issue in TCR transgenic mouse models (35). Another factor supporting the increase in the memory T cell pool is cytokines within the peripheral environment, such as IL-7. IL-7 is a survival factor for both CD4$^+$ and CD8$^+$ memory T cells. IL-7 also promotes CD4$^+$ memory T cell division, whereas both IL-7 and IL-15 supports CD8$^+$ memory T cell division (57-59). IL-15 is produced by APCs, whereas IL-7 is produced by stromal cells, both which help regulate peripheral T cell homeostasis (59). Both CD4$^+$ and CD8$^+$ express high levels of IL-7Rα (CD127). CD8$^+$ memory T cells also express IL-15Rα. Another cause of memory T cell expansion is the conversion of naïve T cells to a memory T cell phenotype (60). Naïve T cells require TCR interaction with MHC ligands complexed with foreign or self peptides for survival. In the absence of foreign antigens,
naïve T cells interact with self peptides/MHC ligands. In an aged animal, where naïve T cell numbers are low, homeostatic proliferation occurs in an effort to maintain the dwindling naïve T cell pool. Homeostatic proliferation results in naïve T cells interacting with self peptides/MHC ligands and low levels of cell proliferation (61). In this way naïve T cells are converted to a memory phenotype in the absence of antigen stimulation (60). In summary the increase in memory T cells in Foxn1 Tg mice is a result of a combination of events that are related to the peripheral T cell pool, and are not entirely dependent on the size of the naïve T cell pool.

The memory T cell pool is a heterogenous group of cells that consists of central (T_{CM}) and effector (T_{EM}) memory T cell subsets. T_{CM} cells express CD44^{hi} CD62L^{+} CD127^{+} CCR7^{+} and produce IL-2 upon stimulation. T_{CM} proliferate and differentiate into effector T cells in response to antigen and IL-2 stimulation (17). T_{CM} are located in the T cell areas of secondary lymphoid organs. In contrast, T_{EM} display CD44^{hi} CD62L^{-} CD127^{+} CCR7^{-} and produce IFN-\gamma upon stimulation (17, 62). T_{EM} demonstrate immediate effector functions and are found in non-lymphoid tissues, such as liver, gut, and lungs, as well as in the spleen, and blood (17). Overall, the results for T_{CM} and T_{EM} showed similar results to CD4^{+} and CD8^{+} memory T cells. Interestingly, for CD4^{+} central memory T cells, there was no effect of age in Wt or Foxn1 Tg mice (Figure 7A). Also, the total cell number of CD4^{+} central memory T cells in young and old Foxn1 Tg mice was less than in young and old Wt mice, respectively (Figure 7A). The number and percent of CD4^{+} effector memory T cells increased with age in both Wt and Foxn1 Tg mice (Figure 7B). In Wt and Foxn1 Tg mice, CD8^{+} central and effector memory T cells
increased in number and percent (Figure 7C and D). In summary, CD4$^+$ effector and CD8$^+$ central and effector memory T cells increased with age in Wt and Foxn1 Tg mice.

