Title
Characterization of Sphingosine-1-phosphate receptor 1 in the human thymus: Implications for T cell reconstitution in HIV infection

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Characterization of Sphingosine-1-phosphate receptor 1 in the human thymus:

Implications for T cell reconstitution in HIV infection

A dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy
in Microbiology, Immunology and Molecular Genetics

by

Rachel Samantha Resop

2014
Emigration of mature naïve CD4 SP T cells from the human thymus to the periphery is a field that remains not fully understood, although elucidation of the mechanisms that govern egress of T cells is crucial to understanding both basic immunology and the immune response in disease states such as HIV infection. In this dissertation work, I have examined the expression and function of a novel requisite T cell egress receptor expressed within the human thymus, as well as characterized changes observed in the expression and function of this receptor during HIV infection, and finally examined additional markers of maturation stages of human thymocytes. In Chapter 2, (“S1P/ S1P-R1 signaling is required for migration of naïve human T cells from the thymus to the periphery”) I investigated whether Sphingosine-1-phosphate receptors (S1P-Rs) are expressed on human thymocyte populations and whether they function in the egress of mature human thymocytes from the thymus to the periphery. In Chapter 3 (“HIV-1 infection results in upregulation of sphingosine-1-phosphate receptor 1 on mature human thymocytes and functional naïve T cell response to S1P”), I examined whether
Human Immunodeficiency Virus 1 (HIV-1) infection affects the expression and function of S1P-R1 utilizing humanized (NSG thy/liv) mice as well as various ex vivo assays to investigate the function of S1P-R downstream signaling. Finally, in Chapter 4, (“Alternative markers for maturation stages of human thymocytes: Expression of CD31 (PECAM-1) during T cell differentiation in the human thymus”), work I performed in collaboration with others within the AIDS Institute at UCLA, I contributed to the characterization of additional markers of developing thymocytes within the human thymus. Interestingly, a novel marker characterized may play a role in modulation of TCR signaling during T cell development in the thymus and may moreover be coexpressed with S1P-R1 on mature human thymocytes. Taken together, this work should advance the fields of basic T cell Immunology as well as HIV Immunology and open new avenues for exploration into therapeutics for HIV infected individuals.
The dissertation of Rachel Samantha Resop is approved.

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The work presented herein is dedicated to the Biology and Chemistry professors and teachers who have inspired, mentored and encouraged me from fourth grade through graduate school; to my parents who have supported me unconditionally; and to my beloved Grandma, who was my best friend and ally. She knew before I did that I would succeed and I know she would have been proud.
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Christel, my mentor, has been the most supportive, caring and knowledgeable doctoral mentor any graduate student could hope for. She saw in me a potential that I hope I have lived up to, albeit when I arrived in her lab my first year in graduate school I had been trained as a microbiologist and felt lost in the slew of Immunology terms and concepts. I cannot thank her enough and have come to love her as family. Marta Epeldegui, Marc Douaisi, and Joshua Craft have been invaluable colleagues who each contributed significantly to the completion of this work, whether though instruction or working by my side. I would also like to acknowledge my thesis committee, the members of which met with me for yearly meetings and were instrumental in my progress and development as a critically-thinking scientist: Dr. David Brooks, Dr. Rita Effros, Dr. Paul Krogstad, and Dr. Otto Martinez-Maza.
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SELECTED ABSTRACTS

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- Resop, R. S.*, Craft, J. and Uittenbogaart, C. HIV-1 infection changes expression of S1P receptor 1 and affects naïve T cell egress from the human thymus. International AIDS Society Meeting 2013. (Oral Presentation.)
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- Resop, R. S.*, Douaisi, M., Craft, J. and Uittenbogaart C. The role of S1P and S1PR1-5 in the egress of mature thymocytes from the human thymus. Midwinter Conference of Immunologists 2013. (Poster presentation.)
- Resop, R. S.*, Craft, J., Douaisi, M. and Uittenbogaart, C. The interplay of S1P and S1PR1-5 in the egress of mature thymocytes from the human thymus. Seaborg Symposium 2012. (Poster presentation.)
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CHAPTER 1:
INTRODUCTION
I. History and Basics of Immunology of T cells

The field of Immunology is relatively young as compared to other Biological sciences. Edward Jenner, an English physician-scientist and discoverer of the concept of vaccination, which continues to be employed to this day, is credited with spurring the origination of the field and is often referred to as one of the “Fathers of Immunology,” along with Paul Ehrlich and Louis Pasteur. Although the theory of vaccination had circulated for some time prior to his work, Jenner cemented the validity of the notion when he intrepidly attempted to protect individuals from deadly smallpox infection by utilizing a closely related infectious agent, cowpox, administering the non-fatal agent to healthy individuals in 1796 and documenting that these individuals were as a result immune to smallpox. Although he was successful, it would be some time before the underlying mechanisms of his discovery were fully understood. This pattern of an observation followed by toils to uncover a mechanism would set the tone for the field in which researchers continue to strive to understand the processes governing the human immune system and the mechanisms of our incredibly intricate and complex response to pathogens that threaten to perturb the delicate balance of a healthy state.

Our current understanding is that our immune system is comprised of two branches, known as innate and adaptive immunity. *Innate immunity* refers to the cells of the immune system that are always ready to respond to a wide range of pathogens. This response is non-specific and involves cells such as granulocytes, Natural Killer (NK) cells, and macrophages (phagocytic cells capable of engulfing many potentially harmful microorganisms); Elie Metchnikoff, a Russian immunologist, primarily characterized these types of reactions. *Adaptive immunity* refers to a more specific response that the immune system generates over time. “Memory” is developed upon encounter with a particular antigen (harmful microorganism or other substance)
such that if the antigen presents again, specific cells are primed to deal with it. Adaptive immunity is further divided into two branches, humoral and cellular.

B lymphocytes (B cells, from “bursa” of the chicken, where they were discovered) are responsible for humoral immunity and generate antibodies that recognize antigens and are able to neutralize recurring pathogens. T lymphocytes, or T cells (from “thymus,” where these cells complete their development) are key players in adaptive immunity, specifically cell-mediated immunity. Briefly, T cells are comprised of several subsets, each of which is responsible for a specific task in the immune response. These jobs involve cytotoxic activity (direct cell killing by CD8+ T cells or cytotoxic T lymphocytes (CTLs)), regulation of other immune cells (Regulatory T cells, or Tregs), helper activity for lymphocytes (Th1, Th2 and T follicular helper cells or Tfh) and protective immunity against extracellular bacteria and fungi (Th1, Th2, Th17, Th22, Tfh, and γδ T cells). Development of these various subsets begins in the thymus but differentiation into the specific subsets above occurs post-thymic egress in the periphery. T cell development in the thymus will be discussed below.

II. T Cell Development in the Thymus and T cell Selection

As discussed in the preceding section, the adaptive branch of the human immune system is quite specific and potent. Therefore, the participants in adaptive immunity must be trained to avoid self-reactivity and to identify and target foreign invaders with an adequately strong response. T and B cell subsets of lymphocytes undergo intense selection processes during development; herein we will focus on T Cells, although it bears mentioning that B Cells undergo an analogous process of selection and maturation within the bone marrow and within the germinal centers of the secondary lymphoid organs (spleen and lymph nodes).
T cells ultimately develop from CD34+ hematopoietic stem cell progenitors that originate in the bone marrow\(^9\). In fact, both T and B cell precursors originate within the bone marrow; however, while B cells continue their development therein, T cell precursors emigrate from the bone marrow and home to the thymus, a lymphoid organ located in the thoracic cavity over the heart, where they progress through several stages of development\(^1\). Upon entry of these precursors to the thymus, they have not yet undergone rearrangement of the genes encoding the T Cell Receptor, or TCR (a heterodimer consisting of an \(\alpha\) and \(\beta\) chain embedded in a CD3 complex that allows recognition of Major Histocompatibility Complex (MHC)-bound antigen presented to T cells and the expression of which is a defining characteristic of T cells\(^{10}\)), a process that occurs during their maturation in the thymus. Additionally, at this stage they lack most of the surface molecules used to characterize mature T cells. Various surface-expressed receptors and other molecules are used to characterize the maturation stages of T cells, as in varied combinations they are quite specific to stages of T cell development in the thymus. T cell precursors in the thymus undergo extensive proliferation, but the majority of these cells die and do not progress along the differentiation pathway to become mature T cells\(^{11}\).

Within the thymic cortex, or the outer portion of its lobes, initial differentiation toward the T cell pathway occurs, spurred by the interaction between T cell precursors and thymic stromal cells. The T Cell Receptor (TCR) gene begins to rearrange and CD4, a coreceptor for the TCR, is expressed on thymocytes. T cells are considered double positive cells once CD8 is also upregulated. The first selection process, termed positive selection, selects for CD4+/CD8+ thymocytes that recognize MHC displayed by specialized antigen-presenting cells in the thymus\(^{12,13}\). Next, thymocytes upregulate CCR7, which is necessary for their translocation to the thymic medulla\(^{14,15}\), guided by additional cytokines produced by stromal cells\(^{16}\). Therein they
undergo a second selection process, *negative selection*, whereby double-positive cells that react too strongly to self Major Histocompatibility Complex (MHC) fail to receive survival signals\(^\text{17,18}\) and undergo programmed cell death, or apoptosis\(^\text{19}\), whereas cells that react mildly are selected for survival\(^\text{18}\). The selection process is not yet fully understood, but recent evidence suggests that microRNAs in developing thymocytes have a role in selection\(^\text{20}\). Upon loss of expression of CD4 or CD8, thymocytes are considered **single positive (SP) cells**.

Typical developmental markers for thymocytes include CD27 (Cluster of Differentiation 27, a Tumor Necrosis Factor receptor), CD69 (Cluster of Differentiation 69, a C-type lectin transmembrane protein), CD45RA (leukocyte common antigen), and CD62 ligand (CD62L, also known as L-selectin). CD27 is also expressed on all thymic medullary cells and is used as a marker of relatively mature human thymocytes\(^\text{21,22}\). CD45 is found in two isoforms, CD45RA and CD45RO, which are expressed differentially on various developing thymocyte subsets. CD45RA, which I used most frequently in my work as a developmental marker, is expressed during the most immature stage of thymocytes development, not expressed on intermediate stages, and finally re-expressed in mature thymocytes. CD45RO is expressed on the majority of CD4+CD8+ (double positive), intermediate-stage thymocytes\(^\text{23}\). CD69, an activation marker that begins to be expressed during positive selection\(^\text{24}\) and is also expressed on activated peripheral T cells, has recently been found to play a role in regulation of T cell egress\(^\text{25}\). CD69 is downregulated on mature thymocytes before egress and is not present on recent thymic emigrants\(^\text{24}\). Finally, thymocytes upregulate CD62L and are able to egress the thymus for the periphery to encounter their antigens as mature single positive T cells. Egress from the thymus will be discussed in the following section. See **Figure 1** for the markers used to identify maturing thymocyte populations.
The transient expression during thymocyte maturation of **Kruppel-Like Factor 2 (KLF2)**, a master transcriptional regulator, has been shown in the mouse to control the expression of several migration-associated surface molecules expressed on thymocytes, including CD62L and CCR5\(^{26-28}\). As KLF2 begins to be expressed on mature thymocytes nearly prepared to egress the thymus for the periphery, and has been demonstrated to regulate transcription of **Sphingosine-1-phosphate receptor 1 mRNA (S1P-R1)**, an egress-associated receptor discussed in detail below and throughout the dissertation) in murine models\(^ {26}\), I examined the potential role of KLF2 in human thymocytes in **Chapters 2 and 3** of the dissertation.

**III. Mature Thymocyte Egress from the Thymus**

Upon completing their development in the thymus, thymocytes that are prepared to egress the thymus for the periphery are considered **mature naïve T lymphocytes**. The work of the Uittenbogaart laboratory and others has determined the phenotype of these cells to be CD3\(^{hi}\)CD27\(^{+}\)CD45RA\(^{+}\)CD62L\(^{+}\)CD69\(^{-}\)\(^{29-31}\). As shown by Vanhecke *et al.*, CD69 is lost prior to thymocyte egress of mature naïve cells\(^ {24}\) and can therefore be utilized reliably as a marker of fully mature thymocytes prepared for egress, in conjunction with the additional surface markers above. Interestingly, these mature T cells are refractory to death by apoptosis (programmed cell death), in contrast to less mature subsets, which are susceptible to apoptosis. Instead, mature SP cells respond to TCR stimulation by proliferating\(^ {32}\). They also must be able to respond to signals that "tell" them it is time to egress the thymus for the periphery. Once they do leave the thymus, these cells are known as **Recent Thymic Emmigrants (RTE)**. See **Figure 2** for a schematic of thymocyte egress from the thymus.
Many of the chemokine receptors and chemokines involved in egress from the thymus have been well characterized, but this work has been performed predominantly in murine models and may not necessarily reflect the situation in the human. Therefore, it is important to consider that the current understanding of the process of thymic egress in humans is quite fluid, and thus subject to fine-tuning as methods emerge to permit in-depth work in humans or a greater number of researchers begin to work with human tissue samples. Briefly, it is known that the chemokine receptor CXCR4 and its ligand CXCL12 are important molecules involved in egress from the thymus to the periphery. CXCL12, also known as Stromal-Derived Factor 1 (SDF-1) is a potent lymphocyte chemoattractant and is known to retain thymocytes at the corticomedullary junction, the region of the thymus microenvironment where thymocytes enter and exit the thymus, until the cells are “told” to leave. Additional factors such as early growth response gene (Egr1, a transcriptional regulator involved in differentiation and mitogenesis), integrin α5β1 (a mediator of migration and proliferation), aryl hydrocarbon receptor (AHR, a helix-loop-helix transcription factor), laminin-5 (a mediator of migration, organization and attachment in tissues), CCR7 (a mediator of migration from the cortex to the medulla during selection), KLF2 (see above and subsequent chapters of the dissertation as well as), PI3 Kinase (PI3K, a negative regulator of KLF2), and Phosphase and Tensin Homologue (PTEN, a regulator of PI3K) also bear mentioning as they have been shown to play roles in the process of egress (albeit much of this is murine work). According to “textbook knowledge,” the maturation process duration is a relatively lengthy process of about two weeks. However, it has recently been discovered that the time between positive selection and emigration is much less than previously believed: 4 or 5 days as opposed to the earlier estimate of 14 days. Additionally, emigration may not be stochastic as previously believed, but rather some data point toward the most immature thymocytes leaving before less mature medullary cells, although this has not been unequivocally demonstrated.
Prior to the thesis research presented herein, there were very limited data on the mechanisms regulating egress of mature human thymocytes from the thymus to the periphery. Murine studies investigated the S1P/S1P-R1 ligation prerequisite for thymocyte egress, and demonstrated that S1P-R1 is essential for the thymic egress of mature single positive murine thymocytes\textsuperscript{48,49}. However, no reports to date had demonstrated whether the S1P/S1P receptor pathway plays a role in the response of mature thymocytes to S1P or egress from the human thymus to the peripheral blood and lymphoid tissues; moreover, the exact population of thymocytes that expresses S1P-R1 had not been fully characterized either in mice or humans.

The following section will treat the role of this particular signaling molecule, **Sphingosine-1-phosphate**, in the egress of mature, single positive CD4 and CD8 thymocytes from the thymus to the periphery in mice and humans, the characterization of which is the main focus of this dissertation.

**IV. Sphingosine-1-phosphate (S1P) and its Role in Chemotaxis**

**Sphingosine-1-phosphate** (S1P) is a signaling sphingolipid molecule, also known as a **lysosphingolipid**, with multiple tasks throughout the body\textsuperscript{50}. The name “sphingosine” in fact originates from the mythological Greek character the sphinx, in reference to the molecule’s promiscuous nature in the human body\textsuperscript{51}. S1P functions in a myriad of roles in humans, from regulation of cell death (specifically, suppression of apoptosis\textsuperscript{52}) and survival\textsuperscript{53,54}, immune responses\textsuperscript{55} as well as autoimmune conditions and allergies\textsuperscript{56-58}, B cell development\textsuperscript{59}, insulin modulation\textsuperscript{60}, cell motility\textsuperscript{61,62}, and response to viral infections\textsuperscript{63,64}. In addition S1P is an “integral constituent” of High Density Lipoprotein (HDL) complexes in the plasma and not only plays a
role in regulation of its bioactivity but also contributes to protection against atherosclerosis.\textsuperscript{65-67} The S1P molecule is comprised of a long saturated carbohydrate chain and one alcohol, amino and phosphate group.\textsuperscript{68} S1P is produced by phosphorylation of its precursor, sphingosine, by sphingosine kinase, which is found ubiquitously in the cytoplasm and endoplasmic reticulum of many cell types\textsuperscript{69,70} and can also be released from ceramides via their conversion to Sphingosine and subsequent phosphorylation\textsuperscript{71}.

Among its many roles, S1P is crucial for proper lymphocyte trafficking between the lymphoid organs and about the periphery\textsuperscript{48,72} as well as the trafficking of various other cell types such as smooth muscle and endothelial cells\textsuperscript{73}. An S1P gradient is present in which S1P is concentrated in the blood (about 100nM) where it is taken up, phosphorylated, stored, transported and released by erythrocytes\textsuperscript{74,75} and activated platelets\textsuperscript{76} in response to factors in the serum\textsuperscript{75}, but is at a low concentration in lymphoid tissues. S1P is degraded by sphingosine lyase in lymphoid tissues, thus keeping tissue concentrations low\textsuperscript{77}; sphingosine phosphatase also aids in breaking down S1P in tissues\textsuperscript{78}. Sphingosine kinase, sphingosine phosphatase and sphingosine lyase together maintain the S1P gradient (See also Figure 3), which promotes the influx of lymphocytes bearing one of its five G-protein coupled receptors to the lymph nodes and other lymphoid tissues. The S1P receptor family is known to include S1P-R1-5; these receptors are present on various cell types\textsuperscript{79}. As will be discussed in Chapter 2 of the dissertation, S1P receptors had not previously been characterized on human thymocytes, although murine work alluded to their importance in emigration from the thymus\textsuperscript{48}.

In murine models, it has been shown that S1P-S1P-R1 ligation is required for mature T cells to egress the thymus to the periphery. Mice with siRNA ablated S1P-R1 have virtually no naïve CD4 or CD8 SP cells in the peripheral lymph nodes; treatment of mice with FTY720, an immune
modulator that targets S1P-R1, 3, 4 and 5, recapitulates this effect.\textsuperscript{48,49} S1P-R1 also plays a role in migration of T cells to and from peripheral lymphoid organs.\textsuperscript{80} S1P-R2 has been shown to inhibit migration of maturing B cells in the germinal centers when ligated to S1P.\textsuperscript{81} However no studies existed prior to this dissertation work demonstrating the role of S1P and its receptors in human thymocyte egress. This topic is the subject of Chapter 2 of the dissertation.

