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Michelle L. Bolner

EFFECTS OF THYMUS SIZE AND INVOLUTION ON THE CONTRIBUTION OF RECENT THYMIC EMIGRANTS TO THE PERIPHERAL T CELL POOL

BY

Michelle Lynn Bolner, Bachelor of Science

APPROVED:

_____
Ellen R. Richie, Ph.D., Supervisory

_____
Bradley McIntyre, Ph.D.

_____
David G. Johnson, Ph.D.

_____
Mark Bedford, Ph.D.

_____
Richard D. Wood, Ph.D.

APPROVED:

_____
Dean, The University of Texas

Graduate School of Biomedical Sciences at Houston

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A

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Michelle Lynn Bolner, B.S.
Houston, Texas

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EFFECTS OF THYMUS SIZE AND INVOLUTION ON THE CONTRIBUTION OF RECENT THYMIC EMIGRANTS TO THE PERIPHERAL T CELL POOL

Michelle Lynn Bolner, B.S.

Supervisory professor: Ellen R. Richie, Ph.D.

The contribution of recent thymic emigrants (RTEs) to the peripheral naïve T cell population is necessary to maintain diversity of the T cell receptor (TCR) repertoire and produce immune responses against newly encountered antigens. The thymus involutes with age, after irradiation or chemotherapy, and due to severe viral infections. Thymus involution results in decreased thymopoiesis and RTE output leading to a reduced diversity of peripheral T cells. This increases susceptibility to disease and impairs immune responsiveness to vaccines. Therefore, studies aimed at maintaining or regenerating thymic function are integral for maintaining and restoring peripheral TCR diversity.

Mice that express a K5.CyclinD1 transgene expression have a severely hyperplastic thymus that fails to undergo involution. Both thymocyte and TEC development appear normal in these mice. We have used the K5.CyclinD1 transgenic model to test the hypothesis that preventing thymus involution will sustain RTE output and incorporation into the peripheral T cell pool to prevent naïve T cell depletion with age. The K5.CyclinD1 transgene was crossed to the RAG2p-GFP transgenic model so that RTEs could be tracked by the intensity of the GFP signal. The frequency and number of RTEs in naïve CD4 splenic T cells was analyzed at monthly intervals to 5 months of age. Using this double transgenic approach, we determined that preventing thymus involution does maintain or enhance the number of RTEs in the peripheral T cell pool before and after thymus involution.

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CHAPTER ONE: INTRODUCTION AND BACKGROUND

Chapter 1: Introduction

The immune system is indispensible for the protection of the body from foreign insults. In 1961, Miller reported that thymectomy of newborn mice resulted in decreased weight, systemic infection and death within weeks showing for the first time that the thymus is necessary for production of defensive factors after birth (1, 2). Miller also noted that antibody production was not affected in thymectomized mice, which led to the hypothesis that cell-mediated immunity and cells capable of producing antibodies were separate populations (3). T cells, so named for their development within the thymus, convey cell-mediated immunity. This population mediates immunological responses by direct cell-to-cell interactions. Each T cell clone possesses a unique and highly specific T cell receptor (TCR) that is the product of multiple rearrangements among TCRα and TCRb gene segments during development within the thymus. To guard against autoreactivity and select for responsiveness, all developing T cells, termed thymocytes, undergo an extensive screening process within the thymus by interacting with epithelial cells. Once this program is complete, newly minted T cells emigrate from the thymus to join the peripheral T cell pool encompassing an estimated 10^{12} unique TCR clones (4). Vast TCR diversity is necessary for generating an immune response against almost any non-self antigen. Receptor diversity is compromised with age by the substantial reduction of naïve T cell production as a result of naturally occurring thymic atrophy (5). The decline in T cell output from the thymus causes changes in the peripheral T cell pool, resulting in decreased immune responsiveness to newly encountered antigens (6). This loss of affinity leads to poor immune responses to vaccines and greater susceptibility to disease. Therefore, understanding how to retain or regenerate a competent thymus in order to preserve naive T cell output would provide a new insight to improving immune responses in aging and immunocompromised individuals.

Thymus Organization

The thymus is a highly specialized organ of the immune system, tasked with producing T cells that are both specific for foreign antigens and tolerant of self-antigens. In fact, nearly 98% of the cells within the thymus are thymocytes at various

stages of T cell development. The remaining cells comprise the stromal microenvironment and consist of primarily of thymic epithelial cells (TECs) but also include dendritic cells, macrophages and endothelial cells (7). Structurally, the thymus is organized into two distinct regions, each of which is required for T cell development. The cortex is the outer region of the thymus, and is home to immature thymocytes and cortical thymic epithelial cells (cTECs). The cTECs process and present self-peptides in the context of major histocompatitbility (MHC) antigens required for positive selection and release cytokines necessary for thymocyte survival and growth (8). The medulla is the inner region of the thymus where semi-mature thymocytes and medullary thymic epithelial cells (mTECs) interact. The mTEC subset is required for negative selection because these cells have the unique ability to express a broad array of tissue restricted antigens that can induce apoptosis of self reactive thymocytes (9). TECs and thymocytes participate in an active crosstalk necessary for the survival of both cell types. TECs provide survival, proliferative and differentiation signals necessary for thymocyte maturation (10); without TEC interactions, thymocytes do not develop within the thymus, as evidenced by the Foxn1-deficient *nude* mouse in which the failure of TEC progenitors to differentiate abrogates T cell development (11). Similarly, TEC differentiation depends on thymocyte-derived signals (12).

T Cell Development

Hematopoietic precursors migrate from the bone marrow through the circulation to the thymus. It is difficult to identify thymus-seeding cells as they do not express any known unique hematopoietic differentiation markers, only the hematopoietic stem cell markers SCA1, and KIT (13). Thymus seeding cells enter the thymus through blood vessels at the corticomedullary junction (CMJ), are not committed to the T cell lineage and retain B cell potential. Expression of the ligand delta-like 4 on TECs initiates the Notch pathway necessary for ensuing T cell development (14), along with pre-TCR signaling. Thymocytes migrate from the CMJ through the cortex to the subcapsular portion of the organ, and then back through to the medulla. During this process, they interact with cTEC and mTEC subsets that provide signals for continued survival and development. Four major thymocyte subsets are defined by the expression of CD4 and

CD8 surface molecules during this process (Figure 1). The most immature thymocytes are double negative (DN) for both CD4 and CD8 expression. The DN1 subset still displays the lymphoid stem cell marker c-kit. The interleukin-2 receptor alpha chain (CD25) is transiently expressed through the DN2 and DN3 stages in the cortex, while c-kit expression is lost at the DN3 stage. TCRβ gene rearrangement is initiated at the DN2 stage and the pre-TCR consisting of a TCRb and pre-Ta heterodimer is expressed at DN3. Pre-TCR expression results in rescue from apoptosis, arrest of β chain gene rearrangement, and a hyperproliferative burst during which CD25 expression is lost. Expression of both CD4 and CD8 peptides indicates transition to the double positive (DP) stage of thymocyte differentiation. The α chain of the TCR then rearranges to create a viable αβTCR heterodimer. Only those cells that recognize self-peptides in the context of self-MHC are stimulated to survive – most DP thymocytes will not respond and thus fail positive selection and die from neglect. Positively selected thymocytes down-regulate either CD4 or CD8, and commit to the T helper (CD4+) or cytotoxic (CD8+) T cell lineages (15). These single positive (SP) thymocytes migrate into the medulla where they undergo negative selection in which TCR affinity is tested. Those that react with high affinity to self-antigen expressed by mTECs are deleted while those that have a moderate affinity for self-peptide/MHC are exported to the periphery. Only 2% of all DP thymocytes survive to the SP stage and are released as naïve T cells to the periphery.

Thymic Emigration

Upon appropriate thymocyte development and selection in the thymus, newly minted T cells emigrate through the vessels at the CMJ, and become recent thymic emigrants (RTEs). Factors involved in emigration center around sphigosine-1-phosphate receptor ($S1P_1$), a G protein coupled receptor expressed by mature SP thymocytes and shown to be integral in the exit strategy of a mature thymocyte from the thymus. The ligand sphingosine-1-phosphate (S1P) is produced by pericytes along the CMJ vessels, creating a chemotactic gradient by which the thymocytes home to the vessels and exit the organ (16). The $S1P_1$ receptor is positively regulated by the transcription factor Kruppel-like factor 2 (KLF-2), and knockout studies have shown that

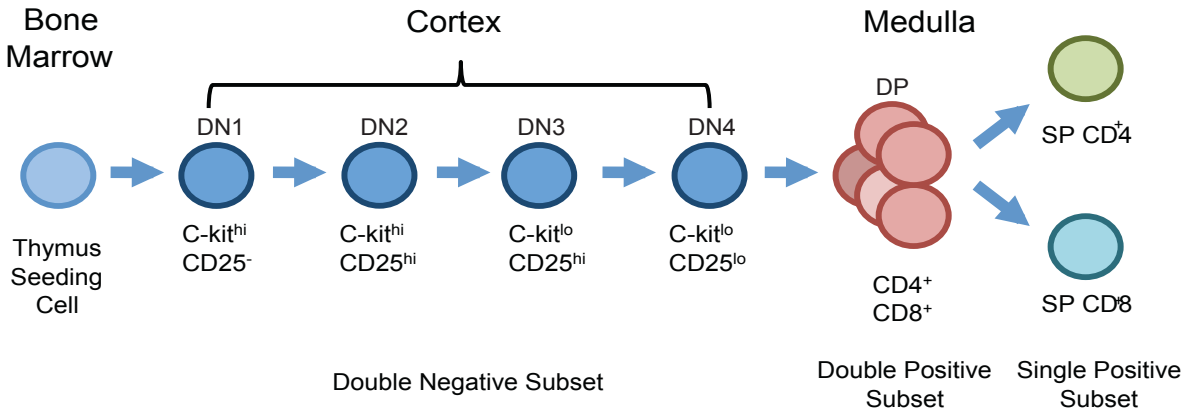

Figure 1. Progression of normal thymocyte development. Thymus-seeding cells enter the thymus and give rise to the double negative (DN) subset of developing thymocytes. Upon TCR rearrangement, thymocytes express both CD4 andCD8 molecules and become part of the double positive (DP) subset. DP thymocytes must lose surface expression of either CD4 or CD8 to be deemed single positive (SP) thymocytes, before preparing for export from the thymus.

both are necessary components for T cell emigration from the thymus (16, 17). Additionally, TCR engagement is a strong activator of the PI3K-AKT pathway and results in nuclear export of FOXO1, a transcription factor that positively regulates KLF2 and thus, $S1P_1$ (18). Therefore, studies suggest that TCR engagement inhibits KLF2 and $S1P_1$ expression, which retains SP thymocytes in the thymus during the final stages of maturation. Additionally, the C-type lectin CD69 is transiently upregulated in response to TCR stimulation, and inhibits $S1P_1$ expression by inducing internalization of the protein (19, 20). These pathways of inhibition serve as an important checkpoint, as TCR engagement ensures the retention of SP thymocyte by way of restriction $S1P_1$ expression until proper maturation in the thymus is complete (18). Therefore, the TCR influences the exit strategy of RTEs in such a way as to ensure only capable, mature thymocytes are allowed exit to the peripheral T cell pool.

1.2 Recent Thymic Emigrants

The population of peripheral T cells is kept at a relatively constant number throughout life by the constant addition of newly produced T cells from the thymus and homeostatic control of peripheral T cell number. In general, peripheral T cells can be classified as naïve T cells which have not yet encountered and undergone activation by recognition of specific antigen, and memory cells which result from clonal expansion and resolution after this recognition. Mature naïve (MN) T cells constitute the virgin T cell population, and express low levels of self-reactivity, which is not enough to cause autoimmunity, but necessary to ensure TCR sensitivity for foreign antigen. Furthermore, the MN T cell population is maintained by MHC interactions and signaling from the cytokine Interleukin-7 (IL-7). Upon antigen recognition, naïve T cells proliferate and mount an immune response, with some cells remaining after infection clearance to serve as memory T cells. Recent thymic emigrants (RTEs) are a distinct T cell subset that sustains TCR diversity of naïve T cell repertoire through the addition of unique TCRs to the population. Upon emigration from the thymus, RTEs are mature with respect to thymocyte development, yet not fully capable of the functional maturity of their MN T cell counterparts (21). Both CD4 and CD8 RTEs express distinct cell surface phenotypes and altered functionality compared to the MN subset. RTEs exhibit

reduced proliferation and production of cytokines IL-2, IL-4 and interferon gamma in both man and mice compared to MN T cells (22). RTEs of newborn mice serve the unique role of establishing the peripheral T cell pool, since there are few peripheral T cells present at this age (23). These RTEs mature and give rise to the MN T cell subset present after birth, and show that this population thrives in a lymphopenic environment. However, thymic emigrants of the healthy adult enter a lymphoreplete environment upon thymic exit, and must compete to share the same niche as MN and memory T cells (24). While RTEs are capable of survival without exogenous factors for several weeks, TCR signaling is essential for long-term survival (25). Many RTEs don't survive well in the periphery, and studies suggest that RTEs and naïve T cells compete for MHC receptor and IL-7 interactions that mediate critical survival signals. RTEs display a reduced affinity for these stimulators (26, 27), which is reflected in poor incorporation into the naïve population (28). Cytokine signaling has been shown to specifically play a role in reduced RTE survival – transgenic expression of the IL-7 receptor on RTEs rescues these cells from death and increases competitiveness with MN T cells of the periphery (21).

The influx of the RTE population is necessary for enriching peripheral T cell diversity, as successful viral clearance in mouse studies is enhanced by the presence of RTEs in the peripheral T cell pool. In chronic viral infections, new naïve T cells promote antigen-specific diversity and homeostatic proliferation that is lost in T cells that have chronically responded (29). The preservation of antiviral CD8+ T cell responses to infection is enhanced by the heterogeneity thymic emigrants contribute to the naive T cell pool (30). Furthermore, individuals recovering from lymphopenia induced from severe illness, HIV, and chemotherapeutics benefit from RTEs, which reconstitute a diverse T cell repertoire. RTEs are clearly required to sustain immune responsiveness to new antigens. Additional studies are required to determine precisely how these cells mature and affect immunological responses.

Peripheral Maturity & Incorporation

In 1970, Stutman suggested that some type of additional developmental process for naïve T cells in secondary organs might be necessary for final maturity to be

achieved (31). To test this hypothesis, an intrathymic fluorescein isothiocyanate (FITC) injection labeling method was designed to label all thymic cells and to identify RTEs. After injection, FITC positive cells were observed in the spleen showing no phenotypic difference from FITC negative cells for CD4 and CD8 expression, and suggesting that RTEs do not undergo further maturation in the periphery (32). It was not until the use of additional cell surface markers in conjunction with intrathymic FITC labeling that differentiation between RTEs and naïve T cell were finally observed (33-35).

RTEs are mature in some aspects including CD4 and CD8 expression, yet not others when compared to naïve T cells (35). RTEs are initially larger than their T cell counterparts, and are functionally competent by 16 hrs in the periphery (32). RTEs lose expression of Thy-1 and CD69 upon emigration. CD69 is an early activation antigen that relies on TCR engagement for expression and suppresses thymic exit through $S1P_1$ inhibition (36-38). MHC class 1 molecules are commonly expressed at higher levels on RTEs than MN T cells. Specifically, the MHC class Ib molecule, Qa2, is upregulated as RTEs develop to mature naïve T cells in the periphery (34, 35). Family members of Qa2 confer antiviral immunity to host defense when expressed on CD8+ T cells (39). Analysis of cytokine receptor IL-7 receptor α (CD127) and co-stimulatory molecule CD28, respectively, show increased expression with $CD8^+$ RTE maturation (40). Heat stable antigen (CD24) expression is decreased in a step-wise pattern from RTE to naïve T cell, and is required for homeostatic proliferation on T cells in a lymphopenic environment (41, 42).