The results on CD4$^+$ and CD8$^+$ naïve T cells and T cell functions in Foxn1 Tg mice were particularly puzzling for two reasons. First, in Wt mice the number and percent of CD8$^+$ naïve T cells were reduced with age. However, in both Wt and Foxn1 Tg mice, no age-related change was found in CD8$^+$ T cell proliferation. While these results were initially surprising, research shows that age-associated decreases in CD8$^+$ T cell proliferation were not present until CD8$^+$ T cells from culture were re-stimulated with antigen (63). Secondly, we predicted that if the age-related decrease in naïve T cells was prevented, it would prevent the age-associated decrease in T cell functions as well. Old Foxn1 Tg mice demonstrated an age-related decrease in IL-2 production and proliferation of CD4$^+$ T cells (Figures 9 and 10). While the number of CD4$^+$ naïve T cells in old Foxn1 Tg mice 28-35 months were decreased compared to young Tg mice, the number was greater than in old Wt mice 28-35 months (Figure 5B). The number of CD8$^+$ naïve T cells in aged Foxn1 Tg mice 28-35 months was greater than in aged Wt mice 28-35 months (Figure 5C). Furthermore, the number of CD8$^+$ naïve T cells in aged Foxn1 Tg mice was also maintained at a level comparable to those seen in young Wt and young Foxn1 Tg mice (Figure 5C). These results indicate that attenuation of the number of thymocytes lessened the decrease of the naïve T cell pool, but did not prevent a decrease in T cell functions. The age-related decrease in IL-2 production and CD4$^+$ T cell proliferation demonstrated by both aged Wt and Foxn1 Tg mice may be explained by the age-related decline in the expression of the CD3 molecule on the cell surface of CD3$^+$
T cells, particularly because T cells were activated through the CD3 receptor (Figure 11). While an age-related reduction in the expression of CD3 by peripheral T cells has also been shown in the elderly, it was previously undetermined if this occurs in mice (50, 64). However, the mechanism for reduced expression of CD3 on peripheral T cells ex vivo has not yet been determined. Surprisingly, activated T cells from young Foxn1 Tg mice also produced less IL-2 and demonstrated less CD4\(^+\) and CD8\(^+\) T cell proliferation, compared to young Wt mice (Figures 8 and 9) even though CD4\(^+\) and CD8\(^+\) naïve T cell levels in young Tg and young Wt mice were comparable (Figure 5). These seemingly contradictory results may also be due to lower expression of CD3 in peripheral T cells in young Foxn1 Tg mice compared to young Wt mice (Figure 11). The lower levels of CD3 expression in CD3\(^+\) T cells in young and old Foxn1 Tg and old Wt mice may be due to different reasons. For example, reduced expression of CD3 in old Wt mice may be due to age-related alterations in the molecular mechanisms involved in CD3 expression. In comparison, lower expression of CD3 in young and old Foxn1 Tg mice may be due to the Foxn1 transgene negatively affecting CD3 expression. Furthermore, old Foxn1 Tg peripheral T cells may show reduced CD3 expression due to both negative effects of age and the Foxn1 transgene.

While the requirement for Foxn1 expression during fetal development has been established during thymic organogenesis and functional TEC maturation, the role of Foxn1 in the adult thymus is not yet known (39, 40). Research shows that downregulation of Foxn1 results in rapid thymic atrophy, and decreased thymocyte number and proliferation of medullary TECs (42). TECs are a critical component of the
thymic stroma and are required for development of the thymus. The thymic stroma forms a three-dimensional network that interacts with and supports thymocytes for normal T cell development. Maintenance of the thymic microenvironment and TEC differentiation requires instructions or “crosstalk” between TECs and thymocytes (65). Foxn1 mediates one pathway of this crosstalk communication, with the N-terminal domain of Foxn1 being required for crosstalk mediating TEC differentiation during fetal development (66).

In Foxn1 Tg mice the interaction of thymocytes with TECs over expressing Foxn1 may negatively influence CD3 expression. Further experiments are needed to investigate the underlying mechanisms between Foxn1 over expression and reduced CD3 expression, such as measuring the expression of the CD3 γε, δε, and ζζ, or ζη dimers in young and aged Foxn1 Tg mice. It would be interesting to analyze the expression of CD3 in the spleen and thymus in a mouse model that down regulates Foxn1 expression to see if the expression of CD3 is higher in young and old mice, compared to the results found in this study, or if changes in CD3 expression are unique to Foxn1 over expression.

Foxn1 is expressed in TECs, making a potential interaction between Foxn1 and CD3 most likely to occur in the thymus. Foxn1 may influence CD3 expression during interactions and crosstalk between TECs and T cells. Cytoplasmic CD3 is first expressed during the double negative 2 (DN2) stage of T cell development, making a potential negative interaction between Foxn1 and CD3 more likely to occur after the DN2 stage. In Foxn1 Tg mice, Foxn1 over expression may negatively influence CD3 expression during positive selection, as contact is required between single-positive T cells and
cortical TECs. During positive selection CD4+ and CD8+ T cells interact with cortical TECs, selecting for T cells whose TCRs recognize self-MHC molecules (67). This interaction prevents positively selected T cells from undergoing apoptosis. CD4+ and CD8+ T cells that survive positive selection then undergo negative selection, which involves T cells interacting with medullary TECs. Negative selection eliminates T cells whose TCR has too high of an affinity for the self-MHC receptor, preventing self-reactive T cells from being exported into the periphery (67).