V. Additional markers for maturation stages of thymocytes in the human thymus

As described above, various markers are used for identification of the stages of thymocyte development, including CD3, CD4, CD8, CD45RA, CD27, CD69 and CD62L. CD31, also known as PECAM-1, is well-known as a marker of recent thymic emigrants (RTE) and interestingly as a marker of CD4+ (but not CD8+) RTE in HIV patients,\textsuperscript{82} although additionally it has been reported that CD31 is expressed on human thymocytes.\textsuperscript{83} In the work in Chapter 4, which I performed in collaboration with M. Douaisi, I contributed to the examination of CD31 on thymocyte subsets, making use of 9-color flow cytometry to precisely define the CD31-expressing populations within the human thymus. This work has implications for the field of T cell development, as CD31 has been shown to have the ability to modulate the TCR activation threshold via activity of tyrosine phosphatases and may therefore play a role in the actual T cell selection process. Additionally, the characterization of CD31 expression on thymocytes applies to the field of thymocyte egress and the characterization of receptors and ligands involved in human thymocyte egress (the subject of my work in Chapters 2 and 3 of the dissertation) as CD31 may additionally be expressed on mature thymocytes prepared for egress and thus its expression may correlate to that of other factors responsible for egress. Perhaps CD31 may function synergistically with other chemotaxis receptors to allow egress of mature thymocytes from thymus to periphery. Chapter 4 focuses on the in-depth characterization of CD31 on
thymocytes from the CD34+ hematopoietic progenitor stage through the final stage of thymocyte development.

**VI. Murine Models and Their Relevance for Human Immunology**

The adaption of mouse models to the study of mechanisms of immune function was a key step forward in beginning to achieve a greater understanding of the human immune system, as well as a scientific milestone. According to H. C. Morse, a researcher who was at the forefront of the development of murine models for research, “Our current realization of the potential of the inbred mouse has developed over a period of more than 70 years and reflects the dedicated research of many skilled and imaginative scientists.” The majority of the facts discussed above, including the characterization of T cell development in the thymus, the various facets of the lymphocyte repertoire and the necessity for cytokines such as S1P for T cell egress from the thymus, were made possible by the use of mice in research. Murine models began to be employed in 1929 with the establishment of the Jackson Laboratory and the development of the first inbred mouse strain, which eventually led to the development of various models of inbred mice designed to gain a better understanding of human diseases. With a repertoire of model systems in which we may manipulate various aspects, such as, for example, knocking out the expression of a specific gene, researchers are able to observe the resultant phenotype and thus infer the function of a particular facet of the immune system. Additionally, murine models of a wide range of diseases and autoimmune conditions have been developed. Moreover, mice closely resemble humans with regard to many aspects of the immune system (however, there are indeed many differences, as will be described below), are relatively simple to work with, and have a short reproductive time frame, thus they are invaluable tools for modern investigators.
Murine work is an adequate model of the immune system of humans in many ways. Like humans, mice develop T, B and Natural Killer (NK) cells, as well as dendritic cells, macrophages, monocytes and other important immune cells. Many of the human cytokines studied in immunology, or analogous cytokines, are also produced in mice, although the effects of cytokines can vary\textsuperscript{90,91}. Recent reports indicate that only approximately 300 genes differ between mice and humans\textsuperscript{92}. However, there exist several discrepancies between the mouse and human immune system, evidenced in both the adaptive and innate branches of the immune system\textsuperscript{93}. These differences may influence interpretation of results of immunological research aiming to understand the basics of immune system development, activation and response to challenge. Some of the key areas in which differences are observed between mouse and human include: balance of lymphocyte subsets, B and T cell signaling pathways, T Helper 1 and 2 cells (Th1 and Th2), certain cytokines and chemokines and their receptors, Toll Like receptors, antigen presentation by endothelial cells, and expression and function of costimulatory molecules\textsuperscript{94}. A recent report also demonstrated that genes that are up or downregulated in response to various immunological challenges (i.e. infectious agents/exotoxins, burn trauma, etc.) differ significantly in mice and humans; the authors conclude that genomic responses in mice “poorly mimic response in humans”\textsuperscript{95}.

In an attempt to address the above issues, humanized mouse models have been developed. Notably, the majority of humanized mouse models evolved from HIV research laboratories, as researchers needed a way in which to examine the effect of HIV on humans and found traditional murine systems inadequate. The \textbf{Severe Combined Immunodeficient (SCID) mouse} was the first such model to be utilized\textsuperscript{96,97}. Today, several humanized mouse models exist, but the underlying shared feature of all models is a reconstitution in the mouse of a
“human-like” immune system. In general, genetic manipulation renders certain aspects of the murine immune system ineffective; components such as murine T, B and NK cells do not develop, and human lymphocyte precursors are normally introduced, which develop into human immune cells in the mouse\textsuperscript{98}. The \textbf{NSG thy/liv model}, which is described in detail in Chapter 3 and which I employed for my studies on HIV infection of the thymus, is generated by: 1) A NOD genetic background, which reduces innate immunity in the mouse [result: macrophages and dendritic cells are not functional], 2) a SCID mutation (loss-of function mutation in the \textit{Prkdc} gene, which is responsible for repairing the double-stranded DNA break that occurs during v-d-j recombination in T and B cell development) [result: adaptive immunity- comprised of T and B cells- does not develop], and 3) a Common Gamma Chain mutation (the \textit{IL2rγ} gene), which compromises a common key component of the receptor for six interleukins (IL-2, 4, 7, 9, 15 and 21) [result: murine dendritic cells do not develop.] These mice may be implanted with human fetal thymus and liver tissue and injected with human fetal CD34+ hematopoietic progenitor cells, which develop in the thymus into functional “human-like” T lymphocytes (this type of mouse is commonly known as a bone marrow, liver, thymus (BLT) mouse.) B and NK cells of human origin also develop in the animal, allowing a strikingly accurate reconstruction of a humanoid immune system within the mouse\textsuperscript{99,100}. For a schematic of the generation of the NSG thy/liv mouse I used for the studies in Chapter 3, see \textbf{Figure 4}.

Thus, the model utilized for this dissertation work as well as other humanized mouse models have come into favor for modeling HIV infection, and are beginning to be taken advantage of to model other human diseases as well, as they appear to more accurately represent the human immune response to challenge: in HIV infection of the BLT mouse, for example, the cellular response observed, as well as the ability of the virus to escape the immune response mounted by the host, is similar to that observed in humans\textsuperscript{101-103}. However, in some fields these models
are still underutilized, particularly in the field of cancer immunology\textsuperscript{104}. This is concerning, considering that several therapies that have been demonstrated to work in mouse models have failed to have the desired effect in human trials\textsuperscript{105-110}. Additionally, multiple differences exist between murine and human thymus immunology, which must be taken into consideration when using animal models to investigate human thymocyte development and egress. The human response to HIV infection, particularly in the thymus, will be discussed below.

\textbf{VII. HIV Infection and Infection of the Thymus}

\textit{Human Immunodeficiency Virus}

Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981, as a mysterious cluster of infections that appeared to disproportionately target gay men, injection drug users, hemophiliacs, and Haitians\textsuperscript{111-116}. The disease remained an enigma during the early 1980s and caused considerable fear among the public as well as controversy over extremist sentiments regarding the groups of people affected\textsuperscript{117,118}. The infectious agent responsible for AIDS, a virus with similar characteristics to known \textit{lentiviruses}, was reported simultaneously in 1983 by French and American research groups led by Luc Montagnier and Robert Gallo\textsuperscript{119,120}, who would later vie for credit for the discovery. Following its initial characterization, virologists and immunologists raced to learn more about the infectious agent. It was soon discovered that the Human Immunodeficiency Virus (\textbf{HIV}) targets lymphocytes, or cells of the immune system, especially T cells, within which it replicates\textsuperscript{121} and that the virus utilizes CD\textsubscript{4}\textsuperscript{122} as well as one of two co-receptors, CXCR\textsubscript{4} or CCR\textsubscript{5}\textsuperscript{123} for infection of T cells. Further characterization of the virus itself revealed it to be a \textit{retrovirus}, more specifically a \textbf{lentivirus} (a subset of retroviruses), comprised of two copies of single-stranded RNA that are capable of reverse transcription (creation of DNA from RNA) and integration into the host genome\textsuperscript{123}. The virus wreaks havoc on
the immune system in many ways, but most significantly by depleting CD4+ T cells, which impairs cell-mediated immunity and eventually allows opportunistic infections that the host would normally be able to clear to do irreversible damage\textsuperscript{124-126}.

Transmission of HIV most commonly occurs via sexual contact resulting in exposure to either cell-associated or free infectious virus particles\textsuperscript{127}. This route of infection includes male-to-female, female-to-male or male-to-male sexual transmission across mucosal (epithelial cell) surfaces\textsuperscript{128}. Less commonly, HIV is transmitted via intravenous drug use or sharing of needles for other purposes\textsuperscript{129}, intrauterine or mother-to-child transmission during birth\textsuperscript{130}, or contact with infected bodily fluids including blood\textsuperscript{131}, semen\textsuperscript{132}, breast milk\textsuperscript{133,134} or vaginal fluid\textsuperscript{135}. The basic mechanisms of HIV infection, epidemiology of the disease, and the immune response to infection are reviewed in several manuscripts\textsuperscript{127,136-142}; however, the subject of this dissertation is the effect of established HIV-1 infection on a specific requisite thymocyte egress-mediating cell-surface receptor, S1P-R1.

Perturbations in lymphocyte count, cell populations, and cytokines during HIV-1 infection all have the potential to affect expression of cell-surface receptors. This had not been examined as pertains to thymocyte egress receptors prior to this work, but has been considered in other systems\textsuperscript{143,144}. Cytokine perturbations during HIV infection have for the most part been characterized in the periphery and not within the thymus proper. For example, it has been well documented that many pro-inflammatory cytokines peak several days after an initial infection event, and each cytokine has its own corresponding time point post-infection at which it peaks in concentration in the periphery\textsuperscript{145} (See Figure 5 which examines cytokine perturbations during HIV infection.)
Typical infection of the human thymus

HIV infection spreads to various organs of the body, including the thymus\textsuperscript{146-149}. As CD4 is expressed on a large proportion of thymocytes including double positive and single (CD4) positive cells, and CXCR4 is expressed at high levels within the cortical subset (mainly double positive cells) while CCR5 is expressed within the medullary subset (mainly mature single positive cells), there are ample targets for both CXCR4 and CCR5 tropic HIV infection within the thymus\textsuperscript{150}. Interestingly, I observed during the earlier days of my thesis research that CD25+FoxP3+ thymic Treg are preferentially infected by CCR5-tropic HIV-1, whereas CXCR4-tropic virus does not readily infect this thymic subset\textsuperscript{151}. Over the course of infection, susceptible subsets are depleted (Resop, R., unpublished data and see also\textsuperscript{146,152}), leaving immature double negative thymocytes and some single positive cells (especially CD8 SP) remaining. Moreover, CD4 is downregulated on infected T cells by gp120, an HIV accessory protein\textsuperscript{153,154} contributing to not only a depleted thymus but also a significantly altered profile of the remaining cells therein and a skewed peripheral T cell profile as well\textsuperscript{155}. However, the thymus continues to contribute to reconstitution, the extent to which it may perform this duty depending on success of HAART treatment within the individual, the stage of HIV infection, and the age of the infected individual, among other factors\textsuperscript{152}.

As these important changes in the human thymus during HIV infection needed to be examined, a model system was necessary. Fortunately, humanized mice are well suited for the modeling of HIV infection of the thymus. In SCID and RAG-/- thy/liv mice infected with CCR5 (R5) tropic or CXCR4 (X4) tropic HIV-1 (the two tropisms of HIV, utilizing the CCR5 or CXCR4 co-receptor) intraperitoneally or intrathymically, the thymus becomes robustly infected after several weeks\textsuperscript{156} (also see \textbf{Chapter 3} of the dissertation.) I have observed that cytokines, including Interferon-alpha (IFN-\(\alpha\)) and Tumor Necrosis Factor alpha (TNF-\(\alpha\)) are elevated in intrathymically infected
human fetal thymic implants in immunodeficient mice by five weeks post infection (Resop et al, Submitted for publication, see also Chapter 3). Because prior to this work little was known about the effect of HIV infection in the thymus on thymic egress receptors including S1P receptors, I examined whether these elevated cytokines, or other direct or indirect effects of the virus, altered the expression of cell-surface egress-modulating receptors on developing thymocytes, thus impacting functional egress of mature thymocytes from the thymus to the periphery. A transient boost in thymopoiesis following an initial infection event has been observed in models of Simian Immunodeficiency Virus (SIV)\(^{157-159}\), but the mechanisms involved or the duration of this effect of infection have not yet been elucidated. This question is discussed extensively in Chapter 3 of the dissertation.

### VIII. Concluding Remarks and Relevance to the Field

Prior to this work, it was unknown whether S1P and its receptors had a role in the egress of mature human thymocytes from the thymus to the periphery, as the only work on this subject to date had been performed in mice. Specifically, seminal work by Matloubian et al, which reported that S1P\(^{-/-}\) hematopoietic cells remained sequestered in the thymus after development into mature CD4+ and CD8+ T cells and could not leave for the periphery, was performed in a traditional (non-humanized) mouse model that could not be assumed to reflect the function of the S1P/S1P receptor system in humans\(^{48}\). Thus it was imperative to examine the function of the S1P/S1P receptor system in the human thymus and to identify and characterize the populations expressing S1P receptors and responding to S1P. This dissertation describes, in part, my work to characterize the expression and function of S1P receptors within the human thymus (Chapters 2 and 3).
As HIV infection results in extensive T cell depletion, there is an urgent need to examine approaches for reconstitution of the T cell “repertoire” in HIV infected individuals. Reconstitution of a peripheral repertoire depends largely upon functional and enhanced thymopoiesis, or generation of mature naïve T cells in the thymus. The egress of mature T cells following development is critical to reconstitution, and had thus far been examined only superficially, with Dion et al as well as others noting a transient increase in T cell output in the acute (earliest) stage of HIV infection^{160-162}, but little follow-up work having been performed to address whether this mechanism is maintained for a considerable duration or whether T cell output is quickly impaired following a short-lived initial burst. Other groups have noted that the thymus, as it is infected and the cells therein depleted, eventually declines in function in HIV patients^{163,164}. Many HIV patients, compliance with HAART therapy notwithstanding, do not experience ample reconstitution of the T cell/ TCR repertoire^{165}. To my knowledge, the function of S1P in the egress of mature thymocytes during HIV infection had not been examined prior to the work presented in Chapter 3 of this dissertation. S1P and its receptors, if found to be present and functional during HIV infection, could represent a tantalizing new possibility for therapy for HIV patients if the system were able to be modulated to promote enhanced thymic output and controlled so that only mature T cells would egress and repopulate the periphery.
Figure 1: Thymocyte maturation in the thymus
**Figure 2:** Egress of naïve T lymphocytes from the thymus

![Diagram showing the egress of naïve T lymphocytes from the thymus](image)

*Spiegel 2010*
**Figure 3:** The S1P gradient

- **Thymus Lobe**
- **Corticomedullary Junction**
- **Blood Vessel**
- **Thymus**
- **Cortex**
- **Medulla**
- **S1P concentration**
- ~100nM S1P

Resop, R. 2014
Figure 4: Generation of NSG thy/liv humanized mice

- 3 months
- NSG fetal thy/liv implant mice
- Fetal thymus/liver implanted
- Day 0 intrathymic injection HIV
- Week 5 sacrifice, analyze implant
- Week 9 sacrifice, analyze implant
- Retroorbital injection CFSE-labeled CD34+ cells (one day prior)

Resop R. 2012
Figure 5: Cytokine perturbations in HIV infection

<table>
<thead>
<tr>
<th>Cytokine</th>
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<th>Thymus (my NSG mice)</th>
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Stacey et al 2009

Resop R. 2014
CHAPTER 2:

S1P/ S1P-R1 signaling is required for mature human thymocyte egress from the thymus

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**Abstract**

The mechanisms that govern the egress of mature thymocytes from the human thymus to the periphery remain understudied, yet are of utmost importance to the field of basic immunology as well as T cell reconstitution in various immunodeficiencies. We examined expression and function of Sphingosine-1-phosphate (S1P) receptors in human thymocyte egress. We aimed to determine whether receptors to S1P play a role in mature human thymocyte egress and to identify the thymocyte population(s) that express S1P receptors (S1P-R) and respond to S1P by migrating across a concentration gradient. Human postnatal thymocytes were exposed to S1P in transwell-plate migration assays coupled to flow cytometry to evaluate the response of thymocytes of different stages of maturation to S1P. S1P-R expression was quantified by real-time PCR in sorted thymocyte subsets and by flow cytometry. Activation of the S1P signaling pathway was determined by detection of intracellular phosphorylated Akt. S1P-R1 was the prevalent S1P receptor on mature human thymocytes (CD3hiCD27+CD69-), which also demonstrated the greatest response to S1P in migration assays. Pretreatment with FTY720, an S1P-R1, 3, 4, and 5-agonist not affecting S1P-R2, significantly reduced migration, suggesting a role for S1P-R2 in retaining thymocytes in the tissue. Lastly, Akt phosphorylation increased in mature thymocytes upon exposure to S1P, indicating downstream signaling. Mature human thymocytes rely on S1P-R1 to migrate toward S1P. Taken in the context of murine work demonstrating that S1P is required for thymocyte egress to the periphery, our data highlight a new key chemokine for human thymocyte egress.

**Introduction**

Naïve T cells develop in the thymus from CD34+ hematopoietic progenitor cells that are produced in the bone marrow and enter the thymus from the blood. Fewer than 5% of
developing thymocytes survive positive selection which takes place in the cortex, and negative selection, which takes place mostly in the thymic medulla, and exit the thymus as naïve T cells at the corticomedullary junction\textsuperscript{16,166,167}. Stages of thymocyte differentiation are characterized by changes in cell surface antigens such as an increase in the expression levels of the CD3/T cell receptor (TCR) complex and upregulation of CD27, CD45RA and CD62L on mature medullary thymocytes\textsuperscript{161,168}. In addition, transcriptional regulators like the master transcriptional regulator Kruppel-Like Factor 2 (KLF2) play an important role throughout T cell development in the thymus by upregulating surface receptors necessary for migration throughout and egress from the thymus\textsuperscript{27}.

The thymic microenvironment is essential for the generation of naïve T lymphocytes and the production of factors that govern entry of CD34+ hematopoietic precursors from the bone marrow into the thymus, and those that control exit of naïve T cells from the thymus to the periphery\textsuperscript{16}. Since little is known of factors that influence exit of naïve T cells from the human thymus to the periphery, we examined Sphingosine-1-phosphate (S1P) and its receptors which have been shown to play essential roles in T and B lymphocyte migration in the mouse\textsuperscript{48,80,169}.