Studies utilizing FITC labeling in conjunction with cell surface markers have provided a foundation for RTE maturity analysis. However, crude intrathymic FITC injections have been scrutinized for lack of specificity, with concerns regarding contamination of the FITC positive population by recirculating MN T cells (43). By repurposing a genetic mouse model created to examine B cell receptor editing for thymic emigration studies, a faithful method of labeling SP thymocytes for export from the thymus was achieved in a non-traumatic manner (44). Recombinant activating gene 2 (Rag2) promoter-driven GFP expression allows for tagging of late stage DN thymocytes. The *Rag2* gene is required for α and β TCR gene rearrangement, and after which its expression is extinguished (40). This transgenic model allows precise

aging of T cells released from the thymus, with the gradual loss of GFP signal used as a molecular timer within RTEs in the periphery. Since GFP half-life is 16-18 hours (40, 45), and GFP signal is completely lost within three weeks in the periphery (40), peripheral naïve T cells can be divided into three separate populations (Figure 2). Those cells that exhibit high GFP intensity (GFP^{high}), have been in the periphery less than one week according to kinetic studies (40), while those that express low GFP intensity (GFP^{low}) have resided in the periphery for 1-3 weeks. Those that are negative for GFP expression (GFP^{neg}) are considered MN T cells. Additionally, GFP expression in RTEs does not alter development nor proliferative capability in these cells (40). Thus, the naïve T cell population can be studied in unmanipulated mice bearing the Rag2p-GFP transgene, with the separation of new naïve T cells by the amount of time spent in the periphery. This transgenic model, coupled with analysis of cell surface markers has defined a reproducible method of chronicling the maturation of RTEs to functionally competent naïve T cells in the periphery (40, 46). By beauty of the system, thymic emigration can be studied at any age in the mouse, provided there is sufficient thymopoiesis occurring to produce RTEs.

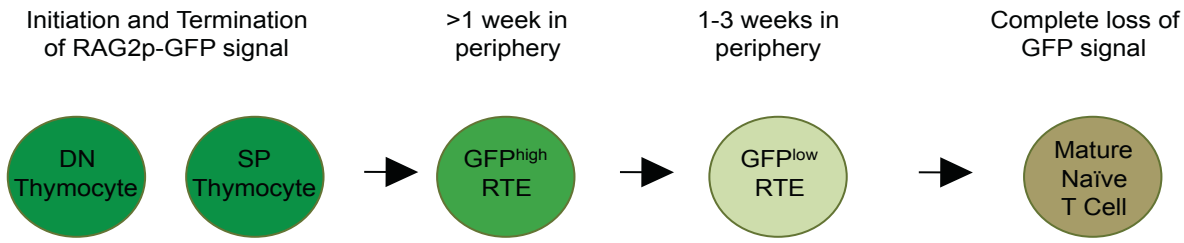

Figure 2. GFP intensity in the RAG2p-GFP model can be used as a molecular timer for RTEs. GFP is expressed during TCR gene rearrangement and is extinguished by the single positive (SP) stage. GFP expression is lost due to proliferation and protein decay. GFP expression is completely lost by 3 weeks in the periphery.

1.3 Thymic Growth and Involution

The thymus originates from the third pharyngeal pouch of the developing mouse embryo. At embryonic day 11.5 (E11.5) the third pouch is divided into thymic fated and parathyroid fated domains. The thymic domain can be distinguished by expression of *Foxn1*, a transcription factor required for TEC development and maintenance (47, 48). Thymic size is largest in children, and peaks at 4-6 weeks of age in the mouse (49,50). Thymic output is also greatest early in life, with RTEs accounting for almost 20% of the total T cell population in mice at six weeks of age (51). Thymus homeostasis is achieved for a short period in young mice as defined by relatively constant cellularity. (7). With age the thymus undergoes progressive involution due primarily to loss of TECs and disintegration of the TEC network required to support thymocyte development. Phenotypic characterization of involution includes disorganization of the stromal compartments, increased fibroblastic populations (7, 52) and accumulation of adipocytes in the tissue (53). As a result of thymus involution, there is a decline in thymopoiesis and naïve T cell output. Involution also occurs independent of age, as a result of cytoablative therapy and after severe viral infections (54).

Effects of Involution on the RTE population

The thymus is still functional in aged mice as shown by the production of RTEs in mice two years of age. The ratio of thymocytes to RTEs is not affected with age, and is independent of involution (51). However due to the overall decline in thymopoiesis, the number of RTEs exported to the periphery decreases up to 30-fold (51). This loss in RTE output leads to a loss of TCR diversity, as the ability to produce T cells with unique affinities is significantly compromised with age. Additionally, production of naïve T cells with defects in cell surface molecule expression and delayed maturation kinetics occurs in old mice (50, 51). Proportions of developing thymocytes do not change in murine thymi as old as 18 months of age, yet the expression of surface molecules are altered. CD3 expression is significantly reduced upon new naïve T cells, and normal down-regulation of CD24 is delayed (55). Responses to mitogen stimulation is also impaired – diminished TCR activation has been observed (51) and the expression of

co-stimulatory molecule CD28 is dampened upon human CD8 T cells in the elderly. Furthermore, activation associated IL-2 secretion is dampened in RTEs of aged mice compared to naïve T cells in the periphery (51). These studies together suggest that naïve T cells derived from involuted thymi are already functionally impaired, adding to the reduced T cell response in the aged.

The peripheral pool cellularity is maintained despite thymic involution, due to tight homeostatic maintenance of T cell populations. There is a shift in the ratio between peripheral populations that is correlated with involution. As the number of RTEs declines with age, the reduction in naïve T cell number prompts clonal expansion of the memory T cell compartment to maintain homeostasis (56). The contraction of the naïve T cell pool has been observed in both humans and rodents with age, and compromises T cell diversity for both CD4 and CD8 T cell populations. Clonal expansion of cells in the memory compartment is responsible for the reduction of 10^8 clones in young adults to 10^6 clones in elderly humans and has been correlated to the reduced ability to mount a successful immune response, particularly to new antigens (57). Additionally, function of old naïve T cells is affected with age. Old CD4 naïve T cells exhibit diminished responses to T cell receptor stimulation, and produce aberrant cytokine secretion compared to naïve T cells from young mice (57). There is an increase in pro-inflammatory CD8 T cells in the elderly, which impedes longevity (58). Low naïve precursor frequency is seen in activated CD8 T cells in aged mice and leads a reduced response of T cells and holes in the repertoire (59). Taken together, decreased RTE production leads to aging in the peripheral T cell pool which results in reduced immune responses in old age.

Prevention of thymic involution and reduced RTE production

The crux of involution and aging studies is to understand the process of involution in order to be able to slow, stop, or reverse thymic involution in aging and immunocompromised patients. Studies focused on androgen ablation have shown that thymic rebound occurs in a transient manner in the aged human and mouse, and T cell output is enhanced upon return of thymic size (60, 61). TECs have been implicated in the degeneration of the thymic environment. *Foxn1* is integral in initial thymic

development and maintenance. Foxn1$^{\Delta/\Delta}$ mice, which are a hypomorphic variant that results in reduced thymic size, produce TECs which are blocked at an intermediate differentiation stage, and results in blocked α:βT cell production (62). Inducible Foxn1 studies in which *Foxn1* expression is targeted to the thymic epithelium after involution suggest that TEC progenitors exist even in aged thymi, and can give rise to this expansion of thymic tissue and maintenance of developing thymocyte numbers (63, C Blackburn, personal communication). Meanwhile, knocking out cell cycle inhibitor proteins like the retinoblastoma (RB) family members in TECs in an inducible fashion can achieve sustained thymic rebound in mice up to nine months of age (J Sage, personal correspondence). However, induction of this phenotype in older mice has not been possible, suggesting age restraints RB-mediated thymic rebound. These studies together show that there is not only a potential for thymic regrowth in aged individuals, but also that T cell output can be positively affected too. However, these studies only accomplish rebound that is transient, or cannot be achieved after a certain age, barring continued T cell output in old age. Furthermore, recent speculation suggests issues with the quality of T cells produced by means of thymic rebound. Age-associated changes including medullary islet complexity and decreased levels of tissue restricted antigens are found in rebounded thymi (64). This new report questions the feasibility of thymic regeneration if quality of the thymic emigrants is compromised.

Cyclin D1 is a key cell cycle regulator that activates cdk4 and cdk6 to promote cell cycle progression and also functions in a cdk-independent manner as a transcriptional regulator. Our lab has shown that expression of a Cyclin D1 transgene targeted to epithelial cells by a keratin 5 (K5) promoter results in a hyperplastic thymus that does not undergo involution (65, 66), yet no hyperplasia of the lymphoid organs is observed (Figure 3). Despite the severe thymus hyperplasia in K5.CyclinD1 mice, cortical and medullary organization are preserved (66). Furthermore, the corticomedullary junction is expanded, and it thought to enhance both TEC and thymocyte development, as this region has been implicated in harboring TEC precursor populations (12). Thymocytes develop normally according to phenotypic analysis, though absolute numbers of the major thymocyte subsets are much greater than in non-transgenic littermates (66). Therefore, the K5.CyclinD1 mouse model provides an

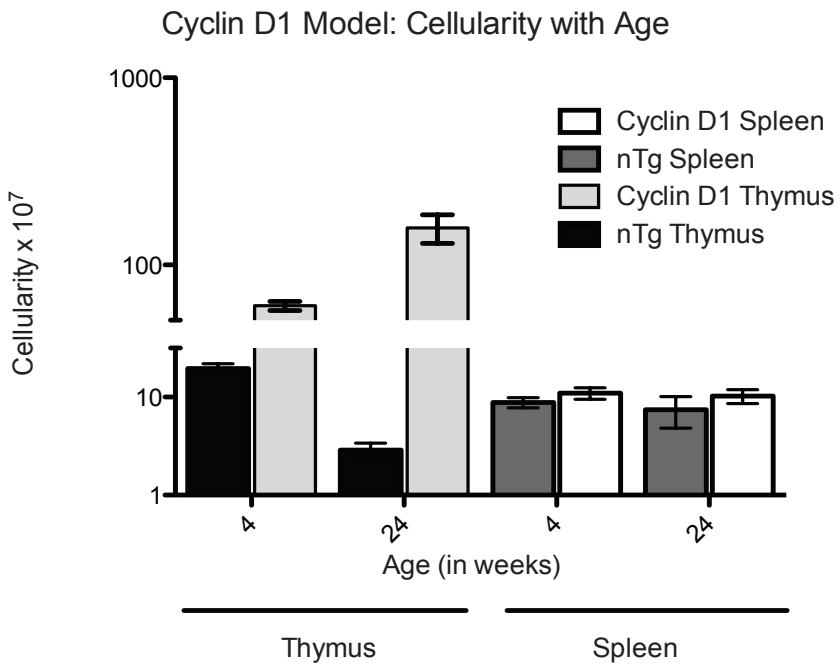

Figure 3 - K5.CyclinD1 thymi develop severe thymic hyperplasia. Thymic and spleen cellularity was taken at 4 and 24 weeks of age from K5.CyclinD1 and nontransgenic littermates. Thymic hyperplasia increases with age, and does not undergo involution. Splenic cellularity is not affected by thymic hyperplasia.

opportunity to study thymic structure and T cell production independent of thymic involution. Determining the alterations made to the peripheral T cell pool by continued production of RTEs, as well as RTE maturity in this model with age will give insight to the idea that continued T cell production and diversity in elderly patients would prevent health issues from infectious agents.

CHAPTER TWO: MATERIALS AND METHODS

Chapter 2: Materials and Methods

Mice

K5.CyclinD1 transgenic breeders were originally provided by C. Conti (MD Anderson Cancer Center, Science Park Research Division, Smithville, Texas) and maintained on the C57BL/6J background. RAG2p-GFP transgenic breeders were purchased from The Jackson Laboratory (Bar Harbor, Maine) on the FVB background and bred with the K5.CyclinD1 model. PCR was utilized to determine K5.CyclinD1 and Rag2p-GFP transgene expression, and visually confirmed upon dissection. All mice were maintained in pathogen-free conditions, and experiments were performed in accordance with all MD Anderson Institutional Animal Care and Use Committee policies.

H&E and Immunohistochemistry

Hematoxylin and eosin (H&E) staining was performed on frozen sections at 5um thickness. Staining was performed as follows: Slides were thawed to ambient temperature for 30 minutes, and fixed using 10% neutral buffered formaldyhyde. Slides were treated with hematoxylin (Fischer) for 3-10 minutes, and rinsed gently under tap water. Slides were then treated with 1% stock eosin (Sigma) for 3-10 minutes, and again rinsed gently under tap water. Slides were dehydrated through a quick series of ethanol dips as follows: 70%, 90%, 96% and 100%. Slides were rinsed in xylene (Fischer) twice for a total of ten minutes. Finally, slides were coverslipped with cytoseal (ThermoScientific).

Immunohistochemistry was performed using frozen sections as follows: Slides were allowed to thaw to ambient temperature for 30 minutes. Slides were fixed in room temperature acetone for 5 minutes, and washed in three 5-minute rinses of TNT (Perkin Elmer). Slides were blocked with TNB block provided in TSA kit (Perkin Elmer). Primary antibodies were added to slides for 1-8 hours at room temperature, and rinsed by three 5-minute rinses with TNT. Secondary antibody was diluted at 1ug/300ul TNB and applied for 30 minutes, and rinsed by 3 5-minute TNT rinses. Sections were treated with DAPI (Vector Labs) at a concentration of 1ul:1500ul ddH20 for three minutes, rinsed a final time for 5 minutes in TNT, treated with VectaShield (Vector labs)

mounting medium, and coverslipped. The following primary antibodies were used: K5 (Covance), K8 (Troma-1, gifted from N. Manley), K14 (Covance), UEA-1 (Vector). Secondary antibodies were donkey-anti-rat-Texas-Red (Jackson) and donkey-anti-rabbit-FITC (Jackson).

Preparation of Tissue for TEC FACS analysis

Thimi were dissected and prepared according to the protocol from Grey et al (67). Thymi were removed and washed in cold, sterile 1x PBS (Hyclone). Thymus lobes were trimmed of connective tissues and fat, separated, and the capsule nicked with scissors. Thymi were digested in 0.125% Collagenase (Sigma), 0.1% DNAse1 (Roche) for 30 minutes at 37°C in a bacterial shaker, and supernatant was removed and stored on ice and freshed collagenase readded every ten minutes. Tissue was then digested in 0.125% Collagenase/Dispase (Roche), 0.1% DNAse1 for an additional 10-20 minutes at 37°C in a bacterial shaker. Any remaining clumps of tissue were disrupted mechanically using a glass pipette. All cells from the same initial thymus were pooled in a 50ml conical tube (BD Falcon). Conical tubes were centrifuged at 1500rpm for 3 minutes to pellet cells, supernatant was removed and cells were resuspended in cold, sterile FACS Wash Buffer to a volume of 10ml. Cell preparations were counted using a trypan blue viability stain (Invitrogen) and a Countess® automated cell counter (Invitrogen). Cells were distributed at 3 million cells per FACs polystyrene tube (BD Falcon) in a volume of 300ul of PBS for antibody staining.

Preparation of Tissue for thymocyte FACS Analysis

Thymi and spleens were removed and washed in cold, sterile 1x PBS (Hyclone). Tissues were trimmed of connective tissues and fat, and pressed through a 70-micron mesh screen (Fisher) using the plunger end of a 1ml syringe (BD Falcon). Strainers and tissue were washed with PBS and collected in a 50ml conical tube (BD Falcon). Conical tubes were centrifuged at 1500rpm for 5 minutes to pellet cells, and resuspended in cold, sterile PBS to a volume of 10ml. Splenic samples were treated with red blood cell lysis buffer (0.002% Tris, 0.0086%NH_4Cl w/v) and washed with PBS. Red blood cell lysis buffer-treated samples were centrifuged again and resuspended in

10ml of cold, sterile PBS. Both thymic and splenic cell preparations were counted using a trypan blue viability stain (Invitrogen) and a Countess® automated cell counter (Invitrogen). Cells were distributed at 3 million cells per FACs polystyrene tube (BD Falcon) in a volume of 300ul of PBS for antibody staining.