Changes in the expression of CD3 may also be related to the structure and production of the TCR-CD3 complex. The TCR-CD3 complex consists of a αβ heterodimer, which determines ligand binding specificity. CD3 is composed of γε, δε, and ζζ, or ζη dimers (67). While either ζζ or ζη dimers are required to form the CD3 receptor, the majority of CD3 receptors include ζζ homodimers (67). CD3 is required for signal transduction after a T cell interacts with a foreign antigen. The ζζ homodimer signals to the T cell interior that a MHC/antigen complex is bound to the TCR. CD3γ and δ are required for assembly of the TCR-CD3 complex. Foxn1 may affect the expression of one or more CD3 dimers during T cell development, which could lead to reduced CD3 expression in peripheral CD4+ and CD8+ T cells.

The age-related reduction in T cell functions could also be due to age-associated changes in the peripheral environment and cell surface expression of T cell activation markers. The age of each naïve T cell, meaning how long a cell remains alive in peripheral circulation, may influence T cell functions. With increasing age, naïve T cells undergo homeostatic proliferation in an effort to maintain the size of the contracting
 naïve T cell pool. Homeostatic proliferation results in an overextended lifespan of naïve T cells causing cells to live longer than they should (32). Newly produced CD4$^+$ naïve T cells from old mice demonstrate expansion, IL-2 production, and cognate helper function comparable to cells from young mice. However, CD4$^+$ naïve T cells in old mice that have aged show significantly reduced T cell functions, indicating that the age of a naïve T cell directly influences its function (68). As cells age, they are exposed to oxidative stress and DNA damage, which may negatively influence the functions of naïve T cells. While the number of CD8$^+$ naïve T cells does not change with age in Foxn1 Tg mice, the turnover rate of naïve T cells may be low, causing naïve T cells to live longer, accumulate DNA damage, and their functions to subsequently decline. Another potential reason for the decrease in T cell functions is possible a reduction in CD25 expression on activated T cells. Studies have shown CD25 upregulation on activated T cells from aged Wt mice to be reduced, which may lead to a decrease in T cell proliferation (18, 23).

The ratio of CD4$^+$ over CD8$^+$ T cells has also been shown to be reduced with age in mice (28). The results in CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells suggest that overexpression of Foxn1 may favor CD8$^+$ over CD4$^+$ T cells with increasing age. In Wt mice the numbers of peripheral CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells decreased with age (Figures 4B and C). In comparison, in Foxn1 Tg mice the percent of CD3$^+$CD8$^+$ T cells increased with age, while no change was found in total cell number of CD3$^+$CD8$^+$ T cells (Figures 4B and C). This resulted in compensatory changes, causing the number and percent of CD3$^+$CD4$^+$ T cells in Foxn1 Tg to decline with age between 2-3 months and 28-35 months (Figures 4B and C). Also, in Foxn1 Tg mice, the total cell number CD4$^+$
naïve T cells did not change between 2-3 months and 20-26 months (Figure 5B). However, the number of CD4+ naïve T cells decreased between 2-3 months and 28-35 months (Figure 5B). On the other hand, no change was found in the percent of CD4+ naïve T cells in Foxn1 Tg mice with increasing age (Figure 5A). In comparison, no difference was found in the total cell number CD8+ naïve T cells in Foxn1 Tg mice as age increased (Figures 5B). Taken together, these data suggest that the age-related decrease in the number of CD4+ naïve T cells in Foxn1 Tg mice was not due to the contraction of the naïve T cell pool, but rather occurred as a consequence to the age-related decrease in the number of total peripheral CD3+CD4+ T cells in Foxn1 Tg mice. Furthermore, in Foxn1 Tg mice the number of CD4+ memory T cells declined between 20-26 months and 28-35 months as a consequence of the increase in the percent of peripheral CD3+CD8+ T cells (Figures 4C and 6B). This decrease in the number of CD4+ memory T cells in Wt mice is also demonstrated as a compensatory change in Figure 8. More evidence supporting the notion that over expression of Foxn1 favors CD8+ over CD4+ T cells is shown in the thymus with the number of single positive CD8+ T cells being higher in young Foxn1 Tg mice than in young Wt mice. However, no difference was found in the thymus in the number of single positive CD4+ T cells in young Wt and young Foxn1 Tg mice (P. Le et. al., Manuscript in preparation).