S1P is a chemotactic sphingolipid molecule with varied roles throughout the body, many of which have been characterized exclusively in murine models\textsuperscript{53,170,171}. S1P is highly concentrated in the blood, where it is produced by erythrocytes, endothelial cells and activated platelets\textsuperscript{170,172,173}, but is low in lymphoid tissues, where it is degraded by sphingosine lyase\textsuperscript{77}. Sphingosine kinase and sphingosine lyase maintain the S1P gradient, which promotes the influx of lymphocytes bearing one of its five G-protein coupled receptors (S1P-R 1-5)\textsuperscript{79,170,174}, to the lymph nodes. In the mouse, CD69, known as an activation marker that is expressed during positive and negative selection in the thymus, has to be downregulated before thymocyte
egress\textsuperscript{25,49}. CD69 expression is also downregulated on mature thymocytes before egress from the human thymus and is not present on recent thymic emigrants\textsuperscript{24}.

S1P/S1P-R1 ligation in the mouse is required for mature naïve T cells to egress from the thymus to the periphery and for memory T cells to egress from secondary lymphoid tissues\textsuperscript{48,49}. In mice, siRNA knockdown of S1P-R1 results in virtually no naïve T cells in the peripheral lymph nodes; treatment of mice with FTY720, an immune modulator that targets S1PR1, 3, 4 and 5\textsuperscript{175}, recapitulates this effect\textsuperscript{48,49}. Additionally S1P-R2 has been shown in the mouse to inhibit migration of maturing B cells in the germinal centers when ligated to S1P\textsuperscript{48} and retain T follicular helper cells in the germinal centers\textsuperscript{176}. However, no studies exist demonstrating the role of S1P and its receptors in human thymocyte egress.

Here we show for the first time that the S1P/ S1P-R1 axis plays a crucial role in human thymocyte egress and identify the phenotype of S1P-R1+ human thymocytes as CD3hiCD69- cells within the CD27+CD45RA+ medullary thymocyte subset. Mature CD3hiCD69-, but not CD69+, human thymocytes migrate toward S1P in \textit{in vitro} chemotaxis assays, and migration is inhibited by a specific agonist of S1P receptors. Expression of S1P-R2 shows donor variability, but our data suggest that it plays an opposite, inhibitory, role to S1P-R1. Other S1P receptors including S1P-R4 and S1P-R5 are expressed at negligible levels throughout all stages of thymocyte development, and S1P-R3 expression is not significantly different across thymocyte populations, supporting our hypothesis that S1P-R1 is the principal receptor responding to S1P for mature thymocyte egress. S1P-R3, 4 and 5 do not appear to be involved in thymocyte migration from the thymus to the periphery; whether these receptors play other roles throughout thymocyte development has to be examined.
Results

Only mature CD3hiCD27+CD45RA+CD62L+ thymocytes lacking CD69 expression migrate toward S1P.

To examine the function of the S1P/S1P receptor system in human thymocytes, we performed in vitro transwell migration assays. Human postnatal thymocytes were added to top chambers of transwell plates and S1P at the optimal concentration (100nM)\(^\text{48}\) was added to bottom chambers. Thymocytes were allowed to migrate for three hours, after which time the migrated cells were collected and stained using antibodies to identify different stages of thymocyte development, including CD3, CD4, CD8, CD27 and CD45RA, by flow cytometric analysis. As shown in Figure 1A, immature CD4/8 Double Negative (DN, mostly CD3-) and CD4+CD8+ Double Positive (DP, mostly CD3lo) thymocytes did not migrate to S1P. In contrast mature CD3hiCD27+ thymocyte subsets expressing either CD4 or CD8 did migrate to S1P (Fig. 1A). These findings indicate that thymocytes responding to S1P are located in the thymic medulla (CD27+).

To further characterize the medullary thymocyte subsets that respond to S1P, antibodies to CD69 and CD62L (L-Selectin) were added to our immunophenotyping panel. CD69 is an early activation molecule expressed during positive and negative selection in the thymus, but not present on thymocytes ready to leave the thymus or on recent thymic emigrants\(^\text{25}\). CD62L is expressed on naïve T cells and is important for homing to lymphoid tissues\(^\text{177}\). We found that mainly mature CD3hiCD27+CD62L+ thymocytes that have lost CD69 expression migrate to S1P (Fig. 1B). No migration to S1P was observed in thymocyte subsets that lacked CD62L expression. Our data confirm that 100nM S1P, reported as the optimal concentration in
mice\textsuperscript{53,178}, is also the ideal concentration for human cells. We tested a range from 0 to 1000 nM S1P and found that indeed cell migration peaked at 100nM (Fig. 1B) while at 1000 nM thymocytes became non-respondent, following a typical bell-shaped dose-response curve\textsuperscript{179}.

Next, we used FTY720, an agonist to S1P receptors 1, 3, 4 and 5, to verify the involvement of the S1P receptors in S1P-promoted migration in transwell plates. FTY720 mimics S1P and binds to S1P receptors, resulting in S1P-R1 receptor internalization\textsuperscript{55} and precluding migration due to lack of functional intracellular signaling. Mature CD4 and CD8 single positive thymocytes (CD3\textsuperscript{hi}CD27\textsuperscript{+}) pretreated with 100 nM FTY720 displayed impaired migration compared to untreated thymocytes (Fig. 1C and D). At the optimal concentration (100nM) of S1P for migration untreated mature CD4 and CD8 SP displayed approximately 4% and 7% migration respectively, whereas less than 1% of FTY720 treated CD4/CD8 SP migrated toward S1P in the lower chamber (Fig. 1C and D). Interestingly the migration of FTY720-treated CD4 SP thymocytes, and to a lesser extent migration of CD8 SP thymocytes, was consistently reduced at 10 nM of S1P compared to the basal level of migration through the transwell membrane without S1P (p<0.01). Since FTY720 does not antagonize S1P\textsubscript{2}, these results strongly suggest that S1P-R2 activation promotes migration away from S1P (S1P repulsion). Moreover it seems that S1P-R2 is more sensitive to S1P than S1P-R1 is as its peak response occurred at 10nM vs. 100nM for S1P-R1.

\textbf{S1P-R1 is the main S1P receptor expressed in the thymus and S1P-R1 mRNA is expressed to the greatest extent within the most mature, CD3\textsuperscript{hi}CD27\textsuperscript{+}CD69\textsuperscript{-}CD45RA+CD62L+, thymocyte subset.}
To determine at which stages of thymocyte maturation S1P receptors are expressed, we sorted total postnatal thymocytes into four populations based on maturation phenotype: CD3-CD27-CD69-, CD3loCD27-CD69+, CD3hiCD27+CD69+, and CD3hiCD27+CD69-. In addition we used CD45RA to ensure that the isolated populations represented homogeneous populations. Others and we have previously shown the CD3hiCD69- subset to be comprised of mature thymocytes presumably prepared to egress the thymus for the periphery\textsuperscript{24,180}, therefore we hypothesized that this subset would express S1P-R1 to the greatest extent. Considering the retentive role of S1P-R2 in B cells and Follicular Helper T cells within germinal centers, we additionally hypothesized that S1P-R2 may have an analogous role in thymocytes\textsuperscript{81,176}. Quantitative real-time PCR (qPCR) for S1P receptors 1-5 revealed that S1P-R1 is expressed to a significantly greater extent within the most mature CD3hiCD27+CD69- subset than the CD3hiCD27+CD69+ subset (p=0.01), which represents the preceding stage of thymocyte development (Fig. 2). S1P-R1 levels were approximately 30-fold the level of GAPDH (average of 5 experiments), whereas expression within CD3hiCD69+ thymocytes was on average less than 10-fold (Fig. 2). Moreover, we found that \textit{S1P}-R1 was the main S1P receptor mRNA expressed in human thymocytes, as qPCR revealed that expression of S1P-R2, 4 and 5 was quite low (Fig. 2). In addition, S1P-R 3, 4 and 5 do not show significant variable expression across populations in the human thymus and do not appear to have a major role in functional egress based on transwell migration assays using a specific antagonist to S1P-R1, W146 (data not shown).

Interestingly, expression of \textit{S1P}-R2 mRNA demonstrates high donor variability (N=3, Fig. 2). In accordance with the above described results, we hypothesized that S1P-R2 expression would be concentrated within the CD3hiCD69+ subset of sorted thymocytes, fitting with its potential role as an inhibitory receptor retaining maturing T cells in the thymus until completion of the final
stage of their development. This was indeed the case in a fraction of thymus tissues assayed, however, in thymi from other donors, \( S1P\)-R2 mRNA expression was higher in the most mature subset. Still \( S1P\)-R2 mRNA was always lower in immature CD3loCD27-CD69+ thymocytes than in mature CD3hiCD27+ thymocytes and was consistently the lowest in the least mature, CD3-CD27-CD69- thymocytes (Fig. 2). The donor variability was corroborated by transwell plate migration assays in which we utilized JTE013, a specific antagonist to S1P-R2, to elucidate the role of this receptor. We reasoned that if S1P-R2 constitutively hinders migration, we should see a relative increase in movement of cells treated with a specific S1P-R2 antagonist. Likely due to donor variability in \( S1P\)-R2 mRNA expression, migration in JTE013-treated thymocytes was quite variable, with heightened migration observed in several thymocyte donors but no effect in thymocytes from other donors (data not shown). Due to donor variability in \( S1P\)-R2 mRNA expression we cannot draw definitive conclusions on the impact of S1P-R2 expression and migration in the thymus.

Kruppel-Like Factor 2 (KLF2) is a purported master transcriptional regulator of S1P-R1 messenger RNA expression and regulator of other receptors necessary for egress of mature human thymocytes or migration throughout the thymus microenvironment, i.e. the C-type lectin CD62L and CCR5 (reviewed in \(^{181,26,28}\)). As it has been reported that KLF2 regulates S1P-R1 expression in peripheral T cells\(^{26,28,46,182}\), it follows that it may maintain a similar role in the human thymus. Thus we hypothesized that the highest S1P-R1 expressing subset should correspond to the subset expressing the greatest amount of KLF2 and examined KLF2 mRNA in parallel with S1P-R1 mRNA. KLF2 mRNA expression increases proportionally to S1P-R1, with the lowest expression being in CD3-CD69- (immature) thymocytes and increasing expression observed as thymocytes mature. As is the case with S1P-R1, CD3hiCD69-CD27+ thymocytes showed the highest expression of KLF2 (p<0.05, Fig. 2).
**S1P-R1 is expressed at the protein level on mature CD3hiCD69-CD27+CD45RA+CD62L+ thymocytes.**

Human postnatal thymocytes (aged 0 days to 3 years of age, median age 9.5 months, 15 donors) were stained with antibodies to S1P-R1 and CD3, CD27, CD45RA, CD62L, CD69, CD4 and CD8. To elucidate the immunophenotype of thymocyte subsets that express S1P-R1 we gated on immature CD3-CD69- and CD3loCD69+ populations within CD27- (cortical) thymocytes and compared these two populations with more mature CD3+CD69+ and CD3hiCD69- thymocytes within CD27+ (medullary) cells (gating scheme Fig. 3A). Confirming qPCR results described above (Fig. 2), we observed low levels of S1P-R1 protein expression within total thymocytes and minimal S1P-R1 expression on CD3-CD27-CD69-, CD3loCD27-CD69+ and CD3+CD27+CD69+ thymocyte subsets, whereas the predominant S1P-R1 expression was observed within the CD3hiCD27+CD69- thymocytes (Fig. 3B). S1P-R1 was expressed on approximately 30% of CD3hiCD69- thymocytes (average of 15 donors, range 5%-60%) as compared to only 1-5% of the three less mature populations (Figure 3C). We additionally examined S1P-R1 expression on CD45RA+CD69- thymocytes within the mature CD3hiCD27+CD62L+ subset and found S1P-R1 expression to be significantly higher within this subset than in CD45RA+CD69+ and CD45RA- immature thymocytes (p<0.01, data not shown).

S1P-R1+ thymocytes were found to be mainly CD3+ and high in CD27 (Fig. 4A.), and a significantly higher percentage of S1P-R1+ thymocytes expressed CD27 than the total thymocyte population (p=0.01, Figure 4B). S1P-R1+ cells in the thymus therefore consist mainly of CD3hiCD27+ mature thymocytes, which are CD4 or CD8 SP, thus in the final stages of their development (Fig. 4A.) The S1P-R1+ thymocytes that do not express CD3 are likely
thymic B cells, as we identified a CD3-CD19+CD45RA+S1P-R1+ population (Suppl. Fig. 1A-B) and to a lesser extent NK cells (CD3-CD19-CD45RA+S1P-R1+).\textsuperscript{183} As a small percentage of S1P-R1+ thymocytes does not fall within the most mature CD3hiCD69-CD27+ population, we determined the exact phenotype of the S1P-R1+ thymocytes. As shown in Figure 4C, within mature CD3hiCD27+CD69- thymocytes, S1P-R1+ cells were comprised of a significantly higher proportion of CD4SP than S1P-R1-, highlighting the correspondence of S1P-R1 with 4SP cells.

**S1P-R1 expression in postnatal and fetal thymocyte subsets.**

We as well as others have established that a high need for thymopoiesis during fetal development and in neonates translates to high expression of migration-associated cell surface proteins such as CD62L\textsuperscript{184}(also M. Epeldegui, unpublished data). We therefore hypothesized that S1P-R1 may be upregulated in fetal thymi as compared to postnatal thymi as a mechanism to promote egress of naïve T cells in the fetus. To examine this question we stained thymocytes prepared from fetal tissues, ages 14-21 weeks, with a median age of 16.5 weeks of gestation across the 18 donors, with the same panel of antibodies as postnatal specimens and analyzed CD3-CD69- and CD3loCD69+ within CD27- cells as well as CD3+CD69+ and CD3hiCD69- within CD27+ thymocytes. S1P-R1 expression in fetal thymocyte subsets was similar to postnatal thymocytes across the four populations of increasing maturation stage (Fig. 5, compare to Fig. 3C). Specifically, S1P-R1 expression was 24% in CD3hiCD69- fetal thymocytes vs. 30% in postnatal thymocytes, and 7% in CD3+CD69+ fetal thymocytes, vs. <5% in postnatal samples. Additionally, we analyzed S1P-R1 expression within CD3/CD69/CD27 subsets (all gated on CD45RA+ thymocytes, as done with postnatal thymus in Figure 3C). Similar to our observations in postnatal thymus, S1P-R1 expression increased throughout thymocyte developmental stages with the highest expression seen on CD3hiCD69-CD27+.
thymocytes (Fig. 5). Although it is intriguing that expression of S1P-R1 appears similar in the fetal and postnatal thymus, we were only able to characterize fetal thymi from the second trimester of gestation and thus we cannot extrapolate those observations to the last weeks of gestation when thymopoiesis may be heightened.

**Thymocytes increase Akt signaling, internalize S1P-R1 and downregulate S1P-R1 and KLF2 mRNA in response to S1P exposure.**

Ligation of S1P to its receptor, S1P-R1, can be reliably detected by measuring downstream Akt/PI3K signaling events\(^\text{185}\). Phosphorylation of Akt reflects S1P-R1 ligation by S1P, S1P-R1 internalization and initiation of intracellular signaling\(^\text{185,186}\). To examine the function of S1P-R1 when ligated to S1P, we performed intracellular flow cytometric detection of phosphorylated Akt (pAkt). As can be seen in Figure 6A, phosphorylation of Akt increased after 30 min of exposure of total thymocytes to S1P. Compared to isotype control-stained thymocytes and untreated thymocytes, mature thymocytes (CD3hiCD27+CD69-) exposed to S1P were significantly enriched in cells expressing pAkt, reflecting activation of the Akt/PI3K pathway (Fig. 6B). Because the Akt pathway signaling has been shown to be a measure of S1P-R1 activity\(^\text{185}\) we are able to conclude that the population with the highest S1P-R1 expression (mature CD3hiCD69-CD27+ thymocytes) is the same population that experiences heightened pAkt signaling in response to S1P. Moreover, upon exposure to S1P for 30 min mature thymocytes displayed reduced surface expression of S1P-R1, consistent with internalization and initiation of downstream signaling (Fig. 6C-D).

Little is known about the regulation of S1P-R1 in the human thymus. To determine whether the S1P-R1 gene is up- or downregulated upon ligation to S1P, we performed in vitro S1P exposure
assays and subsequently examined S1P-R1 and KLF2 expression by qPCR. Thymocytes prepared from human fetal thymic implants from NSG mice\textsuperscript{187,188}, which we show are similar in phenotype to fetal and postnatal thymocytes even after several weeks in the murine environment\textsuperscript{188} were exposed for 30 minutes to 100 nM S1P and analyzed by qPCR for S1P-R1 expression. Following exposure to S1P, S1P-R1 mRNA was significantly downregulated (p=0.0056) relative to untreated donor-matched thymocytes (Fold decrease, Fig. 7A). Interestingly, KLF2 mRNA was also significantly downregulated (p=0.0010) following exposure to S1P (Fold decrease, Fig. 7B). The trend of decreasing KLF2 alongside decreasing S1P-R1 expression after exposure to S1P is reminiscent of that observed for increasing KLF2 expression with rising S1P-R1 expression in developmental stages of thymocytes, and supports a potential role for KLF2 as a regulatory factor of S1P-R1 in human thymocytes as reported in murine models\textsuperscript{189}. To ensure that decreased S1P-R1 expression was due to a specific response to S1P and not the result of the incubation or medium, we examined expression of IL-7 receptor (IL-7R) mRNA in the same donor-matched samples, as this receptor is widely expressed on human thymocytes and is not expected to be directly regulated by KLF2 or directly respond to S1P exposure. IL-7R was not significantly altered by 30 minutes of S1P exposure (Fig. 7C).

\textit{Discussion}

We show for the first time the expression and function of the S1P receptors in the human thymus. Prior to our research there were very limited data on the mechanisms regulating egress of mature human thymocytes from the thymus to the periphery. Studies in the mouse examined the requirement for S1P-R1 in thymocyte egress, and showed that S1P-R1 is essential for the thymic egress of mature single positive murine thymocytes\textsuperscript{48,49}. However, no
reports to date have demonstrated whether the S1P/ S1P receptor pathway plays a role in the response of mature thymocytes to S1P or egress from the human thymus to the peripheral blood and lymphoid tissues; moreover, the exact population of thymocytes that expresses S1P-R1 has not been fully characterized either in mice or humans.

Our studies present an essential role for S1P-R1 in the human thymus and define the subset of mature human thymocytes that expresses S1P-R1 ex vivo and responds to S1P in vitro. We found that the CD3hiCD27+CD69- thymocyte subset displayed the highest percentage of migration toward S1P, in agreement with our observation that S1P-R1 at the mRNA and protein level is enriched in this thymocyte subset. This is the expected profile of mature thymocytes that are selected for egress to the periphery. Additionally, FTY720, an S1P-R1, 3, 4 and 5 agonist, nearly entirely abrogated migration, and a specific antagonist to only S1P-R1 (W146) resulted in a similar decrease in migration, which indicates that S1P-R1 is the main S1P receptor responsible for human thymocyte response to S1P. As anticipated since naïve T cells lack CD69 expression, loss of CD69 was required for thymocyte response to S1P. CD69 has been shown to negatively regulate S1P-R1 when overexpressed in cell lines, acting downstream of Interferon-α (IFN-α). We have previously demonstrated that CD69 is expressed on medullary thymocytes during the positive selection stage, but as shown by Vanhecke et al., CD69 is lost prior to thymocyte egress of mature naïve cells. We also previously showed that plasmacytoid dendritic cells, localized mainly in the thymic medulla, constitutively produce IFN-α in the human thymus, which supports the notion that CD69 may negatively regulate S1P-R1 expression throughout thymocyte development in an IFN-α dependent manner until the final stage prior to egress. Interestingly, Zachariah and Cyster published that S1P produced by pericytes, blood vessel ensheathing cells, contributes to the
egress of mature thymocytes from the murine thymus to the periphery at the cortico-medullary junction\textsuperscript{192}.