Organ Weights and Cellularity

Thymi and spleens obtained for FACS analysis or from dissection were trimmed of connective tissues and fat, and rinsed in cold, sterile PBS. Weights were obtained in measured PBS, on an electronic scale (APX-602, Denver Instrument). Total body weight was taken before dissection, post mortem.

Antibodies used for FACS Analysis

Thymic and splenic cells were stained with the following primary antibodies: anti-CD4-Pacific Orange (Invitrogen), anti-CD8-PE/Cy7 (eBioscience), anti-CD24-PerCP/Cy5.5 (eBioscience), anti-CD25-Pacific Blue (BioLegend), anti-CD44-APC (eBioscience), anti-CD45-PerCP/Cy5.5 (eBioscience), anti-CD62L-Biotin (eBioscience) or anti-CD62L-Alexafluor 700 (eBioscience), anti-CD69-PE (BioLegend), anti-CD127-Biotin (eBioscience) or anti-CD127-APC efluor 780 (eBioscience), anti-IA/IE-Pacific Blue (Biolegend), anti-UEA1-FITC (Vector), anti-Ly51-Biotin (BD Pharmingen), anti-TER119-PE/Cy5 (eBioscience), anti-Qa-2-Biotin (BD Biosciences), anti-Mouse IgG2a,k-Biotin (BD Biosciences). Primary antibodies were added to 3 million cells in 300ul PBS for 15 minutes in the dark, and washed with cold PBS. Samples were centrifuged at 1500rpm for 3 minutes to form cell pellets, and resuspended in 300ul of PBS. Secondary antibody was added if necessary, and SA-Qdot 605 (Invitrogen) was used. Secondary incubation was performed for 15 minutes in the dark, and samples were centrifuged at 1500rpm for 3 minutes to pellet cells. All samples were brought to a volume of 500ul in PBS for FACS analysis using the AriaIIu® cell sorter (BD Biosciences). Analysis for data acquired from FACs was analyzed using the FlowJo (Version 9.4.10) program by Treestar (Ashland, Oregon).

Sorting and qRT-PCR

Cells prepared by TEC FACS method (See above) were sorted on a FACS AriaIIu® cell sorter (BD Biosciences) into $MHCII^{+}CD45^{-}$ or $MHCII^{-}CD45^{+}$ separate populations directly in to TriZol (Invitrogen) RNA reagent, and stored at -80°C. All samples were prepared and qRT-PCR performed by the Molecular Biology Core at MDACC Science Park, Smithville, Texas.

CHAPTER THREE: RESULTS

Chapter 3: Results

Thymus Phenotype in K5.Cyclin D1 mice on a C57Bl/6J background

Although the molecular mechanisms that regulate thymus size and homeostasis are not well defined, it is clear that genetic background is influential (68). Robles et al. reported that the K5.Cyclin D1 transgene produced a severe hyperplastic thymic phenotype that resulted in respiratory distress by twenty weeks of age in mice on a FVB background (65). Since most immunological research is performed in a C57Bl/6J background, we backcrossed K5.Cyclin D1 transgenic mice onto a C57Bl/6J background (F>7 generations). Our initial goal was to determine if the thymus phenotype observed in the original report describing K5.CyclinD1 mice was preserved after crossing the transgene onto a C57Bl/6J background. As in the original report, the K5.CyclinD1 transgenic mice developed hyperplastic thymi that failed to undergo involution with age in contrast to their nontransgenic littermates (Figure 3). We also noted that similar to earlier reports (12, 66), there was only a modest increase in the number of spleen cells in transgenic mice compared to control mice.

In order to analyze thymic architecture in transgenic and control mice, H&E stained frozen sections (Figure 4 A,B) were compared. The medulla and cortex were well defined in the K5.CyclinD1 thymus, with proper delineation between the two compartments. Because H&E stained thymic sections mainly reveal the thymocyte organization within the thymus, immunohistochemistry was performed to analyze stromal organization and maturation. Epithelial cell differentiation is characterized by expression of various keratin (K) markers. In the thymus K5 and K8 were used to distinguish cTEC and mTEC subsets, respectively. We found that 4 week old K5.CyclinD1 transgenic and littermate control thymi contained comparable $K5^{-}K8^{+}$ cTEC and $K5^{+}K8^{-}$ mTEC subsets (Figure 4 C,D). Furthermore, normal expression of the additional cTEC markers CD205 and Ly51 was observed (data not shown). We also analyzed serial sections for expression of K14, a heterodimer binding partner of K5, and binding of the lectin *Ulex europeaus* agglutinin-1 (UEA-1). These two markers identify

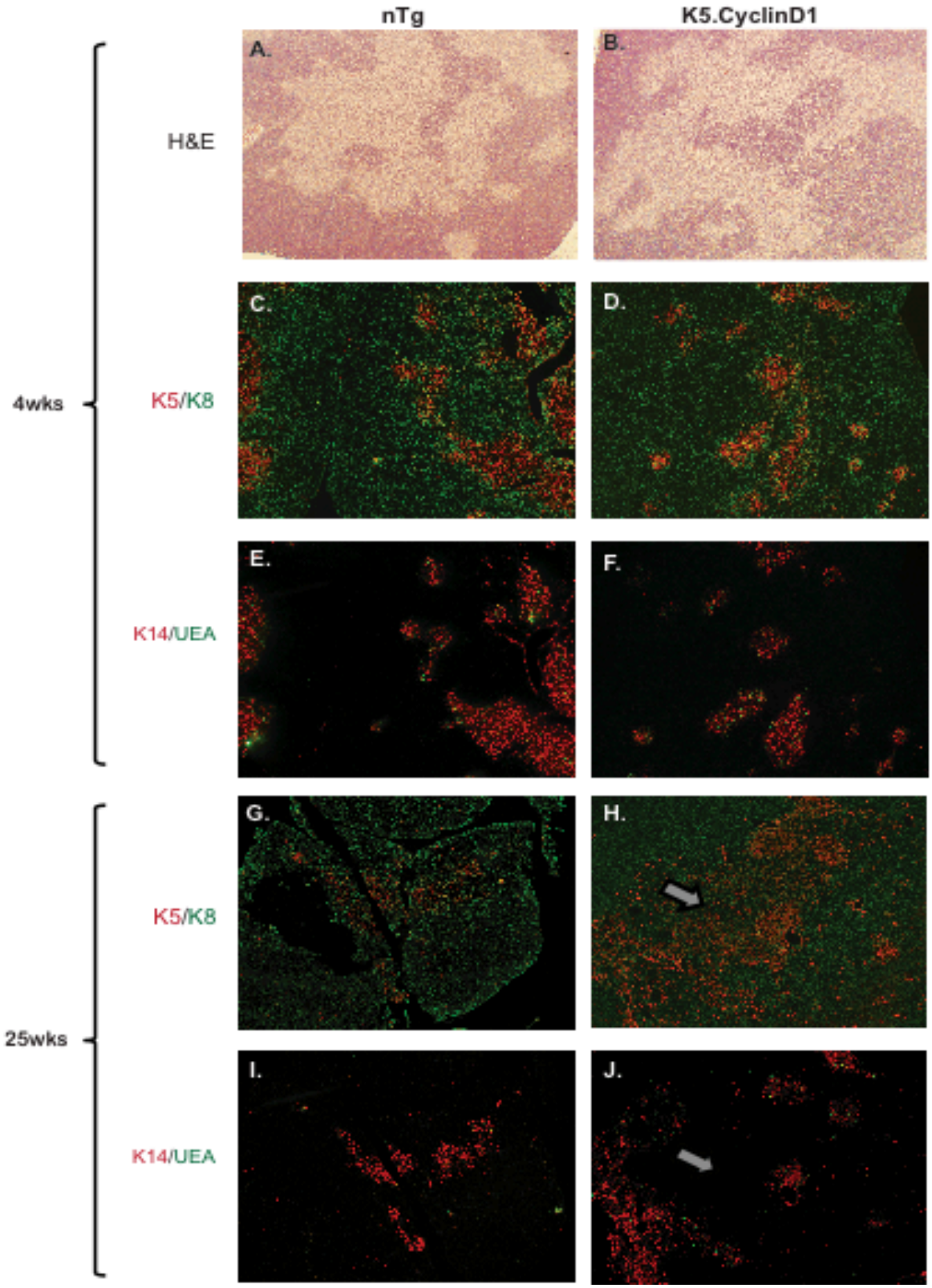
nTg
K5.CyclinD1
A.
B.
H&E
C.
D.
4wks
K5/K8
E.
F.
K14/UEA
G.
H.
K5/K8
25wks
I.
J.
K14/UEA

Figure 4. Thymic architecture is intact in the K5.CyclinD1 model.
H&E staining of 4 week frozen thymus sections shows well defined cortex and medulla in nontransgeneic (A) and K5.CyclinD1 (B) thymi. (C-D) IHC staining of 4 week frozen sections; K5 (red); K8 (green). Serial slides stained for K14 (red); UEA-1 (green). Distinct mTEC ($K8^{-}K5^{+}$) and cTEC ($K8^{+}K5^{-}$) subsets in control (C) and K5.D1 (D) thymi. IHC staining of 25 week frozen sections (G-J), with arrows showing expansion of $K5^{+}K8^{+}$ TECs in K5.D1 thymus. (G) K14 (red) and UEA-1 (green) mTEC subsets.

distinct mTEC subsets (Figure 4 E,F). As in nontransgenic mice, the medullary regions of K5.CyclinD1 transgenic mice contained a major subset of $K5^{+}K14^{+}$ TECs. At 25 weeks of age, however, a distinct subset that did not express K5 or K14, but that binds UEA-1 was observed. These data suggest that K5.CyclinD1 transgenic thymi develop TEC subsets that have typical differentiation markers and are properly localized in cortical and medullary regions. However, as previously reported (12), I noted an expanded $K8^{+}K5^{+}$ subset at the CMJ in K5.CyclinD1 thymi that did not co-express K14 at 25 weeks of age (note yellow 'halo' staining in Figure 4H). We and others have suggested that TEC progenitors reside in this subset (66, 69). These findings imply that the thymus hyperplasia may be due to expansion and/or maintenance of TEC precursors that generate microenvironmental niches that, in turn, support thymocyte development.

Expression of Cyclin D1 is driven by the epithelial-specific K5 promoter resulting in expression of Cyclin D1 in TECs and a profound thymic phenotype. However, the effects of this overexpression on development and maintenance of the TEC compartment have been largely unstudied. To analyze TEC composition, single cell suspensions of enzymatically digested 4 week transgenic and nontransgenic thymi were analyzed by FACS to determine the proportion of TECs to thymocytes. While the thymus is comprised mostly of thymocytes, K5.CyclinD1 transgenic mice exhibited an increase in the percentage of MHC-expressing TECs compared to nontransgenic littermates (Figure 5 A-C). In addition, the absolute number of cells in the TEC population of K5.CyclinD1 thymus was increased compared to nontransgenic littermates (Figure 5D). Thus, the overexpression of CyclinD1 in the epithelial compartment results in an expansion in the overall number of cells in the TEC compartment providing an expanded microenvironment capable of supporting thymocyte development.

TECs and thymocytes participate in an active crosstalk required for development and maintenance of both cell types. In order to determine whether expression of the K5.CyclinD1 transgene altered thymocyte development, we analyzed the frequency of the major thymocyte subsets defined by CD4 and CD8

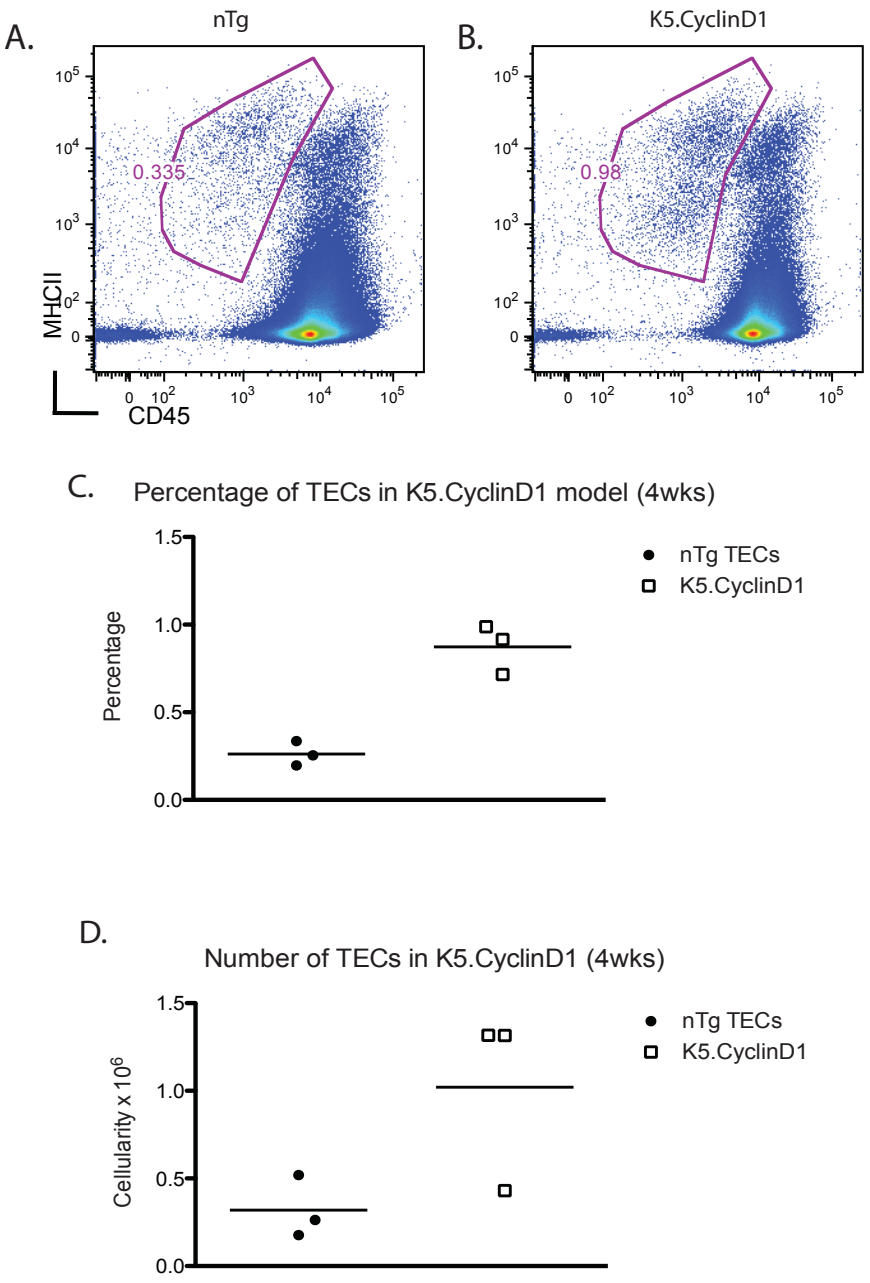

Figure 5. Increased percentage and number of TECs in 4 week old K5.D1 thymi. (A,B) Representative FACS plots from (A) control (n=3 mice) and (B) K5.D1 (n=3 thymi). (C) The percent of TECs is higher in K5.D1 compared to controls. D. The absolute cell number of TECs is higher in K5.D1 compared to controls.

expression. As shown in Figure 6, similar percentages of the major thymocyte subsets were found in transgenic mice and nontransgenic littermates (Figure 6 A-C). However, the absolute number of cells in each thymocyte subset was much greater in the K5.CyclinD1 thymi compared to nontransgenic littermates (Figure 6D). The increased cellularity in the DN thymocyte progenitor subset supports the notion that the expanded epithelial compartment in K5.CyclinD1 transgenic mice supports a greater number of developing thymocytes through the expansion of available microenvironmental niches for thymocyte precursors.