Other models for studying age-associated changes among naïve and memory T cell pools have shown similar results, in terms of memory T cell expansion and T cell functions. One study used two reconstitution models: (1) antibody depletion of peripheral T cells followed by self-regeneration of the T cell compartment and (2) bone
marrow reconstitution of irradiated recipient mice (69). Within the aged peripheral T cell environment in both models, recovery following depletion resulted in a predominance of CD4$^+$ memory T cells in aged mice and low IL-2 production (69). In comparison, in young mice regeneration of the peripheral T cell pool resulted in predominantly CD4$^+$ naïve T cells and high IL-2 production. These results indicated that the mechanisms maintaining the sizes and proportion of the naïve and memory T cell pools differ in young and aged mice. This also confirms that the age-related expansion of the memory T cell pool is heavily influenced by the peripheral environment, compared to age-related changes associated with thymic involution.

Other models have attempted reversal of thymic involution, such as administration of IL-7 and growth hormone (GH) to animals in order to increase T cell development. IL-7 is required for both T and B cell development in mice and T cell development in humans. The IL-7 receptor consists of IL-7Rα (CD127) and the γ common chain (CD132) (67). IL-7 is produced by stromal cells in the bone marrow and thymus. IL-7 expression within the thymus decreases with age in mice (5). Injecting young and aged recipient thymi with an IL-7 secreting TEC cell line restored the age-associated block in thymopoiesis at the DN1 to DN2 transition, but did not prevent a decrease in naïve T cell production, as indicated by the level of sjTREC/10$^5$ cells (70). GH is produced by somatotrophs in the pituitary gland and stimulates the production of insulin like growth factor-1 (IGF-1) (71). IGF-1 is an endocrine hormone produced by the liver and mediates the effects of GH. Administration of GH results in an increase in thymic cellularity in mice and humans by promoting TEC proliferation and T cell
development (72). Removal of GH treatment from patients with adult GH deficiency results in a decrease in naïve T cell production. There also is a positive correlation between sjTREC and IGF-1 levels in patients with adult GH deficiency, indicating that GH and IGF-1 help maintain naïve T cell homeostasis in GH deficient adults (71). In summary, while these models improve age-relate defects in thymopoiesis, neither resulted in an increase in the naïve T cell pool, compared to age-matched unmanipulated controls. Also, both studies used sjTREC levels as their method for quantifying naïve T cells. While this method is reliable, it does not allow differentiation between CD4+ and CD8+ naïve T cells or provide total cell numbers. Furthermore, while over expression of Foxn1 and administration of IL-7 to mice attempt to correct age-related alterations in molecular defects causing thymic involution, administration of GH does not attempt to treat age-related changes associated with thymic involution.

In summary, attenuation of thymic involution by over expression of Foxn1 maintains the number of CD8+ naïve T cells, and improved the number and percent of CD4+ naïve T cells compared to aged-matched Wt mice (Figure 5B and C). Our work confirms previous reports, indicating a direct correlation between thymic involution and the age-associated contraction in the peripheral naïve T cell pool (6, 20). However, maintaining the naïve T cell pool does not prevent the age-associated increase in the number and proportions of CD4+ and CD8+ memory T cells (Figure 6B and C). Yet, the question remains if the difference in results between CD4+ and CD8+ naïve T cells in Foxn1 Tg mice is due to the age-related decrease in the total number of CD3+CD4+, but not CD3+CD8+ T cells in Foxn1 Tg mice. Notably, age-related decreases in IL-2
production and CD4⁺ T cell proliferation were not prevented by attenuation of thymic involution through over expression of Foxn1 (Figures 9 and 10). The decrease in T cell functions demonstrated by aged Wt and Foxn1 Tg and young Tg mice was most likely related to the reduction in CD3 expression (Figure 11).