S1P-R1 mRNA was expressed to the greatest extent within the thymocyte population at the most mature stage of development. Of note, KLF2, a master transcriptional regulator found to dictate S1P-R1 expression in mice\textsuperscript{26,46,182} increased linearly in its expression throughout the four developmental stages of thymocytes we assayed, similar to the increase observed in S1P-R1 mRNA. In the mouse, KLF2 is regulated by FOXO1, a member of the FOXO family, that itself is controlled via phosphorylation by Akt, which prevents its binding to the promoter of KLF2 during intermediate stages of thymocyte development\textsuperscript{193}. The mechanism of the downregulation of KLF2 is not yet fully understood, but thought to be related to the relatively higher levels of TCR signaling during positive and negative selection. Upon the decrease in TCR signaling in the ultimate stages of T cell development, FOXO1 is not phosphorylated and is able to bind to KLF2\textsuperscript{193}, which in turn activates transcription of S1P-R1 and CD62L, two important egress-related molecules. Our data support a role for KLF2 in activation of S1P-R1 expression in the human thymus, and thus potentially a role for FOXO1 regulation of KLF2 and S1P-R1 in a TCR-signaling-dependent manner.

Using multicolor flow cytometry, we were able to identify the population of mature thymocytes expressing the highest percentage of S1P-R1 as CD3\textsuperscript{hi}CD69-CD27+CD45RA+ (mostly CD62L+) thymocytes (average percent expression 40-60\%). While expression of S1P-R1 was high on mature thymocytes at the RNA and protein level, the expression of S1P-R2 mRNA, another receptor for S1P, was low (Fig. 2) and in absence of a reliable antibody S1P-R2 could not be detected at the protein level. Interestingly, in transwell migration assays, migration of cells treated with FTY720, which inhibits all S1P receptors except S1P-R2, was lower at 10nM
of S1P than in the control without any S1P gradient, suggesting that activation of S1P-R2 repels thymocytes away from S1P. Moreover a specific antagonist to S1P-R2, JTE-013, enhanced migration of thymocytes from some donors, in keeping with our hypothesis that S1P-R2 may play an inhibitory role in the migration process in thymocytes (data not shown.) However, the effect of JTE-013 was not consistent and donor dependent. As S1P-R2 is mainly expressed in mature, medullary thymocytes, its function in the human thymus may be to retain mature cells throughout the final stages of selection prior to egress. From our results, it appears that S1P-R2 is activated at 10nM of S1P whereas S1P1 requires 100nM S1P for optimal activity. Therefore S1P-R2 signaling could be triggered in regions of the thymus where there is not enough S1P to activate S1P-R1 (further away from the blood vessels at the corticomedullary junction). In CD3hi69- cells, S1P-R1 expression is increased and as a consequence, signaling through S1P-R1 becomes dominant over S1P-R2 signaling; therefore attraction to S1P mediated by S1P-R1 becomes stronger than repulsion mediated by S1P-R2 and the thymocytes can egress. It has recently been reported that S1P-R2 plays a role in confining germinal center B cells to the follicle\textsuperscript{194} and retaining Follicular Helper T cells in the germinal center\textsuperscript{176}, further supporting a potential role for S1P-R2 as a retention factor during thymocyte development.

In murine models it has been demonstrated that S1P binds to its receptors and initiates a cascade of intracellular signaling via G-protein-coupled second-messengers and the PI3K/Mek/Erk/Akt pathway\textsuperscript{72,186}. Mudd \textit{et al.} used phosphorylation of Akt to show responsiveness of human lymph node T cells to S1P, as they could not detect S1P-R1 expression on these cells\textsuperscript{185}. However, the dynamics of S1P-R1/S1P binding and the molecular regulation of the S1P-R1 gene have not been examined in the human thymus. We found that following exposure to S1P mature populations expressing S1P-R1 (CD3hiCD27+CD45RA+CD69-) increased Akt signaling, decreased S1P-R1 and KLF2
expression and increased S1P-R1 internalization. Together these results imply that S1P-R1 is regulated by a negative feedback mechanism, likely involving the master transcriptional regulator KLF2, upon S1P/ S1P-R1 ligation and subsequent signaling. KLF2 has been reported to regulate the chemokine receptor CCR5 in human CD4 T cells and C-type lectin CD62L, which is expressed prior to thymocyte egress from the thymus, in murine T cells. KLF2 is also transiently expressed throughout thymocyte development in the mouse and upregulated in the single positive stage prior to mature thymocyte egress along with S1P-R1. Therefore it follows that in the human thymus, KLF2 may be a potential key regulator of the timing of thymocyte egress.

In conclusion, we our results suggest that S1P-R1 plays a major role in thymocyte egress. Prior to this work, it was unknown whether human thymocytes expressed S1P receptors or responded to S1P. Although several chemokine receptors, including CXCR4 and CCR5 are well characterized in human T cells, the crucial question of what tells mature naïve T cells to leave the thymus for the periphery remained to be investigated. Our data shed a new light on this question, revealing that, in the human thymus, only phenotypically mature cells expressing S1P-R1 are able to migrate to S1P. Taken in the context of the established requirement for S1P-R1 for thymocyte egress to the periphery in mice, our data highlight a new key requirement for human thymocyte egress.

In addition to its crucial role in thymocyte egress, the S1P/ S1P-R1 axis may have significant implications for immunodeficiencies as well as lymphoproliferative and other diseases in humans, when considered in the context of the thymus. In the case of immunodeficiencies, investigation of the potential to modulate S1P and its receptors in the thymus is warranted. If it would be possible to increase thymic output in individuals with low T
cell numbers or function without subjecting them to an increased risk of autoimmunity this potential therapy is an interesting option. HIV-infected individuals failing to reconstitute adequate functional T cells or patients after bone marrow transplantation may benefit from this approach. On the other hand, dampening the response to S1P, perhaps through novel, intrathymic uses of agonists or antagonists to its receptors, may prove useful in lymphoproliferative diseases, such as certain lymphomas. Local administration of the agonist to S1P-R1, 3, 4 and 5, FTY720 (Drug name: Fingolimod), has been successful in the treatment of Multiple Sclerosis (MS)\textsuperscript{196}, abrogation of asthma\textsuperscript{197} and enhancement of bone allograft incorporation\textsuperscript{198,199}. The potential for local modulation of the mechanisms of T cell egress is as of yet unexplored in the thymus and represents an intriguing new possibility. In addition, a new S1P-R1 specific agonist, Ponesimod has been developed and may have advantages over treatment with FTY720\textsuperscript{200,201}.

\textit{Materials and Methods}

\textbf{Tissue Collection and thymocyte preparation.} 
Postnatal thymus specimens were obtained from children undergoing corrective cardiac surgery at the UCLA Mattel Children’s hospital. Fetal thymus specimens were obtained from the UCLA CFAR Gene and Cellular Therapy Core. Thymocytes were prepared and cultured at $4 \times 10^7$ cells/mL as previously described\textsuperscript{156} in serum-free medium (AT+dBSA) comprised of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 1100µg/mL delipidated bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO), 85µg/mL transferrin (Sigma-Aldrich), 2 mM L-glutamine, and 25µg/mL penicillin/streptomycin.

\textbf{Flow Cytometry}
Surface immunophenotyping of thymocytes was performed as previously described\textsuperscript{202}. Monoclonal antibodies (mAb) were obtained from R&D (unconjugated S1P-R1 and IgG2b control), eBioscience (CD45RA PerCP-Cy5.5, CD8 APC-eFluor 780, CD25 eFluor 450, CD62L eFluor 605\textsuperscript{NC}, CD3 eFluor 650\textsuperscript{NC}), and Becton Dickinson (BD) (CD69 FITC, CD27 APC.) Cells were washed with Nano Crystal (NC) buffer (eBiosciences) with 0.3% Bovine Serum Albumin (Sigma-Aldrich), and resuspended in NC buffer before acquisition on a high throughput (HT)-LSRII cytometer (BD). The data were analyzed with FCS Express (De Novo software).

**Cell Sorting**

Thymocytes were stained with CD69 FITC, CD3 PE, CD27 APC and CD45RA PerCP-Cy5.5 at 10 million cells per tube in 1 mL AT+dBSA (total starting cell count 100 million cells) and sorted on a FACSAnria cytometer(BD). Cells were sorted on the basis of CD3 and CD69 expression utilizing CD27 and CD45RA to ensure purity of populations. Following sorting, cells were suspended in TriReagent (Trizol) (Invitrogen) for RNA isolation and subsequent PCR analysis as previously described\textsuperscript{191}.

**Quantitative Real-Time PCR**

RNA was quantified by Nanodrop and Taqman Quantitative Reverse- Transcription PCR (RT-qPCR) performed to determine the expression of S1P-R #1-5 and KLF2 genes relative to GAPDH internal control. Jurkat cells were utilized as positive controls and to generate standard curves for all S1P receptor genes. Primer-probe conjugates were obtained from Invitrogen\textsuperscript{™} (Life Technologies/Fischer Scientific) for S1PR1 (Assay ID: Hs01922614_s1), S1PR2 (Custom-designed assay using gene sequence from NCBI), S1PR3 (Assay ID: Hs00245464_s1), S1PR4 (Assay ID: Hs02330084_s1), S1PR5 (Assay ID: Hs00928195_s1) and KLF2 (Assay ID: Hs00360439_g1). Target gene expression was normalized to primers amplifying GAPDH mRNA.
on an Applied Biosystems 7300 Real-Time PCR instrument. Data were analyzed in Microsoft Excel and statistics were performed in GraphPad Prism 5 (see below.)

**S1P chemotaxis/migration assay**

Migration of thymocytes to S1P was assayed as described by Matloubian et al. Briefly, $1 \times 10^7$ cells in serum-free medium were loaded in the upper chamber and allowed to transmigrate for 3h at 37°C across 5µm Transwell filters (Corning Costar) to S1P (Sigma) at the indicated concentrations with or without agonists/antagonists, or in medium alone in the lower chamber. Cells from the input and cells that migrated were stained with mAb to CD3, CD4, CD8, CD25, CD27, CD31, CD45RA, CD62L and CD69 for immunophenotyping. Migrated thymocytes were enumerated by collecting events for a fixed time (180 sec.) on an HT-LSRII cytometer. Cells from the input were diluted 1/20 and enumerated in the same way in order to determine the percentage of transmigrating cells.

**S1P exposure assay and phosphorylated Akt intracellular stain**

Postnatal or fetal thymocytes were cultured at $2 \times 10^7$/mL in serum-free medium with or without 100nM S1P (Cayman) for 30 minutes at 37°C. For combined cell surface and intracellular staining to detect phosphorylated Akt after stimulation, cells were first surface stained with CD69 FITC, CD27 PE, CD45RA PerCP-Cy5.5, CD8 APC-eFluor 780, CD3 eFluor 650 NC, CD25 eFluor 450, and CD62L eFluor 605 NC immediately following stimulation, then fixed with 2% Paraformaldehyde for 10 min, permeabilized in PBS 0.2% Tween and stained for intracellular phosphorylated Akt with anti-pAkt- AlexaFluor 647 or IgG control antibody (BD Phosflow) at room temperature, washed with PBS 0.5% BSA in NC buffer and acquired on an HT-LSRII flow cytometer.
**Statistical analysis**

All analyses were conducted with GraphPad Prism 5 (GraphPad Software). Variables were expressed as means with standard error of the mean. One or two-way ANOVA and two-tailed Student’s t tests, paired when appropriate, were used to analyze differences between populations. A p value inferior or equal to 0.05 was considered significant (*: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001, ****: p ≤ 0.0001).

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Figure 1: Mature human thymocytes migrate to S1P.
Migration of thymocyte subsets to S1P was examined with transwell migration assays. A. Percentage of immature CD3- CD4-CD8- (DN) and CD4+CD8+ (DP) and mature CD3hiCD27+ thymocyte subsets expressing CD4 or CD8 that migrate to 100nM S1P in a transwell migration assay (total thymocytes in the upper chamber) (n=9, student’s t-test). B. Further immunophenotypic characterization of migrating mature CD3hiCD27+ thymocyte populations, using antibodies to CD69 and CD62L. C-D. Effect of FTY720, an S1P-R1, 3, 4 and 5 agonist, on migration of mature CD4SP (C) and CD8SP (D) thymocytes (Control: n=9 / FTY720: n=7, Dunnett’s multiple comparisons test, following 1-way ANOVA, to [S1P]=0nM for each condition ± FTY720).
Figure 2: S1P-R1 and KLF2 mRNA are expressed to the greatest extent within the most mature CD3hiCD69- thymocyte subset.

Thymocytes from five donors were sorted into four populations based on maturation phenotype: CD3-CD69-, CD3loCD69+, CD3+CD69+ and CD3hiCD69-. Population phenotypes were verified using CD27 and CD45RA as additional markers of developmental stages. RT-qPCR was performed on mRNA isolated from these four populations to examine mRNA levels of the five S1P receptors and KLF2. A. S1P-R1 mRNA. B. S1P-R2 mRNA. C. S1P-R3 mRNA. D. S1P-R4 mRNA. E. S1P-R5 mRNA. F. KLF2 mRNA.
Figure 3: S1P-R1 protein is expressed to the greatest extent on the most mature thymocyte subset about to egress the thymus.

Human postnatal thymocytes were stained with antibodies to S1P-R1, CD3, CD27, CD69, CD45RA and CD62L. **A.** Gating strategy for B. and C.: Thymocytes were gated on cortical or medullary thymocytes based on surface expression of CD3 and CD27. Cortical, CD27- thymocytes were divided into CD3-CD69- and CD3loCD69+ subsets; and medullary, CD27+, thymocytes into CD3+CD69+ and CD3hiCD69- (most mature) subsets. **B.** S1P-R1 profile of thymocyte subsets of one representative postnatal (2 yr old) thymus, gated as described above. **C.** Summary of S1P-R1 expression on cells from the 15 postnatal thymi, ranging from 0 days to 44 years old with the majority of donors falling between 1 week and 5 years of age (median age 9.5 months). Comparison of S1P-R1 expression on CD27+CD3hiCD69- thymocytes to less mature populations (p<0.0001 for overall trend, and p<0.0001 between CD3hiCD69- and all other subsets; 1-Way Anova followed by Tukey multiple comparison parametric test.)
Figure 4: S1P-R1+ cells are mature CD3hiCD27+CD45RA+CD62L+ CD4 SP and CD8 SP thymocytes.

Human postnatal thymocytes (age 0 days-44 years) were stained antibodies to S1P-R1, CD3, CD27, CD69, CD45RA, CD62L, CD4 and CD8. A. Expression of CD3 and CD27 in S1P-R1+ thymocytes. B. Comparison of CD27 expression within S1P-R1+ cells and total thymocytes. C. Percentage of S1P-R1+ thymocytes that are CD4+CD8+ or CD4/CD8 SP, as compared to S1P-R1- thymocytes (n=10).
Figure 5: S1P-R1 expression on fetal thymocytes.
Eighteen fetal thymi were analyzed, with a median age of 16.5 weeks of gestation. Expression of S1P-R1 on fetal thymocyte populations is shown (p<0.0001 for overall trend, and p<0.0001 between CD3hiCD69- and all other subsets, 1-Way Anova followed by Tukey multiple comparison parametric test.)
Figure 6. Expression of pAkt and S1P-R1 in mature CD3hiCD4+CD69- thymocytes after stimulation with S1P.

Postnatal thymocytes were exposed to 100nM S1P in serum-free medium for 30 minutes or untreated, and immediately stained with an antibody to phosphorylated Akt (pAkt). pAkt and S1P-R1 expression were analyzed in four thymocyte subsets of increasing maturation. A. pAkt expression in CD3hiCD69-CD4SP thymocytes (unstimulated vs. 30 min S1P exposure.) B. Fold change in pAkt following 30 min S1P exposure (p<0.01; Student’s T Test. C. Change in surface expression of S1P-R1 between thymocytes exposed to S1P (dark grey) vs. medium (mock, light grey). D. Fold change in S1P-R1 expression following 30 min S1P exposure of postnatal (PN) and fetal (FT) thymocytes, p<0.01; Student’s T Test.
Figure 7. S1P-R1 and KLF2 mRNA expression decrease after exposure of fetal thymocytes to S1P.
Fetal thymocytes from human thymus/liver implants in immunodeficient mice were exposed to 100nM S1P or medium alone for 30 min and subsequently analyzed for S1P-R1, KLF2 and IL7-R (control) mRNA expression. A. Fold change S1P-R1 mRNA expression with exposure to S1P (p=0.0056). B. Fold change KLF2 mRNA expression following exposure to S1P (p=0.0010). C. IL7-R mRNA expression fold change following exposure to S1P (p=0.3125, without outlier p=0.1).

Supp. Figure 1

Supplemental Figure 1: S1P-R1 expression in additional thymic subsets.
CHAPTER 3:

HIV-1 infection results in upregulation of sphingosine-1-phosphate receptor 1 on mature human thymocytes and functional naïve T cell response to S1P

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Abstract

Lack of T cell regeneration in HIV infection despite successful antiretroviral therapy is likely due in part to an impact on egress of naïve T cells from the thymus to the periphery. However, there are few data available elucidating the impact of HIV on these processes. We studied the effect of HIV on the receptors to Sphingosine-1-phosphate (S1P), a chemotactic sphingolipid. In the mouse it has been shown that S1P plays an important role in the egress of mature thymocytes to peripheral blood. We have shown that human thymocytes migrate to S1P and that FTY720 inhibits migration by functioning as an analogue to S1P, thereby downregulating S1P receptor 1 (S1P-R1), which is important for thymocyte egress. During T cell development S1P-R1 expression is significantly increased at the mRNA and protein level in the most mature CD3hiCD69- thymocyte subset about to exit the thymus as mature naïve T cells. We have also shown that in thymocytes S1P-R1 responds to S1P exposure with increased Akt phosphorylation, internalization of the receptor upon binding, and downregulation of S1P-R1 mRNA. Herein we present data on the dynamics of S1P-R1 expression in the human thymus during HIV infection, which has thus far not been described. Two separate series of NSG mice implanted with human thymus/liver (thy/liv) grafts and infected with both CXCR4- and CCR5-tropic HIV-1 were used to analyze the effect of HIV on S1P–R1 in the human thymus implant. Persistent infection and immune activation were verified by demonstrating that two Interferon alpha secondary genes, MxA and ISG15, are upregulated. Surprisingly, our results show that S1P-R1 as well as its transcriptional regulator, Kruppel-Like Factor 2 (KLF2) were both significantly upregulated in mature infected thymocytes at several time points after HIV infection. S1P-R1 function after HIV infection of the human thymus implants was investigated in vitro and ex vivo by directly measuring Akt signaling induced by S1P/ S1P-R1 binding. Our findings indicate that S1P-R1 signaling is not impaired in infected thymocytes, which is in contrast to
published data in other cell types that point to impaired S1P-R1 response in HIV infection. Moreover, we found that CFSE-labeled CD34+ progenitors developed into mature thymocytes in the human thy/liv implant of infected NSG mice and that a subset expressed S1P-R1 and still migrated to the periphery. Our main objective is to understand how diseases that cause inflammation like infections, cancer and autoimmune diseases, affect migration of lymphocytes from thymus and peripheral lymphoid tissues by changing S1P and S1PR expression. If S1P-R1 remains upregulated and fully functional at various time points post HIV-1 infection, this discovery may offer insight into T cell reconstitution mechanisms during infection as well as provide a potential alternate immunotherapy for patients.
Introduction

One of the most significant issues for HIV infected individuals is the regeneration of functional naïve T lymphocytes following onset of infection. T cell reconstitution after the acute phase of HIV-1 infection is incomplete in many individuals in spite of successful antiretroviral therapy\textsuperscript{203,204} and is associated with persistent immune activation\textsuperscript{205-207} (reviewed in\textsuperscript{206}). Increased levels of pro-inflammatory cytokines, such as Interleukin (IL)-6, IL-7, IFN-α, TNF-α, and chemokines, such as IP-10 (CXCL10), and expression of activation markers CD69 and CD38/HLA-DR on T cells in HIV-1 infection are characteristic signs of continuing immune activation and may contribute to mechanisms hindering T cell reconstitution\textsuperscript{145,206,208}.