Although the specificity of the K5 promoter predicts restricted expression of the transgene to the epithelial compartment, we wanted to verify the fidelity of our model and confirm that expression was restricted to TECs and absent in hematopoietic cells. To test this, single cell suspensions from enzyme-dissociated 6 week transgenic and nontransgenic thymi were sorted into TEC ($CD45^-$MHC class II^+) and thymocyte ($CD45^+$MHC class II^-) populations. RNA was recovered from sorted cells, and qRT-PCR was performed to analyze the expression levels of transgenic and endogenous Cyclin D1 (Figure 7). The data obtained confirmed that K5.CyclinD1 expression is restricted to the TEC compartment in transgenic mice, and is not expressed in the $CD45^+$ thymocyte compartment. Furthermore, endogenous mouse Cyclin D1 expression is not affected by the expression of the K5.CyclinD1 transgene.

Analysis of naïve and memory T cell distribution in CD4+ spleen cells

Although thymocyte cellularity is increased by ~3 fold in young (4-6 week) K5.CyclinD1 mice, the number of splenic cells was not substantially increased (Figure 3). The cells in the spleen consist of B cells, T cells and myeloid cells. With respect to T cells, the spleen houses CD4+ helper and CD8+ cytotoxic T cells, and each type is comprised of a naïve and memory/activated subset. Previous analyses of the spleen did not include an examination of splenic populations, nor were the naïve and memory/activated subsets analyzed. We hypothesized that an increase in thymopoiesis could result in an increase in naïve peripheral T cells that would not be reflected by looking at peripheral T cell

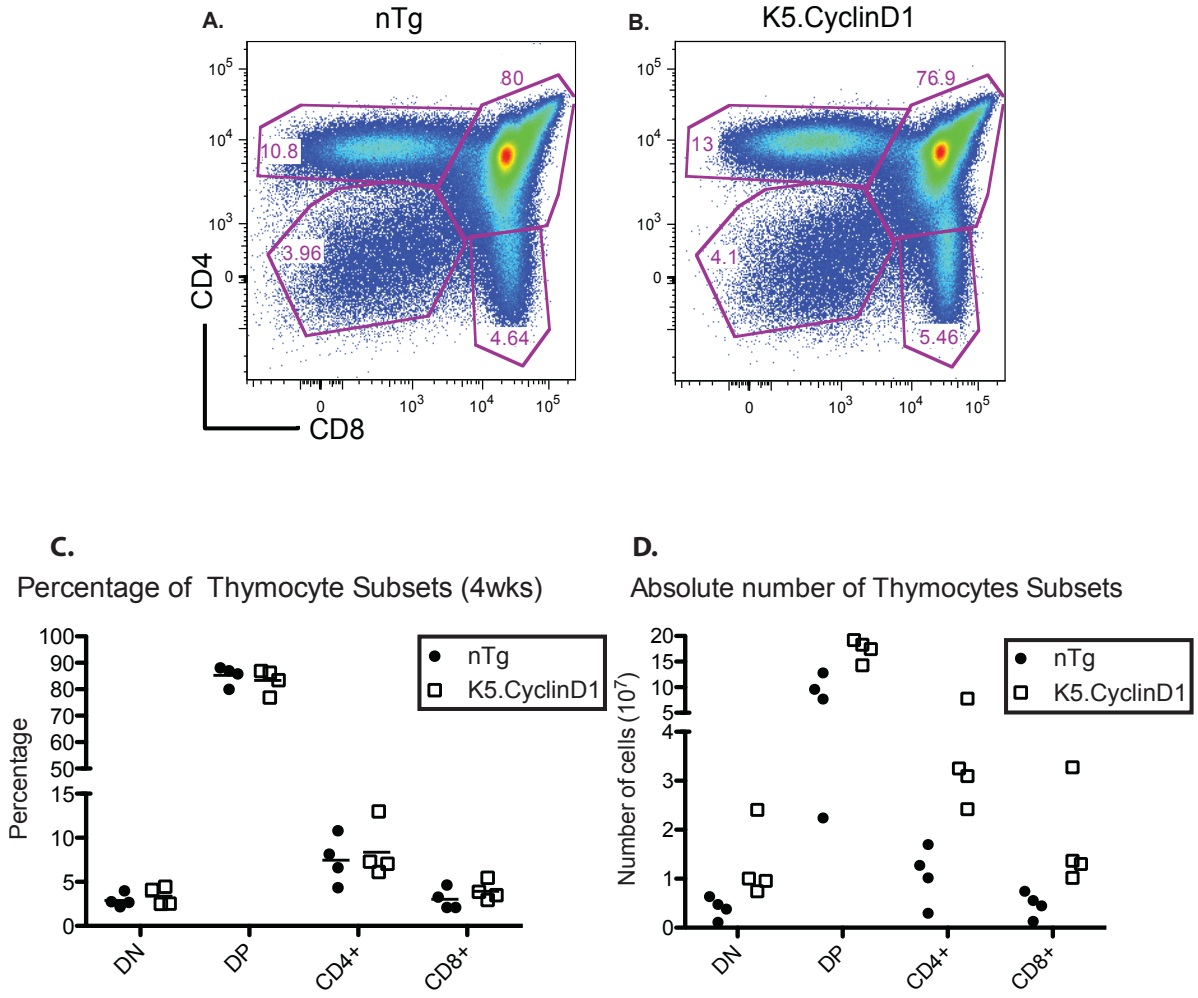

Figure 6. Percent and number of thymocyte subsets in 4 week old K5.D1 thymi. (A,B) Representative FACS blots of thymocyte subsets in nontransgenic (n=4 mice) and K5.D1 (n=4 mice). C. Similar percentages of thymocyte subsets in K5.D1 and control mice. D. The absolute number of thymocytes in each subset is increased in K5.D1 mice.

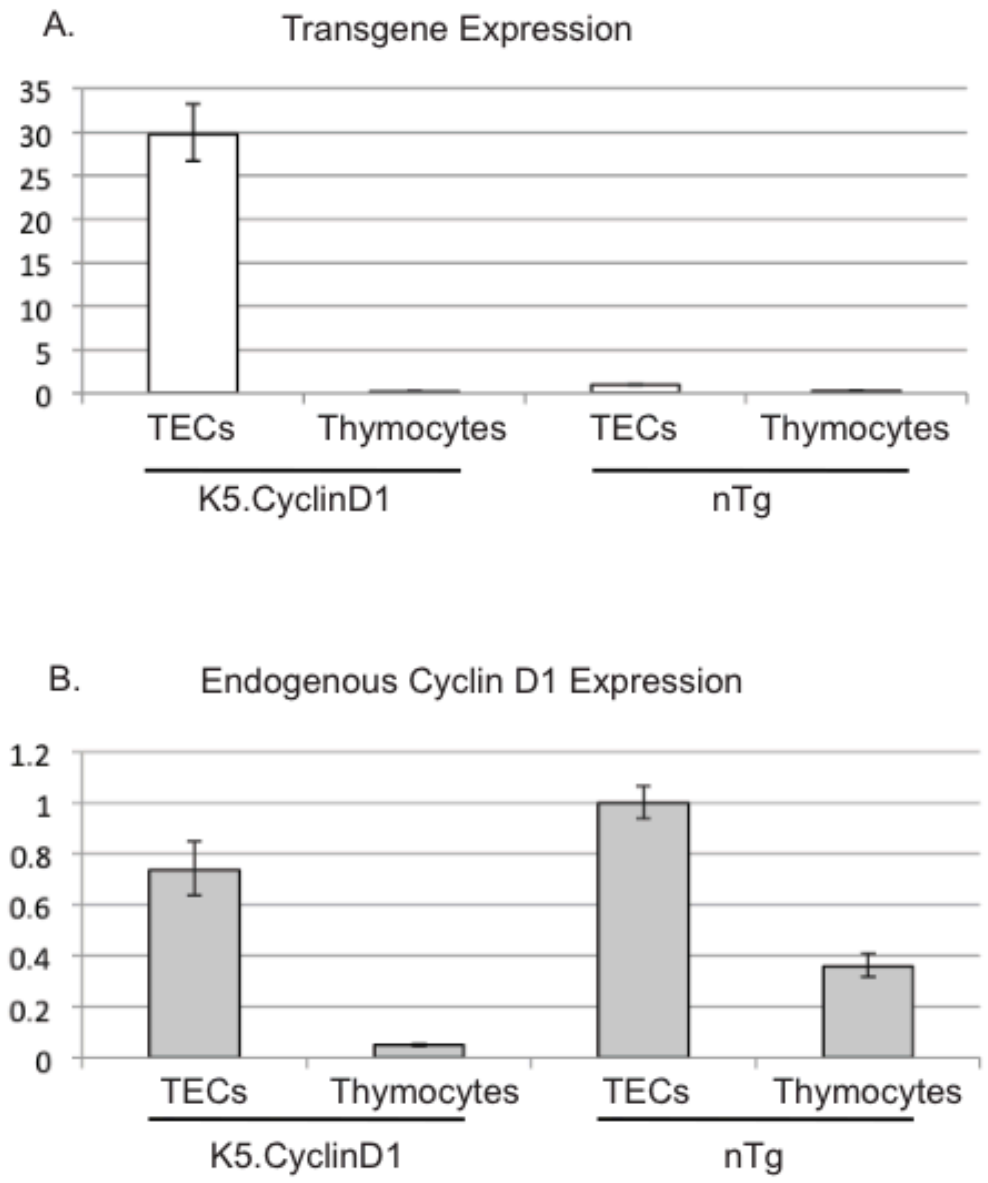

Figure 7. K5.D1 transgene expression is restricted to K5.D1 TECs.
qRT-PCR analysis of K5.D1 (A) and endogenous (B) Cyclin D1 expression in FACS isolated TECs and thymocytes. The data were normalized to α-tubulin. Data show average values for replicates. The experiment was repeated three times with similar results.

compartment as a whole. CD44 and CD62L are generally used to identify naïve and memory T cell subsets in the peripheral pool (26, 70). Naïve T cells exhibit a $CD44^{low}CD62L^{high}$ phenotype, which allows them entry to the secondary lymphoid organs for interaction with peptide-bearing antigen presenting cells. Memory T cells, on the other hand, express a $CD44^{high}CD62L^{-}$ phenotype that permits continual circulation through the periphery. Using these markers, we analyzed the frequency of naïve to memory CD4 T cells in the spleens of 4 week old K5.CyclinD1 and control littermate mice. As shown in Figure 8, we found an increase in the percentages of both CD4 and CD8 T cells in the spleen of K5.D1 mice compared to controls (Figure 8 A,B). After drawing an electronic gate on the CD4 population, we observed a slight increase in the percentage of naïve peripheral CD4 T cells in transgenic spleens compared to controls (Figure 8 C-E). However, there was no difference in the absolute number of naïve T cells at this age (Figure 8F). These data are limited, however, and do not show how the contribution of RTEs from the K5.D1 thymus may be affecting the naïve T cell pool, nor does it determine the frequency of these cells within the population.

Identification of RTEs using the RAG2p-GFP transgenic model system

CD4 T cells that are classified as naïve based on a $CD44^{low}$ and $CD62L^{high}$ phenotype consist of RTEs as well as MN T cells. To determine if the increased thymopoiesis in the K5.CyclinD1 thymus alters the distribution of RTEs and MN T cells, we crossed the K5.CyclinD1 mice with RAG2p-GFP transgenic mice. Prior to analyzing RTEs in peripheral T cells, we determined if expression of the RAG2p-GFP transgene altered the thymus or splenic phenotype typical of K5.CyclinD1 mice. For the purpose of clarity, mice expressing both the K5.Cyclin D1 and RAG2p-GFP transgenes will be referred to as K5.D1 and mice expressing only the RAG2p-GFP transgene will be referred to as control for the remainder of this thesis. One factor to consider was that the RAG2p-GFP mice had been maintained on an FVB background, which could impact thymus or spleen cellularity. Expression of the K5.CyclinD1 transgene still resulted in severe thymic hyperplasia at 4 weeks when crossed into the RAG2p-GFP mouse

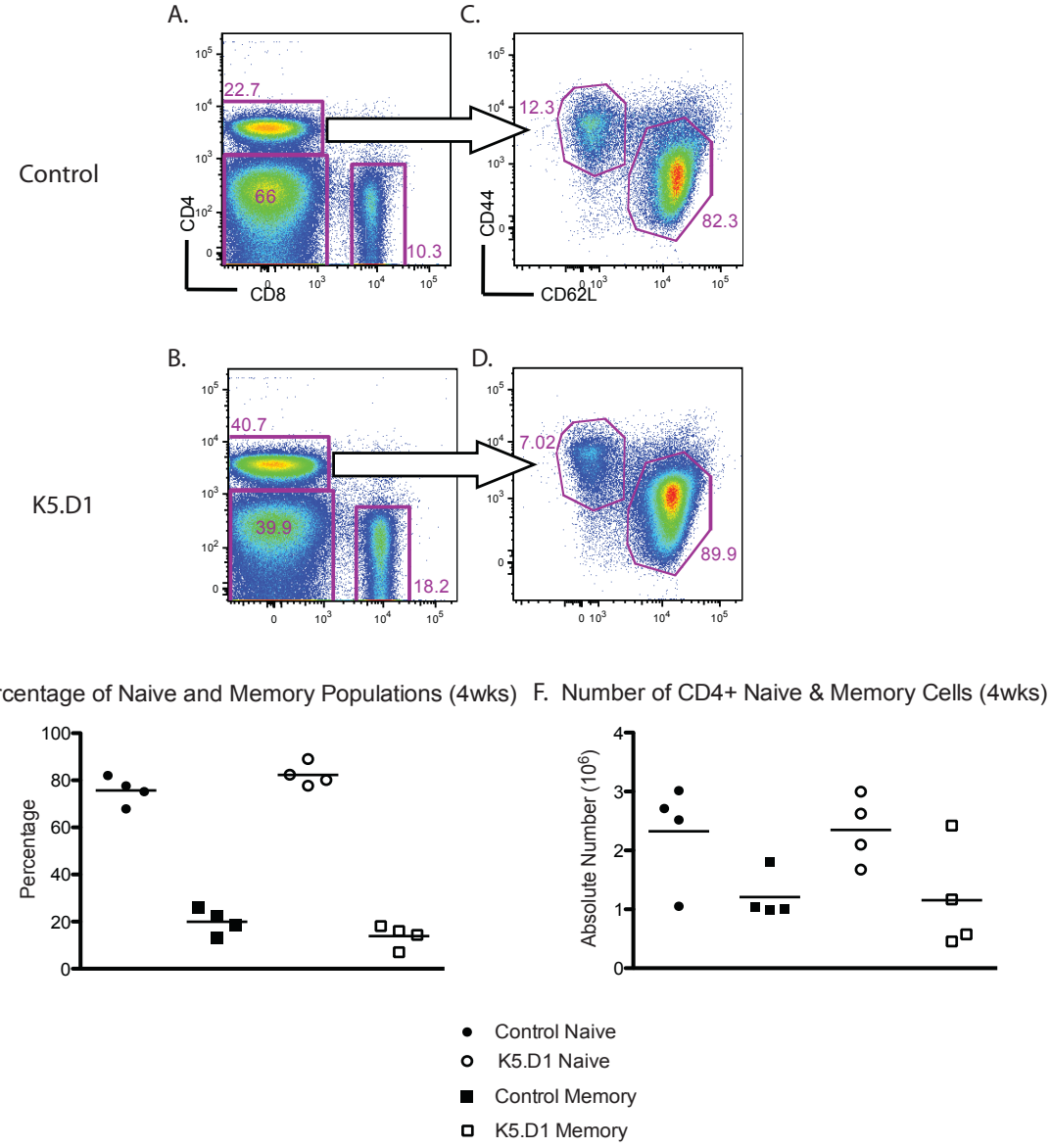

Figure 8. Percent and number of naïve and memory CD4 T cells in 4 week K5.D1 and control spleens. (A,B) FACS plots of CD4 and CD8 expression (C,D) Naïve ($CD44^{low}CD62L^{high}$) and memory ($CD44^{high}CD62L^{low}$) subsets in CD4+ T cells. (E) Percent of naïve and memory T cells. (F) Absolute number of naive and memory T cells.