Future directions are to immunologically challenge young and aged Foxn1 Tg mice with antigen, measure IL-2 production and CD4⁺ and CD8⁺ T cell proliferation, and compare with the results from the in vitro experiments from this study. It would be interesting to further investigate the ability of both young and aged Foxn1 Tg mice to respond to foreign antigens, particularly to determine if young Tg mice continue to demonstrate a hyporesponsive T cell immune response. Another unanswered question is if IL-2 production differs between peripheral T cell subsets in young and aged Foxn1 Tg mice. Another future experiment is to determine if Foxn1 is expressed in the spleen. If Foxn1 is expressed in the spleen, it may be in the spleen that Foxn1 over expression potentially influences CD3 expression. Also, another future experiment is to measure the expression of CD3 receptor γε, δε, and ζζ, or ζη dimers in the spleen of Foxn1 Tg mice. Another possible experiment would be to stain peripheral T cells from young and old Wt and Foxn1 Tg mice for the TCRαβ receptor and activated T cells for CD25. Reductions in the expression of the TCRαβ on peripheral T cells ex vivo and CD25 on activated T cells could also lead to decreased T cell functions. Taken together, our data indicate that attenuation of thymic involution through Foxn1 over expression results in the maintenance of the CD8⁺ naïve T cell pool does not prevent an increase in the CD8⁺ memory T cell compartment or decrease in T cell functions. This suggests the age-
related changes in the memory T cell compartment and T cell functions are more heavily influenced by age-associated mechanisms in the peripheral environment, as opposed to thymic involution.
CHAPTER 6
METHODS

Mice

C57BL/6 Wt mice were between 2-35 months of age. Young and old Wt mice were purchased from Harlan Laboratories and Harlan National Institute of Aging, respectively. Foxn1 transgenic mice generated using the human keratin-14 promoter and bred using a C57BL/6 background, with 12 genetic crosses of homozygous Foxn1 Tg mice. Foxn1 transgenic mice were between 2-35 months of age. All mice used in this study were males. All mice were housed in the Comparative Medicine Facility at Loyola University Medical Center using standard housing conditions, including specific pathogen free conditions, until sacrificed by CO₂ inhalation.

Flow cytometry staining for CD4⁺, CD8⁺, naïve, and memory T cells

Each spleen was surgically removed and splenocytes were isolated. Lymphocytes (1x10⁶ cells) were stained using antibodies anti-CD3e PerCP-Cy5.5 (145-2C11), anti-CD4 PE (GK1.5), and anti-CD8 APC (53-6.7), anti-CD44 APC-AF750 (IM7), anti-CD127 PECy7 (A7R34), and anti-CD62L FITC (MEL-14). All antibodies were purchased from (ebioscience, Inc, San Diego, CA), except anti-CD3e PerCP-Cy5.5 (BD Bioscience, San Jose, CA). Within lymphocytes CD4⁺ or CD8⁺ T cells were selected for. Within CD4⁺ and CD8⁺ T cells, CD3⁺ T cells were selected for. Memory and naïve T
cells were differentiated based on the expression of CD44. In mice, low expression of CD44 on peripheral T cells signifies a T cell that is naïve and has not yet encountered antigen or undergone TCR stimulation. In contrast, memory T cells have previously exposed to antigen and demonstrate high expression of CD44 (15). CD4+ and CD8+ naïve T cells were defined as CD44lo cells within CD3+CD4+ or CD3+CD8+ T cells, respectively. The total cell numbers of CD4+ and CD8+ naïve T cells were calculated by multiplying the percent of CD4+ or CD8+ naïve T cells by the total number of splenocytes.