There are only limited reports on the mechanisms regulating entry of progenitor cells into the human thymus and egress of mature T cells to the periphery, and no data to date exist describing the impact of HIV-1 infection on these processes. Murine studies have shown that Sphingosine-1-phosphate (S1P) and one of its G-protein coupled transmembrane receptors, S1P receptor 1 (S1P-R1) play essential roles in egress of naïve T cells from the thymus to the periphery\textsuperscript{80,169} as well as egress of memory T cells from the secondary lymphoid tissues\textsuperscript{48,49}. We have recently described an analogous role for S1P-R1 in the human thymus, showing that S1P-R1 is the main receptor responsible for response to S1P and S1P-mediated exit of human T lymphocytes from the thymus to blood, and have determined the subset of mature human thymocytes that expresses S1P-R1 and responds to S1P to be CD3\textsuperscript{hi}CD27+CD69-CD45RA+CD62L+ (Resop and Douaisi et al, manuscript submitted for publication). Whether normal function of this system is maintained in HIV infection of the thymus had not been examined prior to this work.
In this work we used our well-established Human Immune System (HIS) NSG mouse implanted with human fetal thymus/liver (thy/liv) tissue, in conjunction with \textit{ex vivo} and \textit{in vitro} assays, to examine the expression and function of S1P receptor 1 in human thymic implants in mice following HIV infection. We describe heightened expression of Interferon-\alpha secondary genes (MxA, ISG15) in HIV infection, establishing that persistent immune activation is present at five and nine weeks of infection and likely has implications for S1P-R1 and CD69 expression. S1P-R1 and its transcriptional regulator, the antiproliferative KLF2, are significantly increased at the mRNA expression level in total infected thymocytes and S1P-R1 protein is significantly increased in the mature CD27+CD3+CD69-CD45RA+CD62L+ thymic subset in HIV-infected thymi. We performed \textit{in vitro} phospho-flow based assays to investigate the functional response to S1P as measured by downstream pAkt signaling and documented cytokine expression changes in the infected human thymus implant as well as the effect of several of these cytokines on S1P-R1 and KLF2 expression in human postnatal thymocytes in order to unearth the mechanism behind the observed increase in S1P-R1.

Our studies suggest that, as S1P-R1 on mature human thymocytes appears to be functional during HIV infection, the observed increase in S1P-R1 expression contributes to regeneration of functional naïve T cells after the acute phase of HIV-1 infection and may therefore be a target for patients otherwise unresponsive to immunotherapy.
Results

S1P receptor 1 is upregulated by mature CD3hiCD69-CD27+ thymocytes in HIV-infected human thymi.

In order to determine whether HIV-1 infection causes changes in S1P-R1 expression, we utilized Human Immune System (HIS) NSG mice implanted with human fetal thymus/liver (thy/liv) tissue. HIS mice were infected intrathymically (direct injection of virus into the implant) with CCR5-tropic (JR-CSF), CXCR4-tropic (NL4-3), or mock (empty vector) HIV-1 and sacrificed at five or nine weeks. Well-established infection was verified by quantitative real-time PCR for multiply spliced tat/rev mRNA within thymocytes (Fig 1 E), and significantly elevated Interferon-α secondary gene expression at both 5 and 9 weeks (MxA and ISG15, Fig. 1A and B) further demonstrated the immune activation characteristic of established infection. To determine the level of S1P-R1 in mock and HIV-infected thymic implants, we performed quantitative real-time PCR for S1P receptor 1 messenger RNA on total thymocytes. S1P-R1 mRNA was elevated in infected thymi relative to mock-infected animals at 5 weeks (Fig. 1C), and significantly elevated (p=0.03) in both CCR5-tropic (JR-CSF) and CXCR4-tropic (NL4-3) infected animals at 9 weeks (Fig. 1F). We also examined KLF2 mRNA expression by qPCR and found it elevated in infected total thymocytes at 5 weeks (Fig. 1D) and significantly elevated (P=0.01) in both JR-CSF and NL4-3 infected whole thymocytes at 9 weeks (Fig. 1G).

In order to elucidate the populations upon which S1P-R1 was upregulated, thymocytes were stained for flow cytometry with S1P-R1 antibody and nine additional thymocyte markers. S1P-R1 protein expression was slightly increased in total infected thymocytes at 5 and 9 weeks relative to mock-infected thymi (Supplemental Fig. 1). Total postnatal thymocyte profiles were
compared to thymocytes gated on several combinations of immature and mature populations in
order to determine the precise populations expressing S1P-R1. We gated on CD3-CD69- and
CD3loCD69+ populations within CD27- (cortical) thymocytes as well as CD3+CD69+ and
CD3hiCD69- thymocytes within CD27+ (medullary) cells (gating scheme Fig. 2B). The greatest
S1P-R1 expression is observed on CD3hiCD69- human thymocytes in healthy, uninfected fetal,
postnatal and adult thymi (Resop and Douaisi et al, in submission). Therefore we anticipated
that this population would be responsible for the observed overall increase in S1P-R1. Indeed,
S1P-R1 expression was significantly increased in CD3hiCD69- thymocytes at both 5 and 9
weeks, with mean expression of S1P-R1 in the population 50% at 5 weeks and 70% at 9 weeks,
as opposed to 10% and 30% in mock, respectively (5 weeks: Fig. 2A and C, overlay of
populations Fig. 2B; 9 weeks: Fig. 3A and C). Of great interest, we also observed an
upregulation of S1P-R1 by a population of thymocytes that normally expresses only negligible
amounts of the receptor, the CD3+CD69+ population. Mock-infected animals expressed minimal
levels of S1P-R1 within this population at 5 and 9 weeks (less than 5% S1P-R1+ within the
CD3+CD69+CD27+ population); however, infected animals at these time points had a markedly
significant increase in S1P-R1+ cells within the CD3+CD69+CD27+ population (mean 51%)
(Fig. 2B and D). The Mean Fluorescent Intensity (MFI) of S1P-R1 within the CD3+CD69+ population
was additionally slightly higher than that of the CD3hiCD69- population (5 weeks:
Fig. 2B). The expression of S1P-R1 during HIV infection in a population that does not
constitutively express this egress receptor in the healthy thymus is of great interest and an area
of continuing investigation. Thus, expression of S1P-R1 is not disrupted but enhanced during
acute HIV infection.
S1P-R1-expressing thymocytes from infected thymi are not impaired in Akt signaling in response to S1P ex vivo.

We next examined whether populations in the human thymus that upregulate S1P-R1 as a result of HIV infection are able to respond to S1P. We used a phospho-flow based pAkt assay to ascertain the level of phosphorylated Akt within thymocyte populations following S1P exposure, as phosphorylated Akt has been previously employed by our lab and others as a proxy for S1P-R1 downstream signaling (Resop and Douaisi et al, manuscript submitted for publication, and185). Whole thymocytes prepared from human fetal thymic implants from NSG mice infected with dual-tropic HIV-1 for two weeks (kind gift from D. Vatakis, UCLA AIDS Institute) were exposed to physiological concentration of S1P100nM53,54,178 or media alone for 30 minutes. Following S1P exposure, thymocytes were immediately fixed and stained with anti-phosphorylated Akt, S1P-R1, and eight additional human thymocyte markers. We gated on several populations of mature thymocytes in order to examine pAkt expression on S1P-R1+ cells. As intracellular staining perturbs surface receptor phenotyping, we corroborated the S1P-R1+ population gating with CD3, CD69, CD27 and CD45RA expression.

We determined the fold change in pAkt following S1P exposure in total thymocytes and compared it to that of mature CD3hiCD69-CD27+ thymocytes. Following S1P exposure, mock-infected thymi increased pAkt signaling by two-fold, whereas there appeared to be a slightly higher, albeit non-significant, fold change in HIV-infected total thymocytes (Fig. 4A and B). By gating on mature CD3hiCD69-CD27+CD4SP thymocytes, the subset expressing and upregulating S1P-R1 in HIV-infected thymi, we were able to determine that there was a no significant difference in the fold change in pAkt expression in 2 week dual-tropic HIV infected thymi relative to mock-infected thymi (Fig. 4A and C). This indicates that the population of
thymocytes that upregulates S1P-R1 retains signaling via the Mek/Erk/Akt pathway and that these thymocytes are functionally responsive to S1P.

**S1P-R1 upregulation on CD3hiCD69-CD27+ thymocytes permits functional thymic entry and egress in vivo.**

Having determined that S1P-R1 is upregulated on mature CD3hiCD69-CD27+ thymocytes during HIV infection, and that this population maintains functional Akt signaling, we examined egress from the thymus in vivo in HIV infected implants in NSG mice. CFSE-labeled CD34+ human hematopoietic progenitors were retroorbitally injected into NSG mice 24 hours prior to the first sacrifice (5 weeks) in order to examine whether functional entry of thymocyte precursors into the thymus occurred with HIV-infected thymi. Thymocytes from implants of mock, CCR5-tropic (JR-CSF) and CXCR4-tropic (NL4-3) HIV infected animals were analyzed by flow cytometry for expression of CFSE, S1P-R1 and various additional surface receptors. We found that a small population of CFSE+ cells trafficked to the thymus by 24 hours post-injection. These cells were CXCR4+ and CD34+ (Fig. 5.)

Additionally, we examined whether thymic egress and T cell development were functional in thymic implants from infected animals by analyzing the spleens for mature T cells expressing CFSE, which would indicate that CD34+ hematopoietic progenitor cells had entered the thymus, developed into mature T lymphocytes, and egressed the thymus for the periphery, then trafficked through the spleen. At 9 weeks post-infection (4 weeks post-retroorbital injection of CFSE labeled CD34+ cells), we observed CFSE+ mature CD4 and CD8 T cells in the spleens of both CCR5 and CXCR4-tropic HIV infected animals. At this time point we also observed CFSE+ cells in the thymus, indicating that entry into the thymus and egress from the thymus to
the periphery were both functional 9 weeks post-infection and 4 weeks post-injection of CD34+ precursors (% data not shown).

**S1P-R1 upregulation is due to an Interferon-α-independent mechanism.**

Interferon alpha (IFN-α) is upregulated in the periphery during acute HIV-1 infection\(^{214-216}\). We expanded on the current knowledge of IFN-α dysregulation during immune activation attributable to HIV infection by examining IFN-α levels in infected thymic implants and the effect of this cytokine on S1P-R1 expression. We analyzed IFN-α secondary gene expression (Interferon-Stimulated Gene 15 (ISG15) and MxA) and found that both were significantly upregulated in CXCR4-tropic HIV infected animals at 5 weeks and CCR5 and CXCR4-tropic HIV infection at 9 weeks indicating heightened IFN-α activity (Fig. 6A and B). We then examined the effect that this important pro-inflammatory cytokine may have on S1P-R1 expression in the thymus. Recent reports indicate that CD69 negatively regulates S1P-R1\(^{49}\), and dogma dictates that CD69, an activation marker, is elevated systemically in HIV infection and correlates with plasma viral load\(^{49,217,218}\). Therefore we anticipated that IFN-α exposure might maintain elevated CD69 levels and thus downregulate S1P-R1. We exposed postnatal thymocytes to two concentrations of IFN-α for 18 hours (1000 and 10000 U) and then examined S1P-R1 expression by qPCR. Indeed, following 18 hours of treatment, S1P-R1 mRNA was downregulated on whole thymocytes, with the greatest decrease in S1P-R1 mRNA expression occurring in thymocytes exposed to the higher concentration of IFN-α (Fig. 6C). This observation was highly interesting considering that even though overall S1P-R1 was upregulated in infected thymi, IFN-α alone downregulated the receptor on thymocytes. In light of the fact that we observed upregulated S1P-R1 in CD3+CD69+CD27+ and CD3hiCD69-CD27+ populations by flow cytometry, these data indicated that there are other mechanisms
contributing the upregulation of the receptor and that these mechanisms are able to override the requirement for CD69 downregulation which allows for constitutive S1P-R1 expression.

**Tumor Necrosis Factor-α is increased in the HIV-infected thymus and may contribute to S1P-R1 upregulation.**

We examined infected and mock-infected thymocytes by qPCR in order to determine whether additional cytokines were perturbed during persistent infection of the thymus. We found that Tumor Necrosis Factor alpha (TNF-α) levels were elevated by 5 weeks in both JR-CSF and NL4-3 infected thymocytes (data not shown). As TNF-α is a pro-inflammatory cytokine that participates in the well-documented cytokine storm during HIV infection and we had previously found that IFN-α, another pro-inflammatory cytokine, did not upregulate S1P-R1, we hypothesized that TNF-α may contribute to upregulation of the receptor and potentially override the dampening effects of IFN-α on S1P-R1 expression. To examine this question, we exposed human postnatal thymocytes to TNF-α in vitro for 18 hours and examined S1P-R1 mRNA by qPCR. We found that following the 18 hour incubation with TNF-α, S1P-R1 was upregulated relative to the untreated donor-matched control. Upregulation of the receptor was observed with three concentrations of the cytokine, 50, 100 and 500 ng/mL, with the greatest effect observed at 100 ng/mL (Fig 7 A).

IFN-γ exposure also resulted in upregulation of S1P-R1 mRNA, albeit less strikingly so than with TNF-α treatment, indicating that both cytokines likely contribute to the elevated S1P-R1 expression we observed in infected thymi, with TNF-α having a greater upregulatory effect, whereas IFN-α has the opposite effect, dampening or controlling S1P-R1 mRNA transcription.
IFN-γ exposure for 18 hours upregulated S1P-R1 at concentrations of 50, 100 and 200 ng/mL, with the greatest effect being at 50 ng/mL (data not shown).

As TNF-α appeared to have the greatest effect on S1P-R1 expression at the mRNA level, we examined S1P-R1 protein expression by flow cytometry following 24 hours of exposure to TNF-α at the same three concentrations. As can be seen in Figure 7C-D TNF-α-treated mature human postnatal thymocytes (CD3hiCD45RA+CD27+CD69-) demonstrated a statistically significant fold increase in S1P-R1 Mean Fluorescence Intensity (MFI) at 100 and 500ng TNF-α, but not at 50ng TNF-α (average of 7 postnatal donors, gated on CD3hiCD27+CD69-thymocytes). Interestingly, S1P-R1 expression was not consistently increased within this subset; however MFI of mature S1P-R1-expressing subset was consistently increased.

IFN-γ exposure also upregulated S1P-R1 at the protein level (peak upregulation at 200mg/mL, however the effect was less striking than TNFα exposure (Fig. 7B). Together these results indicate that TNF-α, and IFN-γ to a lesser extent, contribute to the heightened S1P-R1 expression we observed in infected thymi, and that INF-α moderates S1P-R1 expression, preventing it from becoming too elevated. Pro-inflammatory cytokines other that IFN-α elevated in HIV-infected thymi may override the requirement for downregulation of CD69 prior to S1P-R1 expression, in light of our observation that CD3+CD69+CD27+ thymocytes in infected thymi began to upregulate S1P-R1 (Fig. 2), while this population in mock-infected thymi never expresses S1P-R1.

**Discussion**

The identification of factors that promote egress of mature single positive thymocytes to the periphery is crucial to the field of study of T cell reconstitution during HIV infection. A transient
increase in thymic output following acute HIV infection has been reported\textsuperscript{160-162}, but whether this is maintained for a significant period of time is unknown, and the receptors and chemokines responsible for changes in egress during HIV infection remained understudied prior to this work. We examined S1P and its receptors, a system which has thus far not been examined in T cell reconstitution during HIV infection. To perform these studies we employed the innovative Human Immune System (HIS) NSG thy/liv mouse model implanted with human fetal thymus and liver tissue. Importantly, this advanced \textit{in vivo} model involves essentially complete humanization of the immune system with primary human cells that reside in several anatomic locations and thus closely mimics the situation in HIV infected humans. We observed an increase in the chemotaxis receptor S1P-R1 in HIV-infected thymic implants in humanized mice at 5 and 9 weeks post-infection, which raises the critical new question of whether upregulated S1P-R1 translates to enhanced functionality of thymocyte egress, and, if so, whether this mechanism may be modulated or whether upregulation of S1P-R1 may be prolonged as a means to new therapeutics for HIV patients that, even with HAART, are unable to recover optimal CD4 levels.

We observed by flow cytometry as well as qPCR that S1P-R1, the S1P receptor most prevalent in the human thymus and found on mature CD3hiCD69-CD27+CD45RA+CD62L+ thymocytes about to egress the thymus for the periphery (Resop and Douaisi et al, manuscript submitted for publication), is upregulated on this population during HIV-1 infection of the human thymus at 5 and 9 weeks post-infection. Interestingly, a less mature population of thymocytes, the CD3+CD69+ population, also upregulated S1P-R1 at both 5 and 9 weeks in both CXCR4- and CCR5- tropic HIV-infected thymi, but not in mock-infected thymi. This implies that mechanisms that control S1P-R1 expression are activated during HIV infection, and allow the receptor to be expressed at an earlier stage of development than is observed in the healthy thymus. A transient increase in thymic output during SIV infection of Rhesus Macaques, characterized by
reduced intrathymic proliferation in the thymus and a change in the cytokine profile of the thymus allowing for more rapid progression throughout the thymus microenvironment and egress to the periphery, has recently been reported\textsuperscript{220}. Our observation that S1P-R1, a necessary chemotactic molecule in the human thymus, is increased during infection and expressed at an earlier stage of development than is normally observed, may support the possibility of enhanced thymic output contingent upon speedier development in the thymus. Moreover, we observed that KLF2, an anti-proliferative transcription factor\textsuperscript{221-223} which is also necessary for the regulation of T cell egress from the thymus\textsuperscript{27} and has been shown to regulate the expression of S1P-R1 in other systems as well as additional chemotaxis receptors such as CD62L, CCR3 and CCR5\textsuperscript{28}, was also upregulated in the infected thymus at both 5 and 9 weeks of infection. As KLF2 dampens proliferation and upregulates S1P-R1, it is a likely candidate for effecting the changes we have observed in S1P-R1 expression in HIV infection as well as changes observed by others in intrathymic proliferation time. We are currently exploring the possibility that KLF2 may be directly activated by HIV accessory proteins and that this mechanism may contribute to elevated S1P-R1 in the infected thymus.