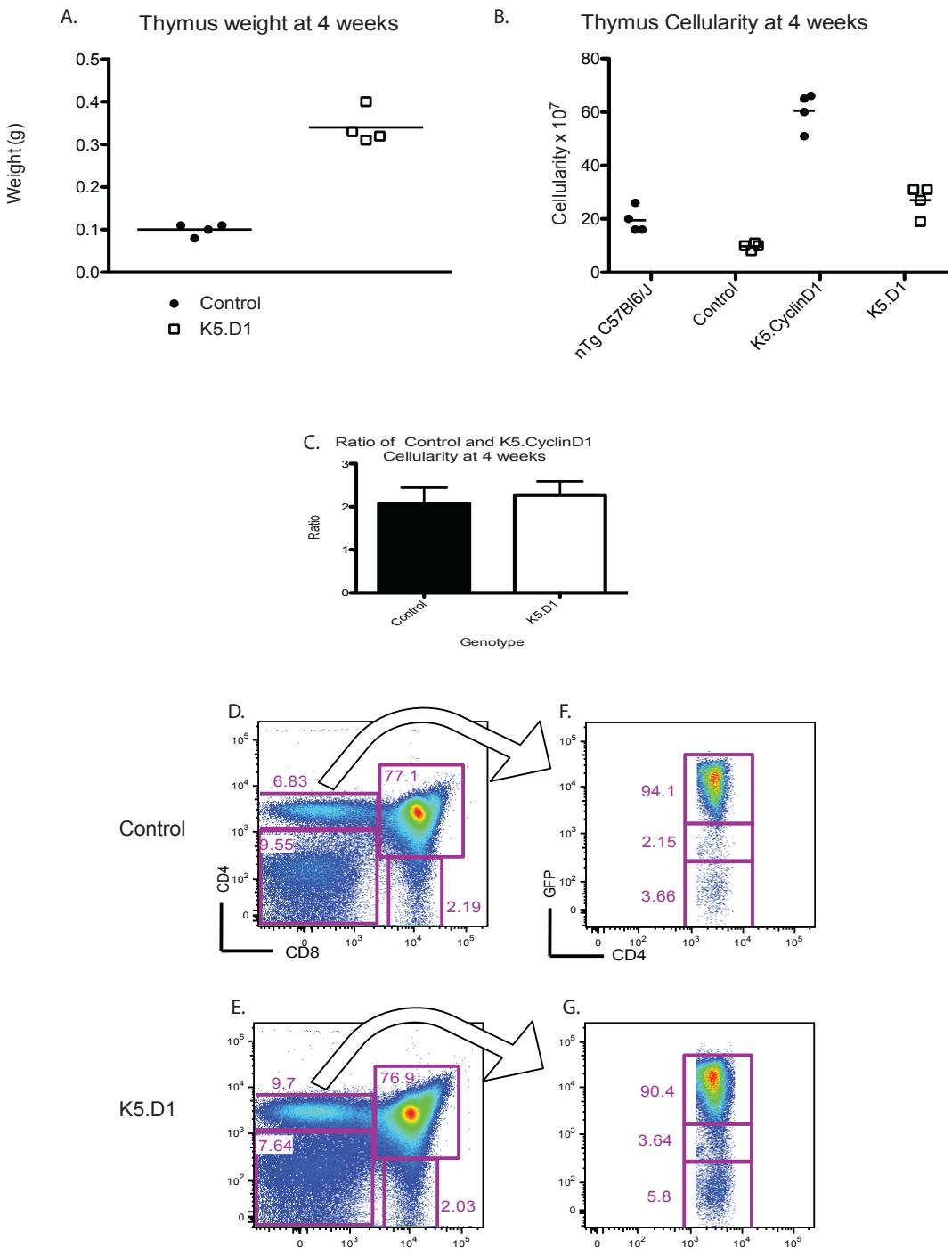

Figure 9. The RAG2p-GFP transgene does not alter the K5.D1 phenotype. K5.CyclinD1 mice were crossed with RAG2p-GFP mice Thymus weight (A) and cellularity (B). (C) Ratio of change in cellularity between controls (black bar) and K5.D1 (white bar) on different backgrounds. (D,E). Representative FACS plots of thymocyte subsets in control (RAG2p-GFP) and K5.D1 (K5.CyclinD1; RAG2p-GFP double transgenic) mice. An electronic gate was set on CD4 SP thymocytes for analysis of GFP expression.

model (Figure 9A,B), suggesting that the K5.CyclinD1 phenotype was unaffected by the RAG2p-GFP transgene. However, when we compared the cellularity of our K5.D1 mice on a mixed background to the K5.CyclinD1 transgenic mice on the C57Bl/6J background, we did find a significant reduction in the cellularity for both control and K5.D1 thymi at 4 weeks of age (Figure 9B). Nevertheless, the relative increase in thymus cellularity caused by the K5.CyclinD1 transgene was not affected by the RAG2p-GFP transgene (Figure 9C). Furthermore, expression of the RAG2p-GFP transgene did not alter the percentages of thymocyte subsets defined by CD4 and CD8 in either K5.Cyclin D1 mice or their littermate controls (Figure 9D,E). This result is consistent with earlier work from the Fink lab showing that RAG2p-GFP transgene expression does not alter proliferation or development of thymocytes (40). Greater than 90% of CD4 SP thymocytes in RAG2p-GFP mice express high levels of GFP (51). We found a similar percentage of GFP^{high} CD4 SP thymocytes in K5.D1 compared to controls (Figure 9F,G). Taken together, these data demonstrate co-expression of RAG2p-GFP and K5.CyclinD1 transgenes did not alter the phenotypes previously described in the single transgenic lines.

Effect of K5.Cyclin D1 transgene on frequency and number of RTEs in peripheral CD4 T cells

Thymus export of naïve T cells is required for diversity of the peripheral naïve T cell pool. Although this process continues throughout life, the number of RTEs in the peripheral T cell pool is greatly reduced with age as a result of thymus involution. RTEs and MN T cells make up the naïve T cell population defined by $CD44^{low}CD62L^{high}$ expression. Because the K5.CyclinD1 transgene results in a hyperplastic thymus, we hypothesized that incorporation of RTEs may be enhanced in our mice. On the other hand, Hale et al reported that RTEs are substantially reduced with age, as the thymus undergoes involution (51). We chose to investigate the composition of the naïve T cell population through the use of the RAG2p-GFP transgene, which labels RTEs for tracking in the periphery. The total naïve CD4 T cell subset was defined by gating on $CD44^{low}CD62L^{high}$ cells and subsequently analyzed for GFP expression (Figure 10A-D).

We found a striking increase in the percentage of GFP^{high} and GFP^{low} cells at the expense of the GFP^{neg} population in young (4 week) K5.D1 mice compared control littermates (Figure 10B,D). These data demonstrate an increase in RTEs being incorporated into the peripheral T cell population (Figure 10B) The increased frequency of RTEs in K5.D1 spleen cells implies that increased thymopoiesis and RTE output leads to greater incorporation to the naïve T cell pool. However, we did not observe a consistent increase in the number of RTEs in K5.D1 mice. This suggests that while RTEs may be present in the peripheral population, they may not be capable of surviving when in direct competition with MN T cells. Similar results were found for CD8 T cells (data not shown). These results suggest that peripheral homeostasis is maintained at four weeks of age in the K5.D1 mice. We also determined the ratio of CD4 to CD8 T cells in RTEs. There was no difference in the CD4/CD8 RTE ratio between K5.D1 and control T cells (Figure 10F).

RTE maturation in young K5.CyclinD1 and control mice

One of the unique benefits of the GFP labeling system used in this study is the ability to distinguish the length of time RTEs have spent outside the thymus. RTEs have been shown to undergo a maturational program that can be monitored by increases and loss of certain cell surface markers during the first few weeks in the periphery. Therefore, the RAG2p-GFP transgene allows us to utilize not only the expression of GFP as a molecular timer for RTEs, but also to assess their maturation status in the peripheral T cell pool by expression of a panel of cell surface molecules (Figure 11). For our purposes, we used the following markers to track RTE maturity: CD24, CD69, IL-7Rα (CD127) and Qa-2. The expression of cell surface markers CD24 and CD69 are lost as RTEs undergo maturation in the periphery. CD127 and Qa-2 expression are upregulated during RTE maturation. Expression of these markers on RTEs and MN T cells from K5.D1 and control spleens was analyzed by flow cytometry. First, we set an electronic gate

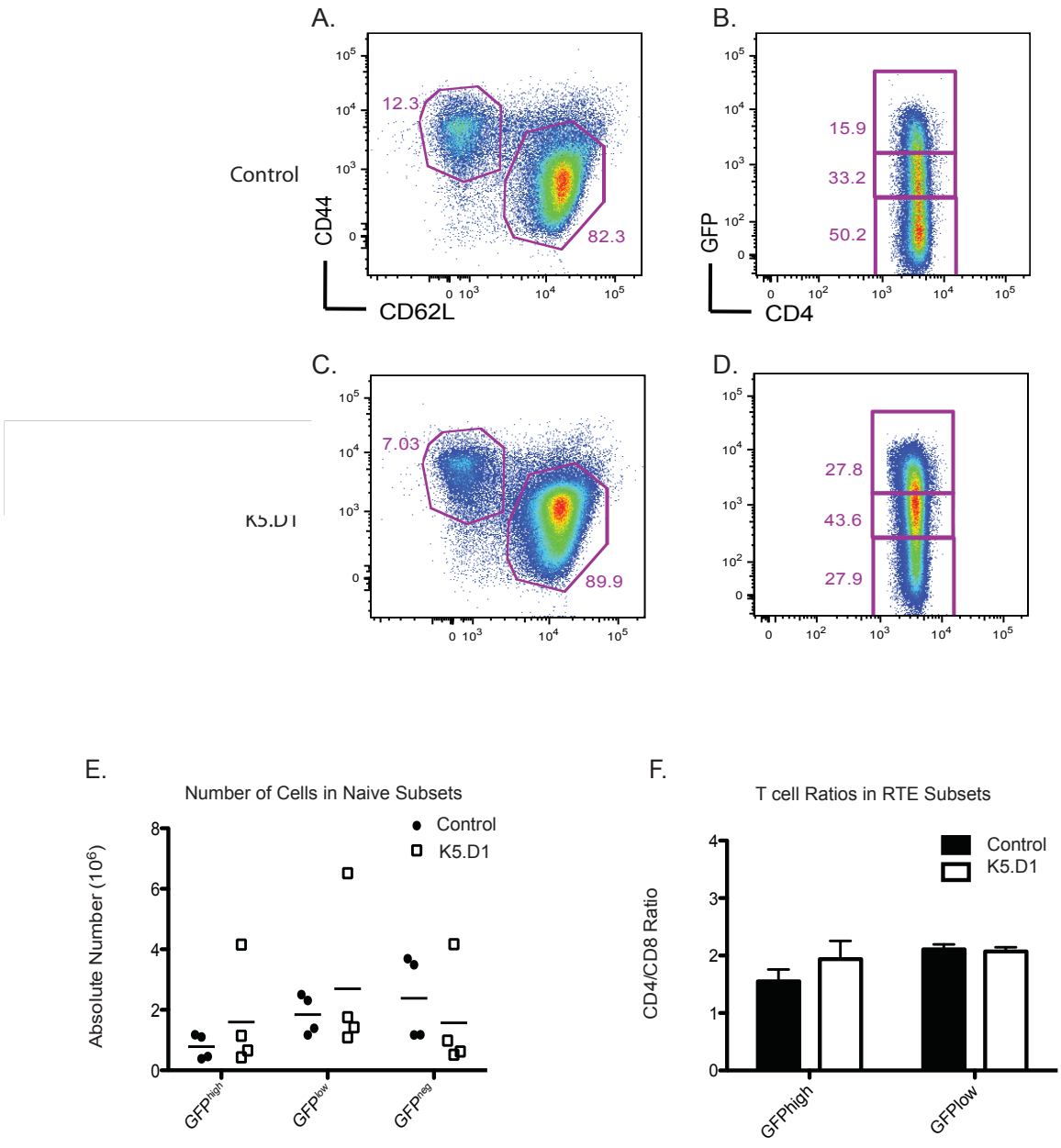

Figure 10 - The distribution of RTEs is altered in 4 week K5.D1 mice. CD4+ T cells were gated from splenocyte samples, gated to isolated CD44lowCD62Lhigh naive T cells, and then distributed by GFP intensity (A&B). K5.D1 naive CD4+ T cells consist of a higher percentage of both GFPhigh and GFPlow RTEs, compared to the control. However, this altered distribution of GFP+ cells is not seen in the absolute number of these subsets (C, N=3/group).

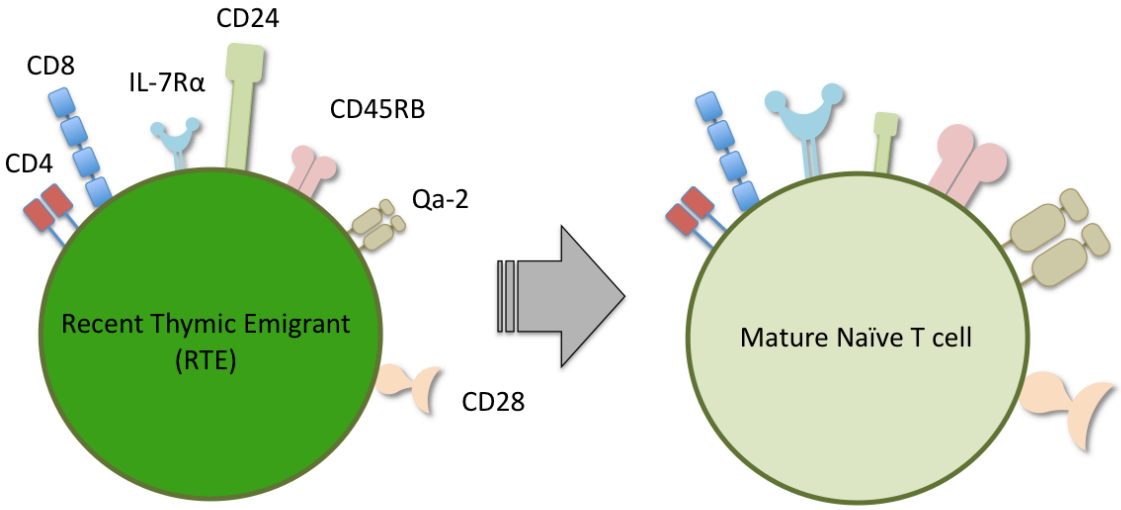

Figure 11. Changes in selected cell surface molecules during RTE maturation. RTE maturation can be analyzed by the upregulation of IL-7Rα (CD127), CD45RB and Qa-2, as well as the loss of CD24 expression.

on the naïve $CD44^{low}CD62L^{high}$ subset of CD4+ T cells. This population was further divided into GFP^{high} RTEs, GFP^{low} RTEs and GFP^{neg} MN T cells. Each of these subsets was analyzed for expression of a particular maturation marker. The resulting histograms were overlaid for comparison of each marker in RTEs and MN T cells from K5.D1 and control mice. At 4 weeks of age, the K5.D1 RTEs exhibited changes similar to control RTEs in the expression of CD69, CD127 and Qa-2 (Figure 12 A-P). In contrast, CD24 expression, which is necessary for homeostatic proliferation (42), was downregulated to a greater extent on GFP^{high} RTEs from K5.D1 compared to controls (Figure 12A-D).