CD4+ and CD8+ memory T cells were defined as CD44hi cells within CD3+CD4+ or CD3+CD8+ T cells, respectively. The total cell numbers of CD4+ and CD8+ memory T cells were calculated by multiplying the percent of CD4+ or CD8+ memory T cells by the total number of splenocytes. Memory T cells were further differentiated into central and effector memory T cell populations. Central memory T cells were defined as CD44hiCD127+CD62L+ within CD3+CD4+ and CD3+CD8+ T cells. Effector memory T cells were defined as CD44hiCD127−CD62L− within CD3+CD4+ and CD3+CD8+ T cells (17). The total cell numbers of central and effector memory T cells were calculated by multiplying the percent of central or effector memory T cells by the total number of splenocytes.

To analyze peripheral T cells for the expression of CD3 lymphocytes were selected for and CD3+ T cells were gated on. The MFI of CD3 was calculated using FlowJo software version 7.5 (Tree Star, Inc, Ashland, OR). Splenocytes were analyzed
using FACS on a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA). All flow cytometry data were analyzed using FlowJo software version 7.5.

**T cell activation for IL-2 production**

The spleen was surgically removed and splenocytes were steriley isolated. T cells were activated using monoclonal, plate bound anti-CD3 (ebio500A2) and anti-CD28 (37.51) antibodies (ebioscience, Inc, San Diego, CA), at a final concentration of 5 µg/ml. Splenocytes were cultured in 96-well, flat bottom plates (Fisher Scientific, Pittsburg, PA), with 500,000 splenocytes/well in culture media. Culture media was steriley prepared by adding 5% fetal calf serum (Invitrogen, Corporation, Carlsbad, CA), 1x Penicillin Streptomycin (Invitrogen, Corporation, Carlsbad, CA), 10 mM Heps (Sigma-Aldrich, St. Louis, MO), and 55 µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) to RPMI-1640 media (Mediatech, Inc, Manassas, VA) (19). Unstimulated splenocytes, cultured without anti-CD3 and anti-CD28 antibodies, were used as a control for each animal. 4 wells of stimulated and unstimulated splenocytes were cultured at 37°C, 5% CO₂ for 24 hours until supernatants were harvested. For each animal, supernatants were pooled for each culture condition, and stored at -20°C until analyzed using mouse IL-2 Enzyme-linked immunosorbent assay (ELISA). A culture time point of 24 hours was chosen because that was the optimal time to collect splenocyte supernatants for analysis of IL-2 production levels (20).
Measurement of IL-2 in supernatants by Enzyme-linked immunosorbent assay (ELISA)

Nunc-Immuno Maxisorp 96-round bottom plates (Fisher Scientific, Pittsburg, PA) were coated with 100 µl of Functional grade purified anti-mouse IL-2 (JES6-1A12, ebioscience, Inc, San Diego, CA), at a final concentration of 2.5 µg/ml. The anti-mouse IL-2 antibody was diluted in 1x phosphate buffered saline (PBS)/0.02% sodium azide (Sigma-Aldrich, St. Louis, MO). The plate was incubated at 4°C overnight. Non-specific binding was achieved by adding 150 µl of 1x PBS/1% Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) to each well. The plate was incubated at 37°C for a minimum of 1 hour. 100 µl of each undiluted supernatant was added to appropriate wells. The IL-2 concentration of each supernatant was measured in triplicates. For a standard curve, 5 standards were prepared using recombinant IL-2 (ebioscience, Inc, San Diego, CA). A 1 to 5 serial dilution pattern was used to prepare the standards, starting at 50 ng/ml to 0.08 ng/ml. Each standard dilution was prepared in replicates. 100 µl of each standard dilution was added to the appropriate wells. Two negative controls with culture media only were prepared. The plate was then incubated at 37°C for 3 hours. 100 µl of biotin anti-mouse IL-2 (JES6-5H4, ebioscience, Inc, San Diego, CA) was added to each well, at a final concentration of 0.8µg/ml in 1x PBS/1% BSA. The plate was incubated at 37°C for 2 hours. 100 µl of streptavidin-conjugated alkaline phosphatase was added to each well, using a 1 to 1000 dilution (Southern Biotech, Birmingham, AL), diluted in 1x PBS/1% BSA. The plate was incubated at 37°C for 30 minutes. 1 mg ρ-nitrophenylphosphate was dissolved in 1 ml mM diethanolamine, 0.5 mM MgCl$_2$·6H$_2$O.
and 100 µl was added to each well. After 40 minutes the absorbance was read at 405 nm using Gen5 version 1.02.8 software (BioTek Instruments, Inc, Winooski, VT). A 4-parameter curve was used to plot a standard curve of recombinant IL-2 standards.