It has been shown that IFN-α, as it is induced after immune activation or viral infection, interferes with the egress of mature murine thymocytes\textsuperscript{49}. In HIV-induced immune activation, increased levels of IFN-α and CD69 are present\textsuperscript{208}, which are likely to influence S1P receptor expression and thereby entry of hematopoietic stem cells into the thymus as well as exit of naïve T cell subsets to the periphery. We expanded on the current dogma that CD69 negatively regulates S1P-R1 expression by examining the effect of the cytokine on postnatal human thymocytes. We observed that following an 18 hour incubation with IFN-α, S1P-R1 mRNA was downregulated. Although we observed elevated IFN-α secondary gene activity in the infected human thymic implants at 5 and 9 weeks post-HIV infection, we surprisingly observed that S1P-
R1 mRNA and protein were significantly elevated and not decreased at both time points. Therefore, there must be a mechanism present that overrides the requirement for downregulation of CD69 in order for S1P-R1 to be expressed. Our observation of S1P-R1 upregulation in CD3+CD69+ thymocytes corroborates this possibility. We are currently examining the role of KLF2 in overriding the requirement for CD69 downregulation prior to S1P-R1 expression.

Additionally, we further tested cytokines present in the thymus for their ability to change the expression of S1P-R1. We observed that TNF-α was elevated in HIV-infected thymic implants by 5 weeks post-infection. We therefore hypothesized that this cytokine may contribute to heightened S1P-R1 expression, potentially overcoming the effects of IFN-α. Indeed, after 24 hours of incubation with TNF-α, S1P-R1 protein was increased on mature CD3hiCD69-CD27+ thymocytes. Peak upregulation occurred at 100ng/mL TNF-α, with the effect dampened at higher concentrations. This effect was corroborated by qPCR, where we observed that 18 hours of TNF-α incubation increased S1P-R1 mRNA expression. IFN-γ incubation also resulted in an increase in S1P-R1 as measured by flow cytometry and qPCR, albeit a less striking increase than TNF-α treatment. Together these results indicate that both TNF-α and IFN-γ likely contribute to increased S1P-R1 expression in the HIV-infected thymus, whereas IFN-α acts to control the receptor’s expression. As there are several additional cytokines present in the thymus, many of which may be perturbed by HIV infection145,224 we are unable to definitively assign roles to these cytokines alone in effecting changes in S1P-R1 on mature thymocytes. The majority of reports on the “cytokine storm” during immune activation secondary to HIV infection have focused on the periphery145,219,225,226; therefore additional work to characterize the changes in cytokine profiles in the HIV-infected thymus and the effects of these changes on chemotactic receptor expression and function is of the utmost need.
Finally, we examined the function of S1P-R1 signaling (by pAkt staining) on mature human thymocytes from HIV-infected thymi and observed that function is maintained during HIV infection. This observation is highly interesting in light of the fact that S1P-R1, were it to be considered as a potential target for therapeutic modulation for HIV infected individuals or persons affected by other immune disorders, would need to maintain its normal function in order to be beneficial. Our results indicate that S1P-R1 upregulated on mature human thymocytes in the infected thymus does indeed retain function and in some donors pAkt signaling post S1P exposure actually increased (this increase was not significant; overall there was no significant difference between HIV infected and mock thymi as pertained to pAkt signaling). This implies that future therapies aiming to maintain adequate T cell count in HIV infected individuals may be a feasible possibility, if S1P-R1 expression can be modulated such that it is appropriately increased on the population desired but not on immature populations not yet ready to egress. We have yet to examine the semi-mature, CD3+CD69+ medullary population of thymocytes that also upregulated S1P-R1 and determine whether downstream signaling is still functional on this population. Taken together, these data contribute to the field of T cell reconstitution in an entirely novel manner. S1P-R1 is required for T cell egress from the thymus, however to our knowledge we are the first to characterize the receptor’s expression and function in the human thymus; observe that HIV alters S1P-R1 expression; report that S1P-R1, while elevated, remains functional during HIV infection of the thymus; and identify cytokine changes in the infected human thymus that contribute to upregulated S1P-R1. As it appears that S1P-R1 in mature thymocytes remains functional, targeting the S1P receptor system may represent an intriguing new possibility for treatment targeting patients who lack immune system reconstitution in response to ART.
**Materials and Methods**

**Murine Infection with HIV**

NOD SCID Gamma (NSG) mice were implanted with human fetal thymus/liver (thy/liv) tissue as described\(^{209}\) and were injected intrathymically with CCR5-tropic (JR-CSF, two series of mice, four animals in each), CXCR4-tropic (NL4-3, 2 series of mice, 4 animals in each) or mock (empty vector, two series of mice, three animals in each) virus. Animals were sacrificed at 5 or 9 weeks post-infection and thymi and spleens were obtained for *ex-vivo* assays and flow cytometric/qPCR analyses. One day prior to the 5 week time point, CFSE-labeled CD34+ hematopoietic progenitor cells were injected retroorbitally into all mice in order to track entry into and egress from the thymic implant.

**Phosphorylated Akt intracellular flow assay and S1P exposure**

Whole fetal thymocytes or splenocytes from humanized mice in suspension were stimulated with 100nM S1P (Cayman) for 30 minutes or untreated at 37C in AT+dBSA media. Immediately following stimulation, cells were fixed in 2% Paraformaldehyde, permeabilized in PBS .2% Tween and stained for intracellular phosphorylated Akt with pAkt- APC antibody (eBiosciences, IgG control also from eBiosciences) in addition to multiple surface markers: CD69 FITC (5µL), CD27 PE (5µL), CD45RA PerCP-Cy5.5 (2.5µL), CD8 APC-eFluor 780 (1µL), CD3 eFluor 650\(^{NC}\) (2.5µL), CD25 eFluor 450 (1µL), and CD62L eFluor 605\(^{NC}\) (2.5µL).

**Flow Cytometry**

Flow cytometry data were acquired on a LSRII analyzer (Becton Dickinson) and analyzed with FCS Express (De Novo software). Surface immunophenotyping of thymocytes with unconjugated and directly conjugated antibodies was performed as previously described\(^{202}\).
Monoclonal antibodies (mAb) were procured from R&D (unconjugated S1P-R1, unconjugated IgG2b control), eBioscience (CD45RA PerCP-Cy5.5, CD8 APC-eFluor 780, CD25 eFluor 450, CD62L eFluor 605NC, CD3 eFluor 650NC), and BD (CD69 FITC, CD27 APC), and were used at the volumes specified above and 5µL S1P-R1 and IgG2b.

Quantitative Real-Time PCR
RNA was isolated from cells suspended in Trizol Reagent by the Trizol® protocol. RNA was quantified by Nanodrop and Quantitative Real-Time PCR performed to determine the expression of S1P-R1-5 and KLF2 genes relative to GAPDH internal control. Jurkat cells were utilized as positive controls for all S1P receptor genes, from which standard curves were generated. Pre-designed primer-probe conjugates were obtained from Invitrogen™ (Life Technologies/Fischer Scientific, Grand Island, NY, USA) for S1PR1 (Assay ID: Hs01922614_s1), S1PR2 (Custom primer/probes using sequence from NCBI), S1PR3 (Assay ID: Hs00245464_s1), S1PR4 (Assay ID: Hs02330084_s1), S1PR5 (Assay ID: Hs00928195_s1) and KLF2 (Assay ID: Hs00360439_g1). Target gene expression was normalized to primers amplifying GAPDH mRNA. Data were analyzed in Microsoft Excel and statistics were performed in GraphPad Prism 5 (see below).

Statistical analysis
All analyses were conducted with GraphPad Prism 5 (GraphPad Software). Variables were expressed as means with standard error of the mean. One or two-way ANOVA and two-tailed Student’s t tests, paired when appropriate, were used to analyze differences between populations. A p value inferior or equal to 0.05 was considered significant.
Figure 1: S1P-R1 and KLF2 mRNA increase during HIV infection in the thymus.
A. Interferon-stimulated gene (ISG) expression increases by 5 weeks post infection in NSG mice. B. Interferon-stimulated gene (ISG) expression is increased by 9 weeks post infection in NSG mice. C. S1P-R1 gene expression increases by 5 weeks of infection in NSG mice. D. KLF2 gene expression increases by 5 weeks post infection. E. S1P-R1 increases by 9 weeks of HIV infection in NSG mice (p<0.05). F. KLF2 increases by 9 weeks post infection (p<0.01).
Figure 2

A. CD3-CD69-

B. CD3loCD69+

C. CD3hiCD69+

D. CD3hiCD69-

S1P-R1 expression in CD3hiCD69-

* Percent S1P-R1+
Figure 2: S1P-R1 and KLF2 protein increase during HIV infection in the thymus (5 weeks).  
A. S1P-R1 protein expression increases by 5 weeks of HIV infection in NSG mice in CD3hiCD69+ and CD3hiCD69- thymocytes.  
B. Gating schematic and overlay of S1P-R1 expression in CD3/69 populations.  
C. S1P-R1 protein expression increases by 5 weeks of HIV infection in NSG mice in CD3hiCD69- thymocytes (summary).  
D. Comparison of S1P-R1 expression (protein) in the four CD3/CD69 populations examined.
Figure 3: S1P-R1 and KLF2 protein increase during HIV infection in the thymus (9 weeks).

A. S1P-R1 protein expression increases by 9 weeks of HIV infection in NSG mice in CD3hiCD69+ and CD3hiCD69- thymocytes. B. Overlay of S1P-R1 expression in CD3/69 populations. C. S1P-R1 protein expression increases by 9 weeks of HIV infection in NSG mice in CD3hiCD69+ thymocytes (fold change, summary). D. S1P-R1 protein expression increases by 9 weeks of HIV infection in NSG mice in CD3hiCD69- thymocytes (raw expression, summary).
Figure 4: Akt signaling is not impaired during HIV infection in the thymus.

A. pAkt increases upon exposure to S1P for 30 min to a similar extent in mock and HIV infected thymi.

B. There is no significant difference in thymic pAkt expression between mock and HIV infected animals (total thymocytes).

C. There is no impairment in thymic pAkt expression between mock and HIV infected animals (mature thymocytes).
**Figure 5:** CFSE+ cells are observed in the HIV infected thymus by one day post injection.
Figure 6: S1P-R1 upregulation is due to an Interferon-α-independent mechanism. A. MxA expression increases by 9 weeks post HIV infection in the thymus. B. ISG15 expression increases by 9 weeks post HIV infection in the thymus. C. Exogenous IFNα treatment decreases S1P-R1 mRNA expression in thymocytes.
Figure 7

A. S1P-R1 expression with TNFα

B. S1P-R1 expression with IFNγ

C. All gated on CD3hiCD69-CD27+

Untreated

24 hours cytokine incubation

TNFα 50 and 100 ng/mL

IFNγ 100 and 200 ng/mL
Figure 7: Tumor Necrosis Factor-α is increased in the HIV-infected thymus and may contribute to S1P-R1 upregulation.

A. Exogenous TNFα treatment increases S1P-R1 mRNA expression. B. Exogenous IFNγ treatment increases S1P-R1 mRNA expression. C. TNFα and IFNγ exposure at various concentrations increases S1P-R1 protein mean fluorescence intensity in mature thymocyte populations. D. Mean fluorescence intensity of S1P-R1 is increased to a statistically significant extent with TNFα treatment of thymocytes.
CHAPTER 4:
Alternative markers for maturation stages of human thymocytes: Expression of CD31 (PECAM-1) during T cell differentiation in the human thymus

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Abstract

Immunologists have often regarded CD31 (PECAM-1) as an endothelial cell lineage marker without an established functional role. However, recent reports have indicated that there may be more to the story of CD31 than originally meets the eye: it is now thought to have roles in adaptive immunity, TCR signaling modulation, T cell homeostasis, and trafficking. Expression of CD31 (PECAM-1) on human thymocytes has been reported; however, an analysis of its expression at various stages of T cell development in the thymus was lacking prior to this work. With this investigation we examine the evolution of CD31 expression from the CD34+ hematopoietic progenitor stages to the CD45RA+ mature CD4+ and CD8+ single positive T cells. Employing 9-color flow cytometry we demonstrate that CD31 is expressed on a high proportion of CD34+ hematopoietic progenitors within the thymus, remaining high throughout the CD3-CD4+CD8+ early double positive stage, at which point expression of CD31 becomes low to negative. CD31 expression peaks on CD3^{high}CD4^{+}CD8^{+} double positive thymocytes and following positive selection its expression pattern dramatically differs between mature CD4+ and CD8+ single positive lineages. Therefore it appears that oscillations in CD31 expression correlate with checkpoints in the T cell selection process, specifically, TCRβ selection as well as positive and negative selection. Taking into account reports that CD31 is able to modulate the activation threshold of TCRs via the recruitment of tyrosine phosphatases, our results suggest that, in addition to its utility as a novel marker for thymocytes at various stages of their development, CD31 additionally has a significant role during T cell development. Intriguingly, there is a substantial subset of semi-mature (CD45RA-) CD4 SP thymocytes that lacks CD31 expression, and remarkably FoxP3+ and ICOS+ cells are overrepresented in this CD31- subpopulation. With the exception of this CD31-CD45RA- subpopulation, the majority of mature CD45RA+ CD4+ thymocytes express CD31 when they are prepared to egress. Therefore CD31 may also correlate with CD62L, S1P-R1 and other egress-associated receptors and may be
used as an additional marker of mature CD4+ and CD8+ thymocytes ready to egress the thymus.
**Introduction**

The utility of various cell-surface markers of developing thymocyte maturation stages, such as CD27 (a marker for medullary thymocytes), CD1a (a cortical thymocyte marker), CD45RA (one isoform of CD45, expressed transiently on various thymocyte populations as they develop), and CD62L (or L-selectin, which denotes mature thymocyte populations prepared to egress, expressed in conjunction with Sphingosine-1-Phosphate receptor 1 (S1P-R1)) has been well documented, both as pertains to basic immunological research and applications such as markers for disease states and diagnostics\(^\text{227,228}\). However, in spite of the plethora of thymocyte markers already established, there is yet a need for additional markers of maturation and functional stages. Such markers are useful to corroborate what we know about various populations, to advance the field by potentially identifying new populations, and to contribute to research of diseases such as certain cancers affecting lymphoid cells. One such potential marker, the expression of which is reported in the human thymus, is CD31 (also known as PECAM-1 or Platelet Endothelial Cell Adhesion Marker 1.) Although known to be present on human thymocytes, the actual role of CD31 and its expression during differentiation of hematopoietic stem cells into mature T cells in the human thymus remains “contentious”\(^\text{229}\).

T cell development within the thymus comprises multiple stages ultimately resulting in the selection of cells that express a functional T cell receptor (TCR) able to recognize MHC class I or II presented antigens (see also Chapter 1: Introduction and reviewed in \(^\text{167}\)); thymocytes that respond too strongly to self antigen are also deleted during negative selection such that central tolerance is established\(^\text{230,231}\). Once the small percentage of thymocytes that successfully completes positive and negative selection is fully mature and prepared to egress, these cells upregulate CD62L and S1P-R1 and can then respond to egress signals that draw them from the thymus to the periphery\(^\text{46,232}\). Naïve T cells that have recently emigrated the thymus, known as
Recent Thymic Emigrants or RTE, have the unique phenotype of CD4/8SPCD45RO-CD62L\textsuperscript{bright}CD11\textsubscript{a}dimCD95\textsuperscript{a}bright\textsuperscript{233}. CD31 is used as an additional marker of CD4+ RTE, as naïve CD4+ T cells are known to downregulate CD31 following homeostatic proliferation or priming\textsuperscript{234-236}, reviewed by Marelli-Berg and colleagues\textsuperscript{237}.

CD31, in addition to potentially being useful as a marker in the thymus for various developmental stages, has been shown to play a role in regulation of T cell receptor mediated signaling\textsuperscript{238,239}. Thus, it is crucial to elucidate CD31 expression throughout the various stages of thymopoiesis. Normally expressed on various leukocytes as well as endothelial cells and platelets (hence the origin of the name Platelet Endothelial Cell Adhesion Factor 1, reviewed in\textsuperscript{83,237}), CD31 has been shown to be capable of engagement in both homophilic and heterophilic binding interactions; of note, heterophilic interaction with CD38, an exctoenzyme expressed on the majority of thymocytes, has been well documented\textsuperscript{24,240}, indicating that CD31 may have an important role in thymocyte development. The CD31 molecule itself is a 130kDa single chain glycoprotein comprised of a cytoplasmic tail containing two ImmunoTyrosine Inhibitory Motifs (ITIMs) and 6 Ig-like extracellular domains separated by a small transmembrane segment. The two ITIMs are phosphorylated followed by subsequent recruitment of the phosphotyrosine phosphatases (PTP) SHP-1, SHP-2 and SHP upon TCR activation\textsuperscript{239} and in turn inhibit TCR activity\textsuperscript{236}. These observations indicate that CD31 may play a role in modulation of TCR activity during thymopoiesis; specifically, CD31 may raise the activation threshold of TCR signaling and thus prevent hyperactivation of developing T cells. An analogous role for CD31 in B cells has been reported\textsuperscript{241}.

The repertoire of established thymocyte maturation stage markers is well known; however, additional markers to further identify stages of development are needed. CD31 is expressed on
human thymocytes, but little is known about the stages on which it is expressed. In this report we demonstrate that CD31 expression fluctuates during T cell development in the thymus, and we provide a detailed analysis of its expression across several phenotypic maturation stages. CD34+ hematopoietic stem cell progenitors express high levels of this transmembrane receptor; however the decision to commit to the T cell lineage rapidly reduces its expression and CD3-CD1a+CD4+CD8αβ- early double positive cells (EDP) are CD31lo. Expression of CD31 then again increases, peaking on CD4+CD8+ (DP) thymocytes. Intriguingly, the CD4/CD8 lineage commitment seems to be a turning point as it marks the time during differentiation where CD31 expression becomes dramatically different on mature CD4 and CD8 single positive (SP) thymocytes. Expression is high on all CD8 SP thymocytes, whereas CD4 SP thymocytes are much more diverse in their levels of CD31 expression; a subset of CD4 SP thymocytes expresses low to negative levels of CD31. Interestingly, this population is enriched within FOXP3+ICOS+ natural regulatory T cells within the thymus. Our results presented herein indicate that CD31 is expressed on distinct subsets at certain stages of development in the thymus, and that it may play a role in the regulation of TCR responses during T cell development.