Comparison of thymus and splenic cellularity in K5.D1 and control mice as a function of age.

A restriction of the T cell repertoire and an increase of memory T cells occur at the expense of the naïve T cell population in aged mice and humans (71). In order to determine if preventing thymic involution would impact peripheral homeostasis with age, K5.D1 mice and control littermates were maintained up to 20 weeks of age. While 20 week mice are in no way old with respect to the normal mouse lifespan, involution is well underway at this age. Increased thymic weights were observed in K5.D1 mice and continued to increase with age (Figure 13A). At all time points, the K5.D1 mice exhibited a significantly higher thymic cellularity than littermate controls (Figure 13B). Our observations on thymic weights and cellularity agree with a previous report on studies conducted in mice on an FVB background (65).

Splenic size and cellularity gradually increased with age in K5.D1 mice (Figure 13C,D), but these modest changes were not significant when compared to control littermates and did not correlate to the relative increase in thymic size we observed in K5.D1 compared to control mice. This result is particularly important to note, as many groups have suggested that a peripheral homeostatic mechanism tightly regulates the absolute number of the peripheral T cell

Figure 12:
4wk CD4 markers

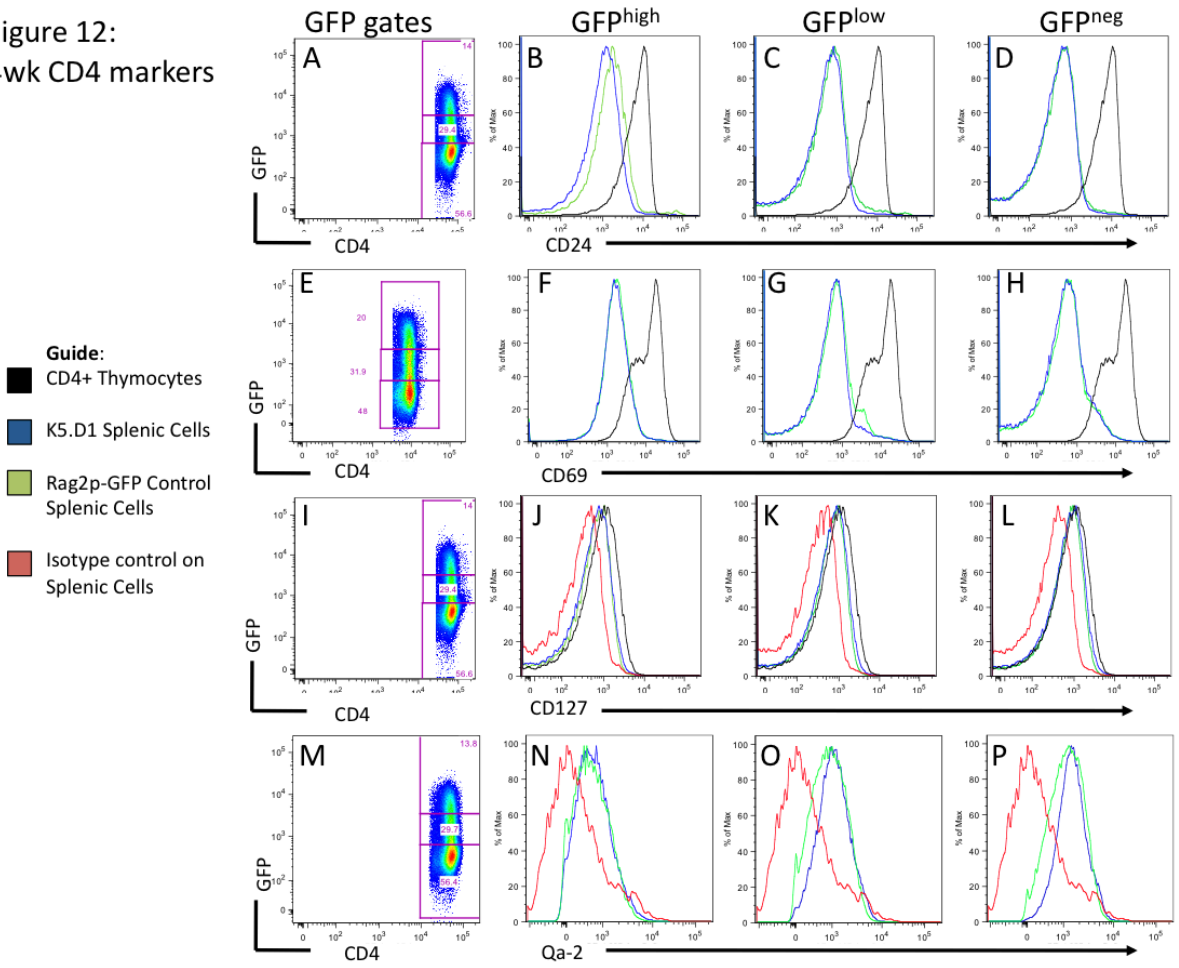

Figure 12. Expression of selected maturation markers on GFP^{high}, GFP^{low} and GFP^{neg} CD4+ naïve T cells from 4 week old control and K5.D1 mice. Electronic gates were set on CD4^{+} CD44^{low} CD62L^{high} naïve T cells. These cells were analyzed for GFP expression level. Representative histograms show expression of CD24 (B-D), CD69 (F-H), CD127 (J-L) and Qa-2 (N-P) by GFP^{high}, GFP^{low} and GFP^{neg} naïve T cells from K5.D1 (blue) and control (green) mice. CD4+ thymocytes (black) were used as positive controls for CD24, CD69 and CD127 histograms, and isotype controls (red) were utilized for CD127 and Qa-2 histograms (n=4 mice/group).

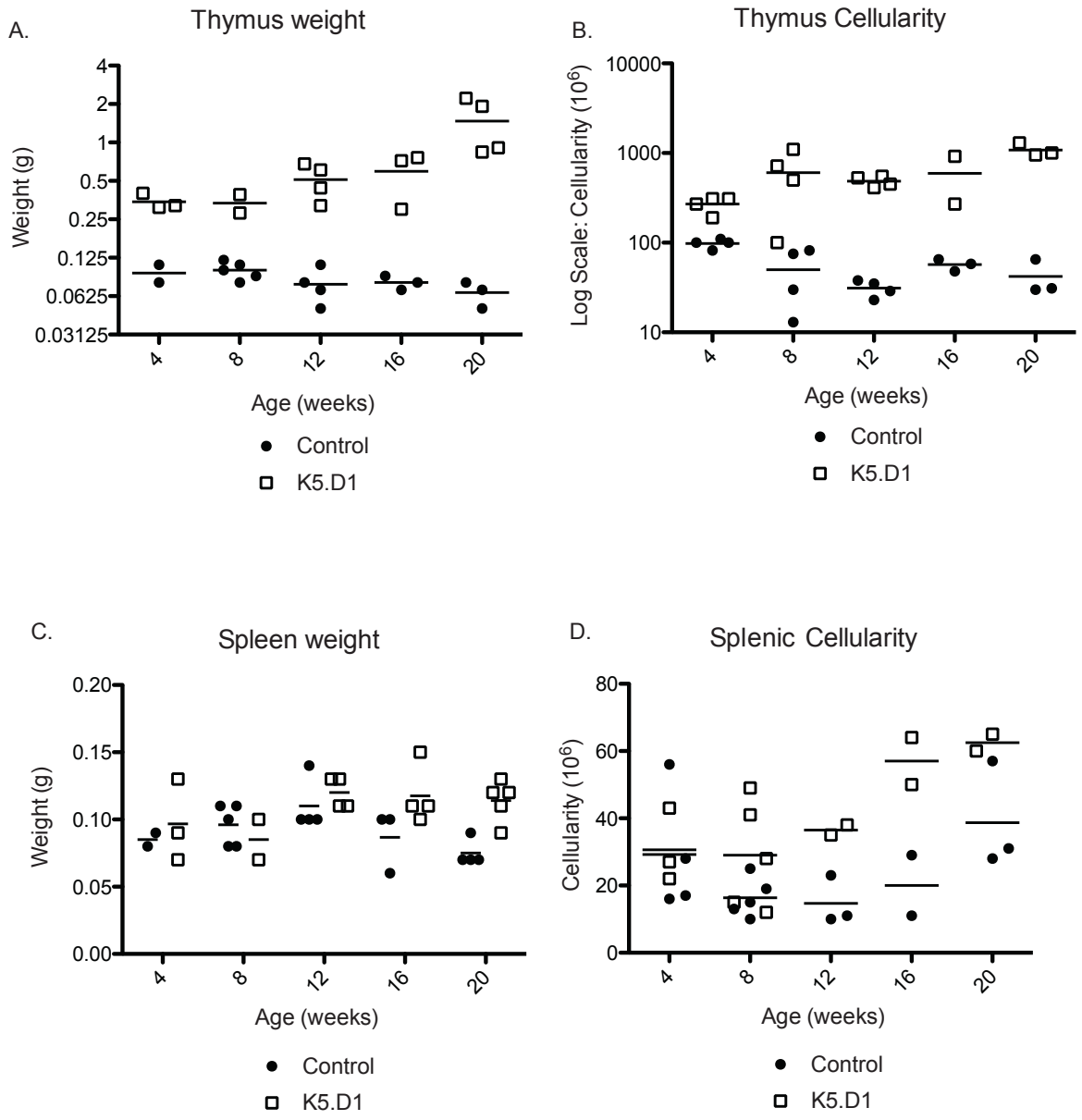

Figure 13 – Weight and cellularity of thymus and spleen with age in K5.D1 and control mice. (A) Thymus weight increases in the K5.D1 mice, as does thymic cellularity (B). (C) Spleen weight increases modestly. (D) Splenic cellularity increases with age (n=2+ mice/group).

population (27, 72). Furthermore, as RTE output declines with age, clonal expansion of existing memory T cells occurs to maintain the number of peripheral T cells. Our thymus and spleen cellularity data taken together suggests that homeostasis is generally maintained in K5.D1 transgenic mice.

Analysis of RTEs in aged K5.CyclinD1 and control mice

Our studies aimed to determine if a lack of involution directly affects the distribution of peripheral T cell populations. Therefore, we investigated post-involution effects on the peripheral T cell population in K5.D1 mice and control littermates. At 20 weeks of age, the K5.D1 transgenic mice displayed an altered distribution that favored naïve CD4 T cells in the spleen (Figure 14A,B). Investigation of the splenic CD8 T cell population showed a similar increase of the naïve T cell population (data not shown). The absolute numbers of memory and naïve T cells showed that there is no change between the number of cells in the memory populations of control and K5.D1 mice; rather, K5.D1 mice exhibit a stark increase in the number of naïve T cells present in the spleen (Figure 14-C,D). This result suggests that the change in distribution of naïve and memory T cells results from an expansion of the naïve population, and is not due to a constriction of the size of the memory population.

We then analyzed the distribution of RTEs and MN T cells in naïve CD4 T cells. In the control mice, the frequency and number of GFP^{high} and GFP^{low} RTEs in the naïve T cell population decreased with age whereas there was a corresponding increase in frequency and number of MN T cells. These data are consistent with previous reports showing that the percentage and number of RTEs declines with age (51). The Fink group has shown that RTEs are still readily detectable in mice up to two years of age, though the percentage of RTEs in the splenic T cell population is significantly reduced (51).

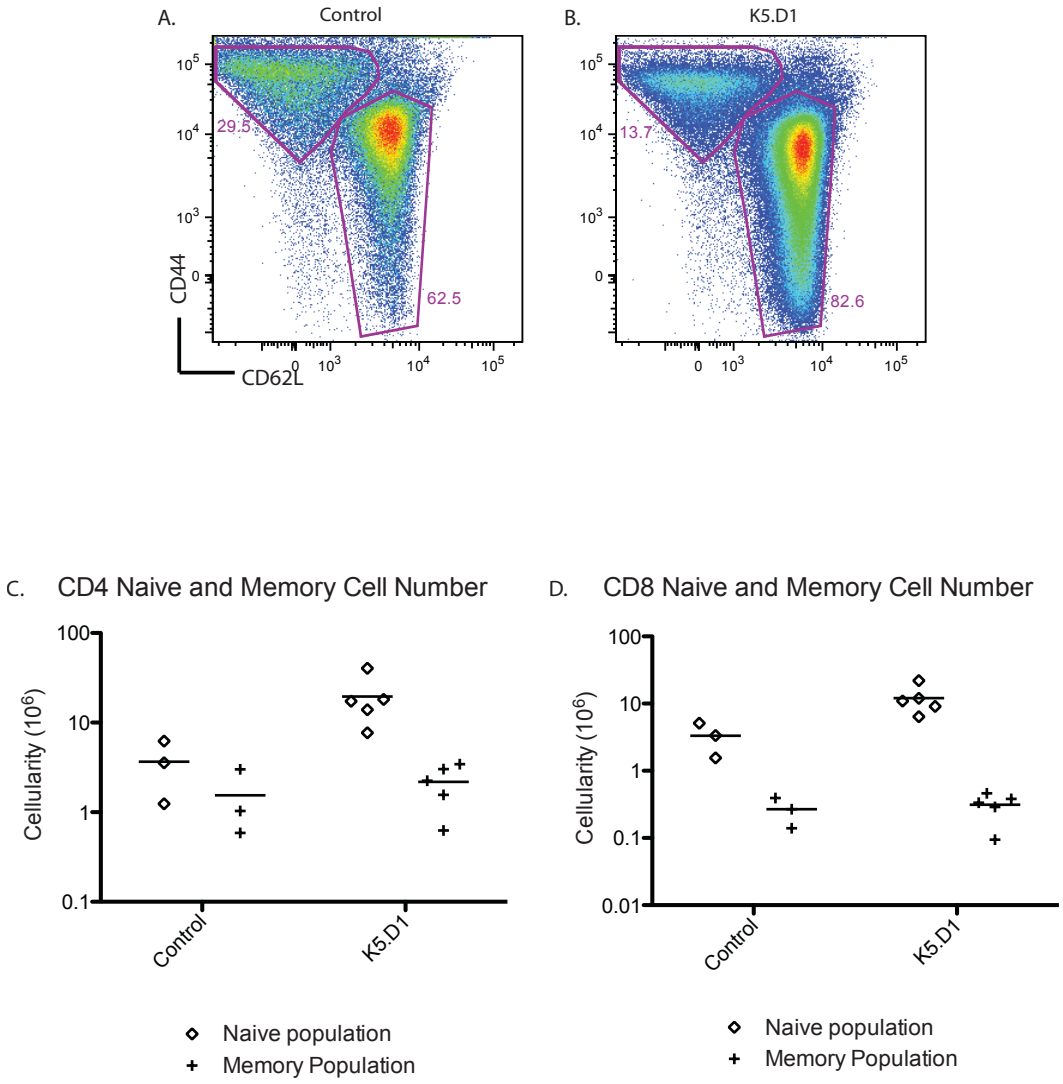

Figure 14. Percent and number of naïve and memory T cells in 20 week K5.D1 and control spleens. (A,B) FACS plots of CD4 T cell memory ($CD44^{high}CD62L^{low}$) and naïve ($CD44^{low}CD62L^{high}$) subsets. (C,D) Absolute number of naïve and memory CD4 T cells.

Therefore, our control data agrees with previous findings.