IL-2 was not detected in the supernatants collected from splenocytes that were cultured without anti-CD3 and anti-CD28 antibodies. For each animal in a given ELISA experiment, the mean and standard error of IL-2 concentration of the triplicate wells was calculated. For each group of mice, the mean and standard deviation of IL-2 concentration was calculated.

**T cell proliferation assay**

Carboxyfluorescein diacetate succinimidyl ester (CFDASE) (Invitrogen Corporation, Carlsbad, CA) was used to analyze T cell division data. CFDASE is a highly membrane permeable fluorescent dye that is taken up by cells due to its lipophilic nature (49). However, once in the cytoplasm intracellular esterases remove the two acetate groups on CFDASE to form 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE), which is far less membrane permeable. The succinimidyl group in CFSE reacts with cytoplasmic amino groups forming intracellular conjugates with 5-(and-6)-carboxyfluorescein. A portion of these conjugates exit the cell, while some conjugates remain within the cell, rendering it stably labeled. If a cell is activated and divides, the CFSE within the cell is divided equally between the two daughter cells. As a result, the intensity of CFSE within a cell correlates well with the number of times a cell divides (73).
To label splenocytes with CFSE, cells were resuspended in sterile, warm 0.1% BSA/PBS (Fisher Scientific, Pittsburg, PA) at 1x10^6 cells/ml. Lymphocytes were labeled with 0.5 µM CFSE and incubated in a 37 °C water bath for 15 minutes. Cold culture media was added to cells (5x the cell volume) and cells were incubated on ice for 5 minutes. Splenocytes were washed two times with culture media, resuspended, and counted before plating. To activate splenic T cells, soluble Concanavalin A (ConA) (Sigma-Aldrich, St. Louis, MO) was added to cultures at a final concentration of 5 µg/ml. ConA is a lectin protein that binds non-specifically to activate T cells through the TCR (74). Unstimulated cells, cultured without ConA and labeled with CFSE, were used as a control for each animal. For each animal, cells were cultured in two to three replicate wells. Splenocytes were cultured in 96-well round bottom plates (Fisher Scientific, Pittsburg, PA) with 1x10^6 cells/well at 37°C, 5% CO2 for 5 days (48, 49, 75). Once cells were harvested, lymphocytes were stained for T cells using anti-CD4 PE (GK1.5) and anti-CD8 APC antibodies (53-6.7). For flow cytometry analysis, lymphocytes were selected for CD4^+ or CD8^+ T cells. Within CD4^+ or CD8^+ T cells, divided T cells were selected for by superimposing and comparing CFSE-FITC histograms of unstimulated cells, cultured without ConA, and stimulated cells, cultured with ConA.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism version 5.02 (San Diego, CA). The F-test was used to test if the standard deviations of 2 populations were equal. If the result of the F-test was not significant and the 2 populations had normal distribution, Student’s t-test was used to determine if the means of 2 populations were
equal. If the result of the F-test was significant and the 2 populations had non-normal distribution, a Mann-Whitney U test was used to determine if the means of 2 populations were equal. Significance for all tests was determined at $p \leq 0.05$. 
REFERENCES


VITA

Paulette Krishack was born to Paul and Celeste Krishack in Chicago, Illinois on December 6, 1984. She attended the University of Illinois at Springfield where she received her Bachelor’s of Science degree in May 2007. Paulette began the Cell Biology, Neurobiology, and Anatomy Graduate Program at Loyola University Chicago in August 2007 as a Master’s student. She joined the Le lab in December 2007 when she began her thesis research on age-associated changes in peripheral T cells. Paulette has one sister, Celeste.