**Results**

**CD31 expression fluctuates during thymopoiesis**

Although CD31 expression on human thymocytes has been reported, a detailed analysis of CD31 expression levels on the various thymocyte populations during the course of T cell development has not been elucidated. With this work we analyzed CD31 expression by 8-10
color flow cytometry of human thymocytes in order to determine its expression across T cell development stages in the thymus.

As shown in Figure 1, the majority of developing thymocytes express CD31 (87.3 ± 1.3% of CD31+ cells) and expression increases as cells proceed through maturation (Fig. 1b). Expression throughout T lymphocyte development follows a non-linear pattern. CD31 expression within the CD3- subset of thymocytes is generally low; however a subset of this population expresses quite a high level of CD31 (19.8 ± 2.7% of CD3- thymocytes, CD31 MFI: 4276 ± 281). On the other hand, a sub-population of CD3hi thymocytes (which globally express a high level of CD31) displays low expression (11.2 ± 1.1%). Therefore expression patterns vary across thymocyte developmental stages, with subsets of populations displaying higher or lower expression than the general population. The variance observed in CD31 expression may be attributed to interactions with CD31 ligands, such as CD38 or CD31 on other cells237,241,243. Such ligands are expressed by cells that thymocytes encounter throughout development and maturation: i.e. not only by other thymocytes, but also by cortical and medullary thymic epithelial cells (TEC) that are mediators of the positive and negative selection processes16.

**Progenitors and early thymocytes express high levels of CD31**

The immature CD3- thymocyte subset is comprised of: 1) CD34+ hematopoietic stem cell precursors which populate the thymus, various transitional populations, and double positive CD4+CD8+ thymocytes that have not yet upregulated a T cell receptor (TCR). Natural killer (NK) cells additionally make up a proportion of this population168. Overall, this population of thymocytes (CD3-) expresses low levels of CD31; however, as described above and demonstrated in Figure 1, a subset of the CD3- population indeed expresses high levels of
CD31. This CD31\textsuperscript{high} cluster consists of CD34+ hematopoietic progenitors as well as their immediate progeny as CD31 expression decreases after CD34 has been downregulated (Fig. 2a and S1).

As demonstrated in Figure 2b, this cluster also contains the CD4+ immature single positive (ISP) thymocytes. Cells within the CD31\textsuperscript{high} cluster display a much more immature phenotype than CD31\textsuperscript{low} CD3- thymocytes (Fig. 2c-d). Thymocytes of various stages of differentiation comprise the CD31\textsuperscript{high} cluster: CD34+CD45RA+CD1a- thymic immigrant cells, CD1a\textsuperscript{low} pre-T cells, CD4-CD8- double negative (DN) thymocytes, CD4 ISP and early CD4+CD8\textsuperscript{α+β-} early double positive (EDP) cells (Fig. 2c). CD31\textsuperscript{high} EDP thymocytes express CD8\textsuperscript{α}, but not CD8\textsuperscript{β}, the expression of which begins after CD31 expression levels have decreased. CD3-CD31\textsuperscript{low} cells are CD1a\textsuperscript{α\textsuperscript{high}} CD4+CD8+, with the majority of the population expressing both CD8\textsuperscript{α} and CD8\textsuperscript{β} chains (Fig. 2d). A minority of the CD31\textsuperscript{low} CD3- thymocytes lacks expression of CD1a and expresses high levels of CD45RA; these cells are likely to be NK cells developing in the thymus\textsuperscript{168}.

Thus we have identified a population of cells in the human thymus that express CD34 and CD45RA, are not pre-committed to the T-cell lineage (CD1a-) and express a high level of CD31. It has been previously demonstrated that peripheral blood CD34+ hematopoietic stem cells express CD31\textsuperscript{244-246}. This suggests that CD31 is a useful marker to identify hematopoietic progenitor cells that populate the thymus, in particular cells that do not yet seem to be committed to T cell lineage (CD1a-). CD31 may contribute to the transmigration of the CD34+ hematopoietic progenitors from the blood stream into the thymus\textsuperscript{247}. In addition, our results suggest that the observed change in CD31 expression levels coincides with TCR\textsuperscript{β} selection as
it has been proposed that TCRβ selection occurs from the CD4 ISP stage through EDP
CD4+CD8α+CD8β- to CD4+CD8α+β+ DP231,248.

**Differential CD31 expression patterns on mature CD4 SP and CD8 SP thymocytes**

In order to examine CD31 expression at more advanced stages of T cell development, we divided CD3high thymocytes into four subpopulations based on expression of CD1a and CD27, from least mature (CD1a+CD27-, gate I) to most mature (CD1a-CD27+, gate IV, Fig. 3a). Commitment to CD4 or CD8 lineage by thymocytes can be seen on the left side of Figure 3b and 3c, and the expression of CD31 and CD45RA is displayed on the right side. Stages I (CD1a+CD27-) and II (CD1a+CD27+) contain mainly late DP thymocytes and cells that show early commitment to CD4 or CD8 lineage; these cells express CD31 (99.3 ± 0.1% and 98.1 ± 0.3% of CD31+ cells at stage I and II respectively.) At stages III (CD1aCD27+) and IV (CD1a-CD27+) all thymocytes have committed to CD4 or CD8 lineages, therefore expression of CD31 and CD45RA is shown for either CD4+ single positive (CD4 SP) or CD8+ single positive (CD8 SP) cells. During stage III, when thymocytes are undergoing negative selection, CD8 SP cells are homogeneously CD31high (MFI: 2782 ± 194) and have begun to upregulate CD45RA (33.5 ± 0.3% of CD45RA+ cells). At stage IV, a majority of CD8 SP cells have acquired CD45RA, a characteristic marker of a mature phenotype (63.5 ± 3.7% of CD45RA+).

The evolution of CD31 expression during the development of the CD4 SP thymocytes is dramatically different from that of the CD8 SP cells. At stage III, CD31 expression on CD4 SP cells is reduced (MFI: 937 ± 79) and a CD31- population lacking CD45RA has appeared (CD31-CD45RA-: 13.2 ± 1.6%). Moreover, only a minor fraction of CD31+ cells expresses CD45RA at this stage (CD31+CD45RA+: 14.1 ± 1.8%). At stage IV, only 42.7 ± 2.5% of CD4 SP
thymocytes express both CD31 and CD45RA, the phenotype of “true naïve” CD4+ recent thymic emigrant (RTE) in the periphery. Remarkably, about a quarter of the CD4+ SP cells at stage IV still lacks expression of CD31 (22.5 ± 2.0% of CD31− cells), the majority lacking both CD31 and CD45RA expression (CD31−CD45RA−: 17.1 ± 1.8%, CD31−CD45RA+: 5.3 ± 0.6%). Thus, in contrast to mature CD8 SP thymocytes, which are a homogeneous population, CD4 SP thymocytes are a heterogeneous population with regard to CD31 expression.

**CD31−cells within the CD4SPCD3**

On peripheral naïve CD4 T cells, CD31 down-regulation appears to be associated with TCR triggering and homeostatic proliferation. Therefore we asked whether mature CD31−CD4+ thymocytes have markers of more extensive proliferation than CD31+ thymocytes. To determine the number of replications each subset had undergone, CD4+CD8−CD27+CD3high thymocytes were sorted into four subsets according to their expression of CD31 and CD45RA as detailed in above and in Figure 3. We measured sjTREC (Signal Joint T cell Receptor Excision Circles, non-replicating DNA circles produced during TCRA gene recombination, reviewed by Douek et al. As sjTREC do not replicate within the cell, they are “diluted” as a cell divides, and may therefore be used as a proxy measurement for the proliferative history of a T cell population.

As can be seen in Figure 4, there is a statistically significant difference in the sjTREC levels of the four CD4 SP subsets defined by CD45RA and CD31 expression (one-way ANOVA, p<10−4). CD31− thymocytes within the CD4 SP population have less than half the quantity of sjTREC per cell as the CD31+ thymocytes (both gated on CD45RA+ (240.5 ± 59.48 vs. 508.5 ± 70.19.) The same phenomenon can be observed comparing CD31− and CD31+ thymocytes within the
CD4SP CD45RA- population (205.5 ± 26.32 vs. 686 ± 89.32 respectively). From these data we are able to conclude that CD31-CD4 SP thymocytes have completed at least one more cell cycle than their CD31+ counterparts.

**A subset of CD31- CD4 SP thymocytes displays markers of maturity**

Expression of CD69, an activation marker in the periphery as well as in the thymus, is upregulated during positive selection in the thymus and expressed until the final stages of development, whereupon it is lost from CD62L+ CD4 or CD8SP cells almost prepared to egress the thymus\(^{46,168}\). As can be seen in Figure 5, we examined the expression of CD69 and CD62L within CD31/CD45RA subsets and found, as expected, that the CD45RA- subsets do not contain mature CD4/8 SP cells or many CD62L+ thymocytes.

Surprisingly, mature CD69-CD62L+ thymocytes are found in similar proportions in both CD31+ and CD31- subsets of CD45RA+CD4SP thymocytes (8.9 ± 2.7% and 8.4 ± 3.6% respectively). This may be explained by the possibility that a minor subset of CD4+ T cells emigrating the thymus has already lost cell surface expression of CD31 and contributes to the CD31- naïve CD4+ T cells in the periphery; such a population would not fit into the "classic" phenotype for CD4+ recent thymic emigrants (RTE).

**FoxP3+ and ICOS+ cells are enriched within the CD31- CD4 SP thymocyte subset**

In order to elucidate specific characteristics of CD31- CD4 SP thymocytes, we tested the expression of FoxP3, the transcription factor specific to regulatory T cells, as natural regulatory T cells arise in the thymus\(^{251,252}\). As shown in Figure 6a, FoxP3+ thymocytes are a population of CD4 SP cells bearing a medullary phenotype: CD3\(^{\text{high}}\)CD27+. Like Treg in the periphery, FoxP3+
thymocytes show low to no expression of the IL-7 receptor α chain (CD127). However, unlike Treg in the periphery, they are not homogeneously CD25^{high} and, although the majority of the FoxP3^{+} cells in the thymus are CD25^{+}, some lack CD25 expression. As for other CD4 SP thymocytes, a small fraction of FoxP3^{+} thymocytes displays the phenotype of fully mature T cells (CD69^{-}CD62L^{+}, Fig. 6a). As for CD31 and CD45RA, their expression patterns differ significantly between FoxP3^{-} and FoxP3^{+} CD4 SP thymocytes: about 40% (40.5 ± 3.3%) of the FoxP3^{+} CD4 SP thymocytes are CD31^{-}CD45RA^{-} whereas only about 19% (19.6 ± 1.9%) of their FoxP3^{-} counterparts display the same phenotype (Fig. 6b).

We measured FoxP3 expression in each of the CD31/CD45RA defined subsets of CD3^{hi} CD4 SP thymocytes as shown in Figure 6c and compared the proportions of FoxP3-expressing cells. A one-way ANOVA test confirmed that FoxP3 expression was not the same across the 4 subsets (p<10^{-4}). Furthermore, semi-mature (CD45RA^{-}) FoxP3^{+} CD31^{-} cells were about 4 times more frequent than FoxP3^{+} CD31^{+} cells (24.4 ± 2.9% vs. 6.5 ± 0.7% respectively, t-test: p<10^{-4}). However, among mature (CD45RA^{+}) CD4 SP thymocytes, the frequency of FoxP3^{+} cells was similar in CD31^{-} and CD31^{+} subsets (23.8 ± 3.1% vs. 17.5 ± 2.5%, t-test: p=0.13) and close to the level observed in the CD31^{-}CD45RA^{-} subset. Therefore, the CD31^{-} CD4 SP subset is characterized by an over-representation of FoxP3^{+} cells.

**CD31^{-}CD45RA^{-} FoxP3^{+} thymocytes display enhanced levels of activation markers**

We further analyzed the expression of markers associated with FoxP3: CD25, CD127 as well as ICOS (Induced COStimulator, a T cell activation marker and member of the CD28 family^{253}). Ito et al. have demonstrated the existence of two populations of FoxP3^{+} Treg within the human thymus, where FoxP3^{+} natural regulatory T cells (Treg) arise^{251,252}, and have characterized them based on function: ICOS^{+} (Induced COStimulator, a T cell activation marker and member
of the CD28 family\textsuperscript{253} Treg make use of IL-10 and TGF-\(\beta\) to suppress immune activation, whereas ICOS- Treg use only TGF-\(\beta\)\textsuperscript{254}. We found that ICOS is expressed by CD3\textsuperscript{hi} thymocytes committed to the CD4 lineage but immature with a late DP, early CD4 SP phenotype (Figure 7a). Interestingly ICOS\(^*\) thymocytes lacked expression of CD31 and, typical of immature thymocytes, they lacked expression of CD45RA as well. Therefore ICOS\(^*\) thymocytes contribute exclusively the CD31\(^-\)CD45RA\(^-\) subset. Additionally, ICOS was mostly expressed on FoxP3\textsuperscript{hi} cells; the rest of the ICOS\(^*\) thymocytes belonged to a group of FoxP3\textsuperscript{lo/-} cells. The phenotype of both groups of ICOS\(^*\) thymocytes was similar for the markers analyzed in this study (data not shown).

Next we analyzed the expression of ICOS as well as CD25 and CD127 on FoxP3\(^+\)CD3\textsuperscript{hi} thymocytes divided into the 4 subsets defined by the expression CD45RA and CD31 (Figure 7b). As expected, ICOS expression was restricted to the immature CD45RA\(^-\) CD31\(^-\) subset, and in addition expression levels of CD25 and CD127 were the highest in the subset. Indeed CD25\textsuperscript{hi} FoxP3\(^+\) cells were only detected in this subset whereas CD25 expression levels were lower in the immature CD45RA\(^-\) CD31\(^+\) subset and even more reduced in the mature CD45RA\(^+\) FoxP3\(^+\) cells (both CD31\(^+\) and CD31\(^-\)). Regarding CD127, its expression on FoxP3\(^+\) CD3\textsuperscript{hi} thymocytes only coincided with high levels of CD25; therefore CD127\textsuperscript{hi}\(\text{lo/-}\) FoxP3\(^+\) thymocytes could be found only in that same immature CD45RA\(^-\) CD31\(^-\) subset.

Altogether these data suggest that the CD31-CD45RA- subset of CD4 SP thymocytes is enriched in activated (ICOS+) Treg (CD25+FoxP3+) cells. Regarding CD127, its expression on FoxP3\(^+\) CD3\textsuperscript{hi} thymocytes only coincided with high levels of CD25; therefore CD127\textsuperscript{hi}\(\text{lo/-}\) FoxP3\(^+\) thymocytes could be found only in that same immature CD45RA\(^-\) CD31\(^-\) subset. Simonetta et al. (2010 and 2012) have reported the existence of CD127/CD25-expressing FoxP3+ cells in
the murine thymus; additionally they demonstrated that IL-7 promotes the survival and proliferation of CD127$^{hi}$ Treg and that CD127 is additionally upregulated on Treg during activation and proliferation$^{255,256}$.

Taken together, our observations support the hypothesis that CD31- semi-mature FoxP3+ thymocytes are activated Treg-like cells. Their fate requires further elucidation and will be the subject of future experiments; however, from our data thus far it appears possible that IL-7 may promote the survival and proliferation of these cells while they undergo selection in the thymus.

**Discussion**

CD31 expression has been reported on human thymocytes$^{240,242}$; however, a detailed analysis of expression throughout the stages of T cell development, which may provide insights into its potential role in thymopoiesis as well as provide an additional useful marker for developing thymocytes, was lacking prior to this work. CD31 is reported to influence TCR signaling$^{238,239}$; thus we considered it imperative to elucidate its presence on developing thymocyte populations as a key first step to understanding its possible role in thymopoiesis. With this research we address the evolution of CD31 expression in the human thymus and demonstrate that expression varies extensively during thymopoiesis and this may correlate to a functional, regulatory role for CD31 in T cell development.

CD34+ hematopoietic stem cell progenitors express high levels of CD31, the expression of which remains elevated throughout the loss of CD34 on CD3- thymocytes, the CD4+ immature single positive (ISP) stage and the CD4+CD8$\alpha$+$\beta$- early double positive (EDP) stage. This is interesting as it reveals that, in conjunction with other well-established markers CD31 may be
used as an additional marker of early thymocyte development stages. CD31 levels fall as CD4+CD8α+ EDP cells upregulate CD8β; it has previously been shown that CD8β and intracellular TCRβ expression are concurrent developmental events associated with the acquisition of CD3 and the pre-TCRα chain on the cell surface\textsuperscript{248} and it has been proposed that TCRβ selection may begin in the CD4 ISP phase and continue in CD8α+ and CD8β+ cells\textsuperscript{231}. This hypothesis is in keeping with reports that CD31 modulates TCR signaling, as variations in CD31 expression coincide with TCRβ rearrangement and selection events.

CD31 expression steadily increases throughout the DP stage of thymopoiesis as CD3 is acquired. The commitment to CD4 or CD8 SP lineage marks a “fork in the road” where CD31 expression levels diverge and become remarkably different between the two lineages. CD8SP thymocytes express a homogenously high level of CD31 whereas CD4SP thymocytes express a generally lower level of CD31 but comprise various subsets, two of which are CD31- thymocytes within both the CD45RA- “semi-mature” and CD45RA+ mature thymocyte populations. Moreover, the CD31-CD4SP thymocytes have fewer sjTREC, which indicates that they have experienced a longer proliferation history relative to their CD31+ counterparts; additionally this population is enriched in ICOS+CD127+loFoxP3+ cells (activated natural Treg) whereas the CD31+FoxP3+ thymocytes are ICOS-CD127-. Thus, the CD4 and CD8 lineages, as they display strikingly different CD31 expression patterns, may experience different negative selection processes, with the CD4 lineage selection being perhaps more stringent.

In addition to emerging as a useful new marker for thymopoiesis stages, CD31 has been shown to modulate TCR signaling through recruitment of PTPs including SHP-1 and SHP-2 upon TCR-mediated phosphorylation of its cytoplasmic tail ITIMs. Engagement of PTPs by CD31 leads to subsequent inhibition of ZAP-70 phosphorylation and a raised activation threshold of the TCR
signaling pathway. Consequently the cell is less responsive to TCR engagement by peptide/MHC complexes. Thus CD31 involvement during T cell development may have implications for the process by which thymocytes with MHC affinity within a narrowly defined range survive positive and negative selection (as per the Classical Affinity Model).