When we similarly analyzed the K5.D1 mice, we found a notably distinct pattern of age-related changes in the frequency and number of RTEs. There was a higher percentage of RTEs and a correspondingly lower percentage of MN T cells in the peripheral T cell pool of K5.D1 compared to control mice at all ages analyzed (Figure 15). However, there was an inversion in the relative frequency of GFP^{high} and GFP^{low} RTEs with age. Thus, whereas ~25% of CD4 naïve cells were GFP^{high} at 4 and 8 weeks, only ~12% were GFP^{high} at 16 and 20 weeks in the K5.D1 spleen. In contrast, the percentage of K5.D1 GFP^{low} RTEs was ~40% at 4 weeks but increased to ~50% by 20 weeks of age. Interestingly, however, we found a marked increase in the absolute number of both GFP^{high} and GFP^{low} RTEs at 20 weeks of age. These data, taken together with the data on total naïve versus memory T cells (Figure 14) suggest that the splenic naïve T cell population in K5.D1 mice is significantly expanded. Even though only a modest increase in splenic cellularity is observed, the naïve T cell population is substantially increased at the expense of other cell populations in the spleen.

RTE maturation in aged K5.CyclinD1 and control mice

The quality of thymocytes from regenerated thymi through castration and KGF treatment has been scrutinized recently in the literature, as neither medullary islet complexity nor the levels tissue restricted antigens seen in young thymi are restored with regeneration (64). These observations suggest that T cells produced after thymus regeneration may be more autoreactive than T cells from young thymi (64). For our purposes, the data suggest that age-related alterations in the T cell microenvironment may not be reversed in the regenerated thymus. This consideration points out that we cannot assume that preventing thymus involution in K5.D1 mice will prevent age-associated changes in the TEC microenvironment that affect T cell development.

We assessed the maturation status of RTEs from 20 week K5.D1 and littermate controls by flow cytometric analysis of CD24, CD69, CD127 and Qa-2

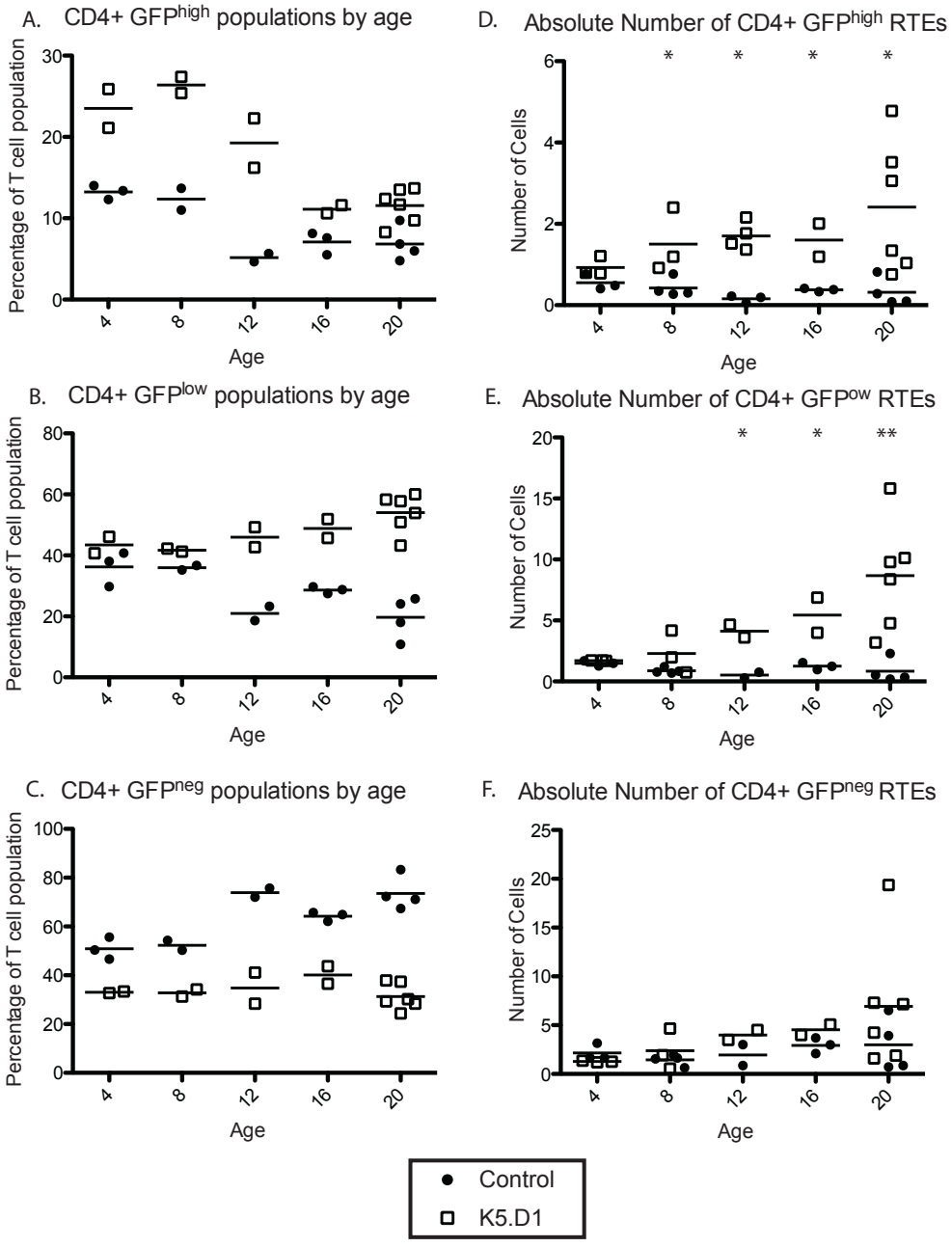

Figure 15. Distribution of GFP^{high}, GFP^{low}, and GFP^{neg} CD4 naïve T cell subsets with age in K5.D1 and control mice. (A-C) Percentage of GFP^{high} (A), GFP^{low} (B), and GFP^{neg} (C) populations of naïve T cells with age. (D-F) Absolute number of GFP^{high} (D, *P<0.05), GFP^{low} (E, *P<0.05 **P<0.001), and GFP^{neg} (F) populations of naïve T cells with age (n=2+ mice/group).

expression to determine if age and/or lack of involution affects RTE maturation. Similar to the data obtained at 4 weeks of age (Figure 12), CD24 expression was downregulated on K5.D1 GFP^{high} RTEs compared to controls (Figure 16A-D). CD69 expression was downregulated on K5.D1 RTEs and MN T cells compared to controls (Figure 16E-H). This result was unexpected, and might be explained by altered regulation of $S1P_1$, which regulates CD69 expression and is necessary for T cell emigration. Alternatively, since CD69 expression is upregulated on TCR activated T cells, this result could suggest changes in the TCR signaling. Interestingly, CD127 (IL7Ra) expression was elevated at 20 weeks on K5.D1 GFP^{low} and MN RTEs compared to controls (Figure 16I-L). CD127 is downregulated upon interaction with IL-7, which is one of the main molecules regulating peripheral homeostasis (50, 73). The increase in CD127 suggests that GFP^{low} RTEs and MN T cells from the K5.D1 thymus may have a survival advantage compared to GFP^{high} T cells. Qa-2 expression showed little change in the K5.D1 compared to control RTEs and MN T cells (Figure 16M-P). Together, these changes in expression of maturation markers suggest that the K5.D1 RTEs are undergoing an altered maturation process in the periphery that is exacerbated with age.

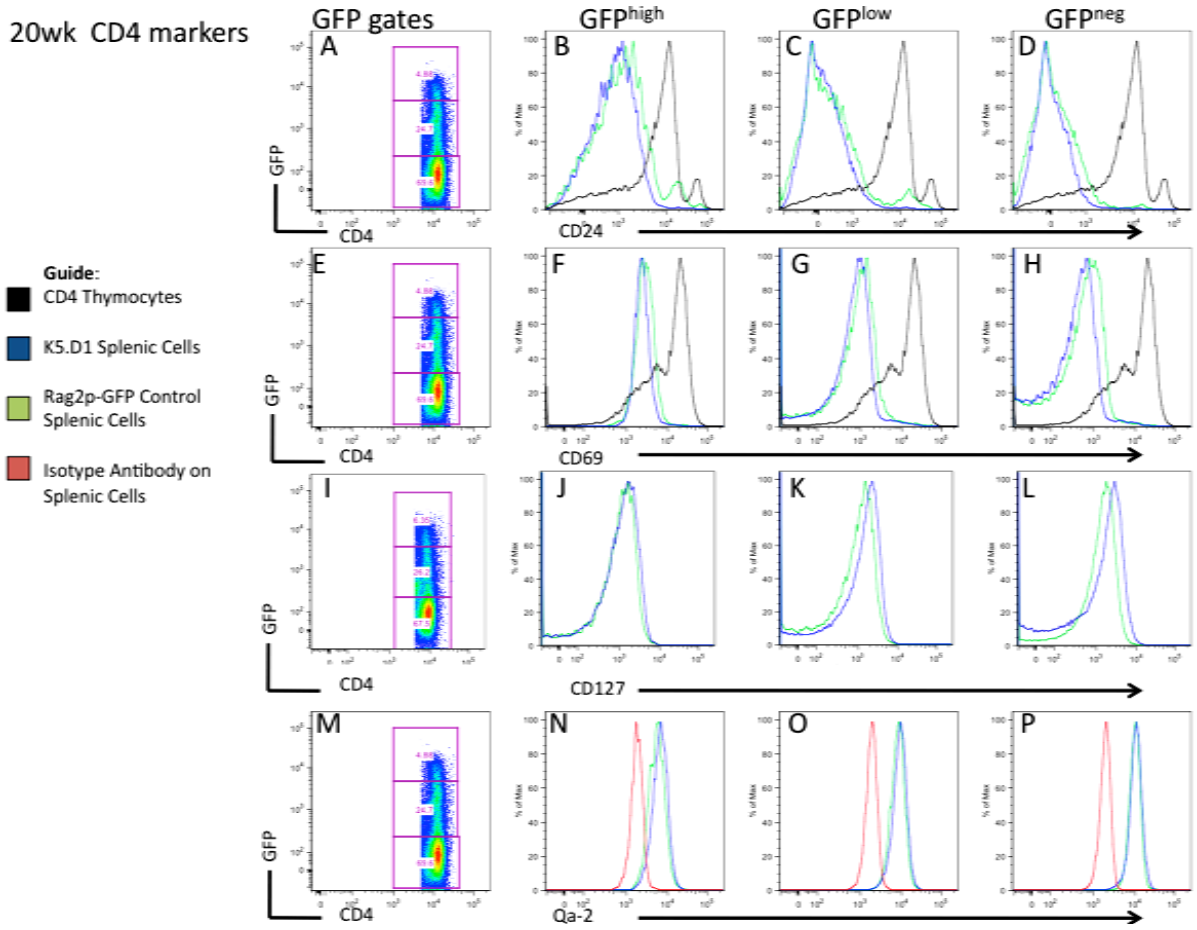

Figure 16. Expression of selected maturation markers on GFP^{high}, GFP^{low} and GFP^{neg} CD4 naïve T cells from 20 week old K5.D1 and control mice. Electronic gates were set on CD4^{+} CD44^{lo} CD62L^{hi} naïve T cells. These cells were analyzed for GFP expression level. Representative histograms show expression of CD24 (B-D), CD69 (F-H), CD127 (J-L) and Qa-2 (N-P) by GFP^{high}, GFP^{low} and GFP^{neg} naïve T cells from K5.D1 (blue) and control (green) mice. CD4^{+} thymocytes (black) were used as positive controls for CD24 and CD69 histograms, and isotype controls (red) were utilized for CD127 and Qa-2 histograms (control n=4 mice/group, K5.D1 n=6 mice/group).

CHAPTER 4: DISCUSSION

Chapter 4: Discussion

The studies of this thesis were designed to address two major hypotheses. The first was that increased thymopoiesis would increase the number of RTEs incorporated into the peripheral T cell pool. The second major hypothesis was to determine if preventing thymic involution would avert the decreased RTE output that occurs with age and compromises immune responses. In summary, our results show that the K5.CyclinD1 transgene on different genetic backgrounds resulted in thymic hyperplasia and promoted thymopoiesis that led to an increase of RTEs in splenic T cells of young mice and sustained the increase in RTEs up to 20 weeks of age. The greater incorporation of RTEs in peripheral T cells of K5.D1 mice maintained a relatively high percentage of naïve T cells and a higher ratio of naïve to memory T cells. Furthermore, the data suggest that new T cells generated from the K5.CyclinD1 thymus may be better suited for survival in the peripheral immune population, especially in older mice. Taken together, our findings suggest that preservation of the TEC population with age maintains thymopoiesis, leading to greater RTE output and incorporation into the peripheral T cell pool.

Phenotype in K5.Cyclin D1 mice on a C57Bl/6J background

The hyperplastic thymic phenotype observed in the K5.CyclinD1 transgenic mouse (65, 66) provides an opportunity to study how prevention of thymus involution and sustained thymopoiesis affect the contribution of naïve T cells to the peripheral T cell pool. TECs provide microenvironmental niches that promote thymopoiesis by providing factors necessary for thymocyte growth, survival and differentiation (74). It has been shown that an increase in the number of TECs provides more microenvironmental niches to support greater thymopoiesis (75). We observed an increase in the $K5^{+}K8^{+}K14^{-}$ (Figure 4) TEC population that harbors TEC precursors. We also found an increased number of TECs by flow cytometry, confirming that the expression of the K5.CyclinD1 transgene drives expansion of the TEC population. We clearly demonstrated that increased thymocyte cellularity is not a result of transgene expression in thymocytes by qRT-PCR data. Thymus size is has been shown to be positively regulated by the number of TECs present (76). Therefore, our results support

the idea that increased percentage of TECs in the thymus promotes expansion of thymocyte cellularity through an increase in microenvironmental niches necessary for thymocyte development. However, to prove this premise would require isolation and functional analysis of TEC progenitors.

Thymus hyperplasia was evident in K5.D1 mice at 4 weeks and increased with age, however, only minimal expansion in the splenic cellularity occurred. One explanation for this observation may be that SP thymocytes are not exiting the K5.D1 thymus, and therefore are not able to join the peripheral T cell population. However, at no point in these studies was a major increase in the percentage of SP thymocytes observed in the thymus to indicate retention. Furthermore, we observed the proper loss of CD69 expression, which is regulated by the sphingosine-1-phosphate receptor in a reciprocal manner (19). $S1P_1$ expression is necessary for thymic emigration, and the downregulation of the CD69 expression suggests that $S1P_1$ was expressed (18). GFP^+ RTEs were observed in the spleen of K5.D1 mice when crossed to the RAG2p-GFP transgene. This confirmed that thymocytes are released from the hyperplastic K5.Cyclin D1 thymus to the periphery, as the expression of *Rag2* is permanently extinguished during thymopoiesis (40). These data combined suggest no observable retention of SP thymocytes. In another study investigating thymic hyperplasia, increases in peripheral T cell population size were also minimal (77), suggesting that the peripheral homeostasis may act restrictively to control the size of the peripheral pool in these cases.

Background Influence on thymus size

Determining the effect of background strain on transgenic phenotype was not an initial aim of our studies, but was a necessary component to our analyses. Because of limited availability, we crossed the K5.CyclinD1 mice on a C57Bl/6J background to RAG2p-GFP females on an FVB background. This cross caused the study mice to be on a 50:50 mixed background, and led to the reduction in thymus size and cellularity. Others have established that genetic background greatly influences thymus size and involution (68). C57Bl/6J mice are considered a ‘slow involution’ strain that exhibits a larger thymus, and therefore, most immunological studies are performed with mice on

this background (78). We observed a reduction in size upon initial dissection of both control and K5.D1 thymi, confirming that FVB background genes result in a smaller thymus size and cellularity, even when expressing the K5.CyclinD1 transgene. Furthermore, when these data were compared to K5.D1 and control data on the C57Bl/6J background, the changes in size and cellularity were similar in both K5.D1 and control genotypes, indicating that the background affected both genotypes to the same extent. FVB mice tend to have smaller thymi and undergo premature involution compared to age matched C57Bl/6J mice (79) supporting our conclusion that our results are due to the background genetics and not to RAG2p-GFP expression. Dr. Pam Fink is providing us with RAG2p-GFP mice on the C57Bl/6J background to confirm this conclusion as well as for future studies as described below.

Frequency and maturation of RTEs in young K5.Cyclin D1 and control CD4 T cells

The naïve T cell compartment is robust at a young age in normal mice, and contains a high percentage of naïve T cells. Our observations in young K5.D1 mice suggest that the transgene may not yet have an impact on the distribution of the peripheral T cell subsets. There was a minimal increase in the percentage of naïve splenic T cells, but no increase in the number of naïve splenic T cells in the K5.D1 mice, which suggests that peripheral homeostasis is maintained in these mice at 4 weeks of age. Expression of the K5.D1 transgene did result in an increased incorporation of RTEs into the CD4 naïve T cell compartment, supporting increased released of RTEs from the thymus. These findings were unexpected, as normal RTEs do not compete as efficiently with MN T cells in the periphery (21). Other groups interested in thymic size and rebound have also seen an increase in RTEs when the thymus is regenerated by administration of exogenous growth factors (77, 80). K5.D1 RTEs may posses a selective advantage compared to control counterparts. This premise can be tested in adoptive transfer experiments described in a later section. Additionally, our cohort was too small in this study, and the 4 week age data suggested that the peripheral T cell population may be impacted more significantly than the power of our study was able to describe. Further investigation with larger cohorts will be

performed to determine more precisely how the thymus hyperplasia in K5.CyclinD1 transgenic mice affects the number and frequency of RTEs in the peripheral T cell population.

In order to understand how transgene expression affected extra-thymic T cell maturation, we investigated the maturation status of newly exported T cells from the K5.D1 transgenic thymus. The results showed little difference in maturation markers between the K5.D1 RTEs and those from control littermates at 4 weeks of age, with the exception of CD24 expression. CD24 is necessary for homeostatic proliferation and transition to memory T cell type, but is not necessary for survival (42). CD24 expression was downregulated in K5.D1 GFP^{high} RTEs relative to control RTEs, whereas CD24 expression in the GFP^{neg} MN T cells is comparable in K5.D1 and control splenic CD4 T cells. While functional consequences of CD24 expression on RTEs have not been specifically elucidated, the lower expression level of CD24 K5.D1 GFP^{high} RTEs suggests that these cells may have defective homeostatic proliferation in a lymphopenic environment. Future studies will analyze phenotypic RTE maturation using additional maturation markers. The cell surface molecules CD28, CD3 and CD45RB have been used to monitor RTE maturation (28), and may provide more information about the maturation of K5.D1 RTEs. In addition, functional analyses such as cytokine production and antigen-induced proliferation, will be performed to determine if thymocyte development in the K5.D1 thymus alters the kinetics and/or degree of RTE maturation

Analysis of the naïve T cell subsets and RTE contribution in aged K5.CyclinD1 and control mice

The second aim of this study was to determine how aging affected thymopoiesis in the non-involuting K5.D1 model. The data presented show that the composition of the naïve T cell population is altered when thymus involution is prevented. In nontransgenic mice age-related thymus involution results in a reduction of RTEs that diminishes the number and proportion of naïve peripheral T cells population and results in clonal expansion of the T cells to maintain total peripheral T cell numbers (56). The total number of splenic cells was not significantly affected in young K5.D1 mice at 20

weeks of age, but there was a very apparent shift favoring the percentage of naïve to memory/activated T cells in K5.D1 compared to control mice. This result attests to the idea that continued thymic output of new T cells from the K5.D1 thymus can impede age-associated reductions in RTEs in the naive peripheral T cell population. At 20 weeks of age, the number of CD4 and CD8 naïve T cells was increased in the K5.D1 spleen; however, the number CD4 and CD8 memory/activated T cells was not significantly different from control. This suggests that the increased numbers of RTEs and naïve T cells present in the K5.D1 mouse model does not actually restrict clonal expansion of the memory population. Instead, the change in distribution of naïve to memory CD4 T cells results from the greatly increased numbers of RTEs and MN T cells present in the periphery – absolute numbers of total naive T cells at 20 weeks were four fold higher than that of naïve numbers at 4 weeks of age. Therefore, we suggest that the K5.D1 mouse model overwhelms the peripheral homeostatic mechanism by the release of sustained and increased output of RTEs to the periphery.

It has been reported that RTEs are at a competitive disadvantage compared to MN T cells for incorporation into the peripheral T cell pool of lymphoreplete mice (21). Interestingly, phenotypic analysis of differentiation markers suggested that RTE maturation is altered in K5.D1 mice, which may provide an explanation for the increase in number of splenic CD4 naïve T cells. Those cells that recently emigrated from the thymus in 20 week mice continued to express CD24 on RTEs at a lower level than their control littermates. This result is consistent with the downregulation of CD24 seen at 4 weeks, and suggests an age-associated downregulation of this molecule on RTEs. Because CD24 is necessary for conversion of naïve T cells to the memory T cell type (42), normal memory T cell expansion may be inhibited, which would agree with the altered distribution of naïve:memory T cells seen at 20 weeks of age. Furthermore, K5.D1 RTEs displayed increased expression of IL-7Rα (CD127) during maturation in the periphery. IL-7 is one of the few known regulators of peripheral T cell homeostasis, and the increase of IL-7Rα expression in K5.D1 naïve T cells may impart a selective survival advantage. Recent studies suggest that TCR engagement during thymopoiesis is integral in determining the level of expression of IL-7Rα on mature T cells, which impacts their survival potential (81). In addition, IL-7 transgenic mice have

an increased number of mature T cells (82), and only T cells with appropriate levels of IL-7Rα expression survive in the periphery (81). Therefore, the naïve T cells in older K5.D1 mice may be expressing higher levels of IL-7Rα and increasing their survival potential as a consequence of enhanced TCR signaling in thymocytes perhaps induced by engagement with self peptides presented on TECs. A corollary to this hypothesis is that age-related changes in the array of self-peptide/MHC complexes presented to thymocytes by K5.D1 TECs and/or their ability to present self-peptides may enhance TCR mediated signaling. However, regardless of the specific explanation, the increased expression of IL-7Rα may allow K5.D1 RTEs to compete more effectively with MN T cells for incorporation into the peripheral T cell pool. This premise will be tested in future experiments summarized below.

Age-related changes in the frequency of naïve T cells in K5.CyclinD1 and control mice

The expansion of the naïve T cell population observed in the K5.D1 periphery was largely due to the increased incorporation of RTEs. However, the frequency of GFP^{high} RTEs dramatically dropped with age in K5.D1 mice, while the RTE^{low} and RTE^{neg} frequencies increased (Figure 15). We considered the possibility that this pattern may be due to retention of RTEs in aging K5.D1 transgenic thymi. However, this was not the case, as total cell number for the RTE^{high} subset dramatically rose with age and there was no increase in the percentage of SP thymocytes in 5 month old mice.

Overall, every RTE population expanded in total cell number the K5.D1 transgenic mouse. This result suggests a few possible circumstances. The first explanation for the results we've observed is that RTEs may be proliferating at a faster rate in the K5.D1 periphery. This is most likely not the case, as we showed that the transgene was restricted to the TEC compartment and not present in developing thymocytes (Figure 7). Additionally, utilization of the time sensitive, and proliferation sensitive GFP label precludes any proliferative expansion of the GFP^{high} and GFP^{low} subsets without these cells losing GFP intensity (40). However, there may still be some type of change in proliferative capability of peripheral T cells in the K5.D1 transgenic

mice that has been conferred by the transgene expressing TECs. This hypothesis should be tested through the use of a proliferation assay coupled with GFP transgenic expression to determine if the GFP^{high} and GFP^{low} RTE subsets are proliferating at an increased rate in K5.D1 mice.

The increased cellularity of GFP^{low} and GFP^{neg} naïve T cells could be a consequence of a survival advantage perhaps as suggested above, due to increased IL-7Rα expression. If this were the case, it would explain why our results show increases in the GFP^{low} RTE population with time – persisting GFP^{high} cells in the periphery would cause an expansion of the GFP^{low} and MN T cell subsets as they matured. There are two known limiting factors involved in peripheral T cell homeostasis: availability of MHC molecules for TCR engagement and the availability of the cytokine IL-7 (26). However, while the increase in receptor expression on the K5.D1 naïve T cell subsets may allow them a better chance of survival in periphery, it doesn't solve the predicament of the limited amount of IL-7 cytokine released by stromal cells. Nevertheless, the hypothesis that enhanced survival improves the ability of K5.D1 RTEs to become incorporated into the peripheral T cell pool can be tested in a competitive adoptive transfer assay. An equal ratio of $CD45.1^{+}$ K5.D1 RTEs would be mixed with CD45.2 control RTEs and injected into an irradiated $CD45.1^{+}/CD45.2^{+}$ recipient (Figure 17). At various times after the adoptive transfer groups of mice will be sacrificed and the splenic T cells analyzed for the contribution of CD45.1 and CD45.2 RTEs in the peripheral T cell pool. This experiment could also be performed with RTEs expressing high versus low levels of IL-7Rα.

The other possible explanation for the observed results in K5.D1 mice is that the thymus releases such a vast amount of RTEs to the peripherally that the naïve compartment is inundated with T cells, overwhelming homeostasis and causing increased cell numbers in every T cell subset. There may be no increased survival potential in K5.D1 RTEs, and our observations could stem from an overwhelming number of RTEs released to the periphery. RTEs can persist for weeks in the periphery without any interaction, while our GFP label is only detectable for three weeks (83). There may be no selective advantage to the RTEs being produced, and the results observed are simply an affect of expanding the peripheral T cell population.

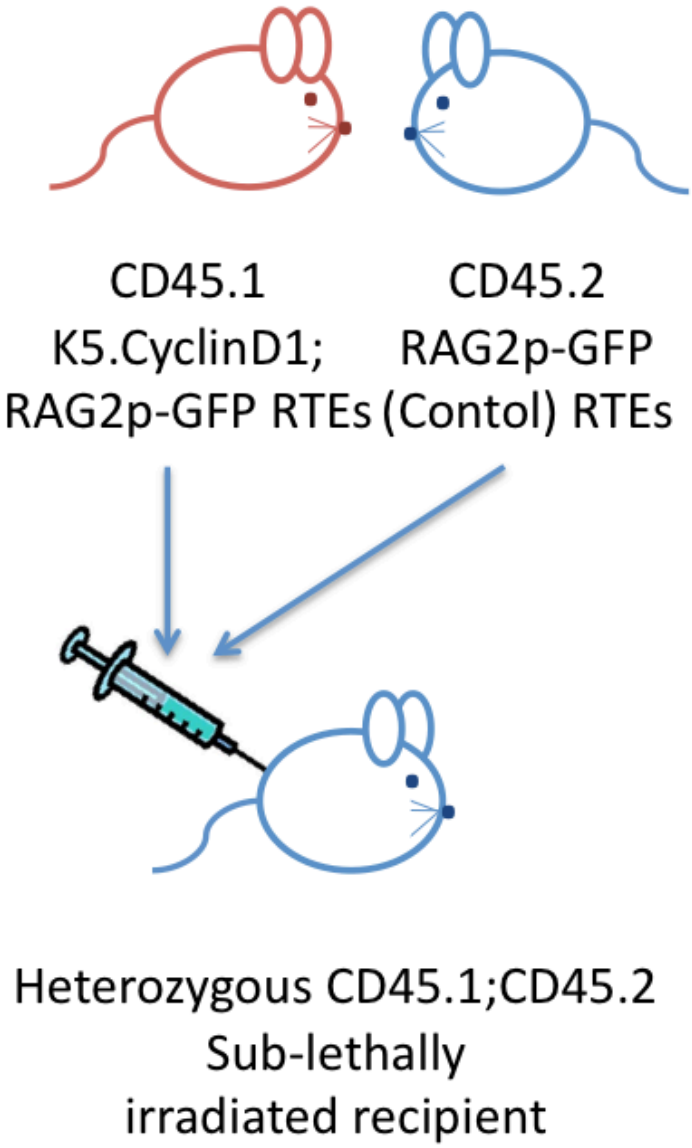

Figure 17. Proposed adoptive transfer experimental setup. K5.CyclinD1 mice will be bred to RAG2p-GFP mice bearing the congenically marked CD45.1 RTEs. RTEs from this cross will be added with control RAG2p-GFP CD45.2 RTEs at a 1:1 ratio to heterozygous (CD45.1,CD45.2) irradiated recipients to determine survival potential. Transferred RTEs will be analyzed 24 hours, 1 week and 3 weeks post transfer. and allowing RTEs from the thymus to lose signal and die in the periphery.

However, previous work analyzing how multiple transplanted thymi affected the peripheral T cell population showed that even with an expanded number of T cells being produced, peripheral homeostasis was capable of controlling T cell number in the periphery, and only a small increase in peripheral T cell size was observed (24), and this agrees with our minimal increase in total splenic cellularity date in the K5.D1 at 20 weeks of age. Therefore, this hypothesis should be tested through a cell death assay coupled with the GFP transgenic expression to determine if there is an increase in cell death in the GFP^{low} and MN subsets. The investigation of IL-7 in secondary lymphoid organs and RTE adoptive transfers should also be performed to rule out other possibilities for the observed results.

Conclusions and Future Aims

In conclusion, the studies presented in this thesis demonstrate that increased thymopoiesis results in a greater proportion of RTEs in the peripheral T cell pool in young mice and prevents age-associated decrease in RTEs. The altered distribution of naïve versus memory peripheral T cell subsets, combined with the continued production of RTEs suggest that the diversity of the peripheral TCR repertoire may be sustained or even enhanced. This is an important advantage since a diverse TCR repertoire is necessary for successful immune responses to new antigens.

Our study did not directly analyze the number of cells/day that emigrate from the thymus of K5.D1 and control mice. Prior studies have directly quantified the number of thymic emigrants by injecting FITC directly into the thymus and counting the number of FITC labeled T cells in peripheral lymphoid organs at 24 hours. We also plan to directly analyze the TCR diversity of naïve T cells in the periphery of K5.D1 and control mice at various ages. This can be accomplished by utilizing CDR3 length spectratyping . Another future goal of this project is to determine whether the increase of RTEs in the K5.D1 mouse confers greater immune responsiveness to new antigens, and future aging studies will include mice up to one year in age. The K5.CyclinD1 mouse model is a valuable asset for studies to discern how thymus hyperplasia and lack of involution affect the composition and function of the peripheral T cell pool.

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Vita

Michelle Lynn Bolner
3908 Yarborough Avenue
Austin, TX 78744

mlbolner@mdanderson.org

Michelle Lynn Bolner was born in Del Rio, Texas on February 26, 1986, and is the daughter of Michael and Tammy Bolner. After completing her work at Canutillo High School, Canutillo, Texas, in 2004, she entered Baylor University in Waco, Texas. She received the degree of Bachelor of Science with honors and a minor in chemistry from Baylor in May 2008. In August 2008 she entered the Molecular Carcinogenesis program at the University of Texas at Houston Health Science Center Graduate School of Biomedical Sciences.

Current Position:
Graduate Research Assistant at The University of Texas at Houston Health Science Center, M D Anderson Cancer Research Center, Science Park. Supervisor: Ellen Richie.

Education:
B.S. Biology, Chemistry Minor, Baylor University, Waco, Texas. 2008