In support of this hypothesis, CD31 levels vary across the selection process concomitantly with discreet selection events: 1) CD31 expression is reduced at the TCRβ selection stage (CD8α⁺β⁻ /CD8αβ⁺ transition); 2) CD31 expression is highest during positive selection (CD3⁺/high CD4⁺CD8⁺DP); 3) CD31 expression is lowest during negative selection of CD4SP thymocytes; 4) CD31 is absent on a subset of CD45RA⁻ CD4SP thymocytes where FoxP3+ cells are over-represented (<20% of the subset), most of them displaying markers of activation (ICOS, CD127). Thus, taking these observations together we hypothesize that CD31 may indeed have a role as a TCR signal modulator during T cell selection in the thymus, and further investigations into its role as a signal modulator on various thymocyte subsets are currently under investigation. Our current working model is that CD31⁺/hi thymocytes have a high affinity threshold whilst CD31⁻ thymocytes have a lower threshold, facilitating the function of CD31 as a modulator of TCR signaling during T cell development. Consequently, CD31⁺ thymocytes require a stronger affinity TCR/MHC-peptide complex interaction to reach the affinity threshold than CD31⁻ thymocytes. Therefore CD31⁻ thymocytes may be more readily depleted via negative selection than CD31⁺ thymocytes.

An additional way in which CD31 may perform a regulatory function is in respect to its downregulation both on thymocytes undergoing TCRβ selection and following positive selection on the CD4SP subset of thymocytes (which includes activated ICOS⁺FoxP3⁺ cells). Similar downregulation of CD31 is known to occur on peripheral CD4+ T cells following activation and
TCR priming\textsuperscript{238,249}. Ergo, in the above situations we posit that CD31 may be functioning as a regulator by itself being downregulated, which may occur following encounter with high affinity peptide-MHC complex. This type of encounter naturally leads to a strong TCR signal that triggers activation and proliferation.

The fate of CD31-CD4 SP thymocytes is as of yet unclear. The CD45RA-CD31-CD4SP thymocytes comprise another intriguing population that is not yet fully understood. It is possible that this population re-expresses CD31 upon successful completion of maturation and subsequent CD45RA acquisition. Future experiments will utilize humanized mouse models or \textit{in vitro} fetal thymus organ cultures (FTOC) to determine whether CD31-CD45RA- CD4SP cells survive, acquire CD45RA and re-express CD31. Additionally, we observed a small fraction of mature CD45RA+CD69-CD4SP thymocytes lacking CD31 expression. It is expected that all CD4+ naïve T cells leaving the thymus are CD31+, as CD31 is a well-established hallmark of CD4+ RTE in peripheral blood lymphocytes\textsuperscript{234,235}. Comparison of TREC levels in mature CD45RA+CD31-CD4 SP thymocytes and published TREC levels in peripheral CD45RA+CD31-CD4+ naïve T cell revealed that the CD45RA+CD31-CD4SP thymocytes expressed 3-5 times as many TREC/cell as the peripheral CD31- naïve CD4+ T cells. Therefore it appears unlikely that these cells are recirculating cells; rather, they may be contributing to the pool of naïve CD31- peripheral CD4+ cells described by Killian \textit{et al.} and Kimming \textit{et al.}\textsuperscript{234,235}. However, it is unclear whether these CD31- cells are able to egress the thymus, as CD31 may be required for transmigration across the corticomedullary junction\textsuperscript{192}. This is a particularly interesting notion as we have recently reported that S1P-R1 is required for human postnatal and fetal response to S1P which likely translates to requirement for S1P/ S1P-R1 ligation for mature human thymocyte egress from the human thymus to the periphery\textsuperscript{232}; these data imply that CD31 may
be coincident with S1P-R1 on this mature thymocyte population and therefore it may be used as a marker for mature thymocytes prepared to egress.

Intriguingly, approximately one fifth of the semi-mature CD45RA-CD31- subset of CD4 SP thymocytes is comprised of FoxP3+ natural regulatory T cells (thymic Treg). We attempted to determine, using antibodies to ICOS, CCR6 and CD200, whether the remainder of cells in this subset could potentially belong to a group of established self-reactive T cells such as natural Th17. We discovered that there is indeed a higher frequency of ICOS+ cells within the CD31- subset; however, the majority of these cells also co-express CD25 and FoxP3 (Figures 6 and 7) and do not express any of the Th17-associated markers we probed for, including RORγ, CCR6, CD200; data not shown). In addition we performed cell sorts to obtain RNA of four comparative populations, which we examined for master transcription factor expression that would indicate cell lineage commitment. We examined T-bet (Th1), GATA-3 (TH2), RORγ (Th17) and FoxP3 (Treg) expression in the following four populations within the CD3+hiCD27+ thymocytes: CD45RA+CD31+, CD45RA-CD31+, CD45RA+CD31-, and CD45RA+CD31+. We did not observe a significant difference in the expression of these master transcription factors across the four populations, indicating that these populations likely retain the potential to differentiate into more than one cell type. Natural Treg and natural Th17 share developmental features such as the requirement for TGFβ and both cell lineages are thought to arise in the thymus and to recognize self peptide-MHC class II complexes. Taken together these results suggest that natural Th17, like FoxP3+ natural Treg, could lack CD31 expression during their negative selection in the thymus; this possibility will be the subject of future investigations. Thus, CD31 expression may correlate with regulation of TCR signaling during thymopoiesis, and its absence on a distinct subset of CD4SP thymocytes defines a population of ICOS+FoxP3+ natural regulatory T cells within the thymus.
**Material and Methods**

**Tissue Collection and thymocyte preparation.**

Postnatal thymus specimens were obtained from children undergoing corrective cardiac surgery at the UCLA Mattel Children’s hospital. Fetal thymus specimens were obtained from the UCLA CFAR Gene and Cellular Therapy Core. Thymocytes were prepared and cultured at $4 \times 10^7$ cells/mL as previously described\(^{29,156}\) in serum-free medium (AT+dBSA) comprised of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 1100$\mu$g/mL delipidated bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO), 85$\mu$g/mL transferrin (Sigma-Aldrich), 2 mM L-glutamine, and 25$\mu$g/mL penicillin/streptomycin.

Cord and adult blood samples (PBMC) were obtained from healthy volunteers through the UCLA Center for AIDS Research Virology Core.

**Flow Cytometry**

Surface immunophenotyping of thymocytes was performed as previously described\(^{202}\). Monoclonal antibodies (mAb) were obtained from R&D (unconjugated S1P-R1 and IgG2b control), eBioscience (CD45RA PerCP-Cy5.5, CD8 APC-eFluor 780, CD25 eFluor 450, CD62L eFluor 605\(^{\text{NC}}\), CD3 eFluor 650\(^{\text{NC}}\)), and Becton Dickinson (BD) (CD69 FITC, CD27 APC). Cells were washed with Nano Crystal (NC) buffer (eBiosciences) with 0.3% Bovine Serum Albumin (Sigma-Aldrich), and resuspended in NC buffer before acquisition on a high throughput (HT)-LSR II cytometer (BD). The data were analyzed with FCS Express (De Novo software).
For detection of intracellular FoxP3, cells were first stained for cell surface markers, fixed and permeabilized with eBioscience recommended buffers following manufacturer instructions, then incubated with FITC or eFluor 450 conjugated monoclonal antibodies against FoxP3 (eBioscience, clone PCH101).

**Cell Sorting**

Prior to separation of thymocyte subsets by flow cytometry, CD27⁺ cells were enriched by immunomagnetic separation. Briefly CD27⁺ cells were separated using an EasySep human “DIY” selection kit (StemCell Technologies) associated to a purified monoclonal antibody against CD27 (eBioscience) on a RoboSep magnetic cell separator. The purity of the positively selected fraction was above 90%. For further isolation of various subsets of mature CD4 single-positive thymocytes, CD27⁺ thymocytes were stained for CD3, CD4, CD8, CD27, CD31, CD45RA and CD69. Cells were sorted on a FACSaria II cell sorter (Becton Dickinson). The purity of sorted subsets exceeded 95%.

Thymocytes were stained with CD69-FITC, CD3-PE, CD27-APC and CD45RA-PerCP-Cy5.5 at 10 million cells per tube in 1 mL AT+dBSA (total starting cell count 100 million cells) and sorted on a FACSaria cytometer (BD). Cells were sorted on the basis of CD3 and CD69 expression utilizing CD27 and CD45RA to ensure purity of populations. Following sorting, cells were suspended in TriReagent (Trizol) (Invitrogen) for RNA isolation and subsequent PCR analysis as previously described¹⁹¹.

**Quantitative Real-Time PCR**
Sorted cells were immediately washed once in phosphate buffered saline (PBS) before DNA and RNA extraction using the AllPrep DNA/RNA Micro Kit (Qiagen). Because of the limited amount of initial material, complementary DNAs were generated with Qiagen’s Whole Transcriptome Amplification kit following manufacturer’s guidelines. Quantitative PCR was performed on an Applied Biosystems 7300 Real-Time PCR System with the QuantiFast Multiplex PCR Kit (Qiagen) and the following primers/probe sets (TaqMan Gene Expression Assay, Applied Biosystems): FoxP3 (FAM), assay ID Hs01085835_m1; S1P1 (FAM), assay ID Hs00173499_m1; eukaryotic 18S ribosomal RNA (VIC, TaqMan Endogenous Controls, Applied Biosystems).

Data were analyzed in Microsoft Excel and statistics were performed in GraphPad Prism 5 (see below.)

**sjTREC quantification**

Signal joint (sj) TREC s resulting from the rearrangement of the TCRA locus were detected in genomic DNA extracted from sorted thymocytes subsets. Protocol was adapted from\textsuperscript{163}. Cell numbers and sjTREC s in each sample were quantified by multiplexed real-time PCR analysis on the Applied Biosystems 7300 system using Custom TaqMan® Copy Number Assays. The sequences of the primers and FAM dye-labeled MGB probe to detect sjTREC sequences were designed with the GeneAssist™ Copy Number Assay Workflow Builder tool available on the Applied Biosystems website to be compatible with the RNase P detection kit used to quantify the cells (TaqMan® Copy Number Reference Assays, VIC dye-labeled TAMRA probe). Each primer is located on one side of the single joint sequence: sjTREC forward,
CCATGCTGACACCTCTGGTTTT; sjTREC reverse, TGCCAGCTGCAGGGTTTAG; sjTREC probe, CAGGTGCCTATGCATCACC.

The 20µL reaction mixture consisted of 8µL of genomic DNA solution, 10µL of QuantiFast Multiplex PCR master mix (Qiagen), and 1µL each of sjTREC and RNase P primers/probe sets. Standards consisted of known concentrations of a mixture of a plasmid containing a sjTREC fragment (7.5 to 6 × 10^3 TRECs per reaction) and genomic DNA from non-TREC containing Hela epithelial cells (37.5 to 3 × 10^4 cells per reaction). All samples and controls were prepared and performed in triplicate on the same plate to reduce error. The mean value for each triplicate was used for further analysis.

**Statistical analysis**

All analyses were conducted with GraphPad Prism 5 (GraphPad Software). Variables were expressed as means with standard error of the mean. One or two-way ANOVA (followed by Dunn’s or Tukey’s posttest as appropriate) and two-tailed Student’s t tests, paired when appropriate, were used to analyze differences between populations. A p value inferior or equal to 0.05 was considered significant (*: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001).
Figure 1: Global CD31 expression in the human thymus.
A. CD31 expression as a function of CD3 expression by flow cytometry on a whole population of freshly prepared thymocytes. B. Median fluorescence intensity of CD31 on CD3$^{-/}$neg, CD3$^{\text{low/dim}}$ and CD3$^{\text{high}}$ populations (n=15, mean ± SEM).
Table S1: Expression of CD31 in thymocyte population defined by CD3 expression.

<table>
<thead>
<tr>
<th></th>
<th>% of total</th>
<th>%31+</th>
<th>CD31 MFI</th>
<th>%CD31neg</th>
<th>%CD31dim</th>
<th>%CD31hi</th>
<th>CD31 MFI of 31hi</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3neg</td>
<td>20.6 ± 2.0</td>
<td>80.2 ± 2.3</td>
<td>617.2 ± 57.7</td>
<td>19.7 ± 2.3</td>
<td>60.4 ± 3.4</td>
<td>19.8 ± 2.7</td>
<td>4276.0 ± 281.3</td>
</tr>
<tr>
<td>CD3low</td>
<td>31.9 ± 2.9</td>
<td>88.1 ± 1.4</td>
<td>790.6 ± 70.7</td>
<td>11.9 ± 1.4</td>
<td>81.7 ± 1.8</td>
<td>11.4 ± 0.8</td>
<td>n/a</td>
</tr>
<tr>
<td>CD3high</td>
<td>48.1 ± 3.8</td>
<td>88.8 ± 1.1</td>
<td>1324.0 ± 115.4</td>
<td>11.2 ± 1.1</td>
<td>59.5 ± 2.2</td>
<td>29.2 ± 2.0</td>
<td>n/a</td>
</tr>
<tr>
<td>Whole thymocytes</td>
<td>100</td>
<td>67.1 ± 1.3</td>
<td>n/a</td>
<td>12.8 ± 1.3</td>
<td>67.1 ± 2.6</td>
<td>20.0 ± 1.8</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n=15, mean ± SEM

Table S1: Expression of CD31 in thymocyte population defined by CD3 expression. Supporting data for Figure 1b.
Figure 2: Phenotype of CD3−CD31\textsuperscript{high} and CD3−CD31\textsuperscript{low} thymocytes. A. CD31 and CD34 expression on CD3− thymocytes; black line: CD31 staining, gray solid plot: isotype control, dotted vertical line indicates lower limit for CD31\textsuperscript{high}. B. CD31 expression of CD4\textsuperscript{+}CD8\textsuperscript{−} single positive thymocytes (red gate in left panel); CD4 immature single positive (ISP) thymocytes are CD3− and express high levels of CD31. C. and D. Phenotyping of CD3−CD31\textsuperscript{high} (c) and CD3−CD31\textsuperscript{low} (d) using early T cell development markers.
Figure S1: Phenotype of CD3⁻CD3¹⁺ thymocytes during down-regulation of CD34 expression.
Figure 3: Evolution of CD31 expression during the development of CD3$^{\text{high}}$ thymocytes.

A. CD3$^{\text{high}}$ thymocytes were divided into 4 subpopulations based on their level of expression of CD1a and CD27, from the least to most advanced maturation stage: CD1a$^{+}$CD27$^{-}$ (I), CD1a$^{+}$CD27$^{+}$ (II), CD1a$^{\text{low}}$CD27$^{+}$ (III) and CD1a$^{+}$CD27$^{+}$ (IV).

B. Expression of CD4 vs. CD8 and CD31 vs. CD45RA on DP thymocytes shortly after positive selection and lineage commitment in subpopulations I and II.

C. Expression of CD4 vs. CD8 on subpopulations III and IV and expression of CD31 vs. CD45RA on CD4 and CD8 SP thymocytes.
Table S2-a: Percentages and level of expression of CD4 and CD8 on thymocytes in the maturation stages I - IV defined by the expressions of CD1a and CD27.

<table>
<thead>
<tr>
<th>Stage</th>
<th>%DP ± SEM</th>
<th>%4SP ± SEM</th>
<th>CD8 MFI of 4SP ± SEM</th>
<th>%8SP ± SEM</th>
<th>CD4 MFI of 8SP ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>87.0 ± 1.8</td>
<td>11.8 ± 1.7</td>
<td>532.9 ± 25.3</td>
<td>0.9 ± 0.1</td>
<td>1078.4 ± 174.4</td>
</tr>
<tr>
<td>Stage II</td>
<td>85.9 ± 2.3</td>
<td>10.4 ± 2.0</td>
<td>416.9 ± 41.0</td>
<td>2.5 ± 0.3</td>
<td>967.2 ± 148.1</td>
</tr>
<tr>
<td>Stage III</td>
<td>30.1 ± 3.2</td>
<td>45.2 ± 2.6</td>
<td>232.7 ± 22.9</td>
<td>21.4 ± 1.7</td>
<td>697.4 ± 101.6</td>
</tr>
<tr>
<td>Stage IV</td>
<td>5.1 ± 0.6</td>
<td>59.2 ± 2.3</td>
<td>129.0 ± 22.7</td>
<td>33.5 ± 2.2</td>
<td>366.4 ± 54.3</td>
</tr>
</tbody>
</table>

Table S2-b: Percentages and level of expression of CD31 and CD45RA on thymocytes in the maturation stages I - IV defined by the expressions of CD1a and CD27.

<table>
<thead>
<tr>
<th>Stage</th>
<th>%31+RA- ± SEM</th>
<th>%31+RA+ ± SEM</th>
<th>%31-RA- ± SEM</th>
<th>%31-RA+ ± SEM</th>
<th>%31+ total ± SEM</th>
<th>CD31 MFI of 31+ ± SEM</th>
<th>%RA+ total ± SEM</th>
<th>CD45RA MFI of RA+ ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>95.9 ± 0.6</td>
<td>3.4 ± 0.6</td>
<td>0.7 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>99.3 ± 0.1</td>
<td>1711.0 ± 123.4</td>
<td>3.4 ± 0.6</td>
<td>1128.1 ± 83.9</td>
</tr>
<tr>
<td>Stage II</td>
<td>74.4 ± 2.8</td>
<td>23.7 ± 2.9</td>
<td>1.9 ± 0.3</td>
<td>0.1 ± 0.0</td>
<td>98.1 ± 0.3</td>
<td>2180.1 ± 182.6</td>
<td>23.8 ± 2.9</td>
<td>1994.5 ± 209.5</td>
</tr>
<tr>
<td>Stage III - CD4 SP</td>
<td>71.9 ± 1.9</td>
<td>14.1 ± 1.8</td>
<td>13.2 ± 1.6</td>
<td>0.8 ± 0.1</td>
<td>86.0 ± 1.6</td>
<td>839.6 ± 79.2</td>
<td>14.9 ± 1.8</td>
<td>1397.8 ± 97.1</td>
</tr>
<tr>
<td>Stage III - CD8 SP</td>
<td>64.0 ± 3.0</td>
<td>33.4 ± 3.0</td>
<td>1.7 ± 0.4</td>
<td>0.1 ± 0.0</td>
<td>98.2 ± 0.4</td>
<td>2782.1 ± 194.2</td>
<td>33.5 ± 3.0</td>
<td>1575.6 ± 138.9</td>
</tr>
<tr>
<td>Stage IV - CD4 SP</td>
<td>34.9 ± 1.8</td>
<td>42.7 ± 2.5</td>
<td>17.1 ± 1.8</td>
<td>5.3 ± 0.6</td>
<td>77.5 ± 2.0</td>
<td>906.9 ± 68.8</td>
<td>48.0 ± 2.5</td>
<td>2866.3 ± 232.5</td>
</tr>
<tr>
<td>Stage IV - CD8 SP</td>
<td>34.2 ± 3.7</td>
<td>65.0 ± 3.7</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>99.2 ± 0.2</td>
<td>3224.2 ± 221.8</td>
<td>65.3 ± 3.7</td>
<td>2851.6 ± 261.7</td>
</tr>
</tbody>
</table>

n=16, mean ± SEM

Tables S2: Expression levels (percentages and MFI) of CD4 and CD8 (A.) and CD31 and CD45RA (B.) on thymocytes in the subpopulations defined in figure 3. Supporting data for figure 3 (n=16, mean ± SEM).
Figure 4: Quantification of sjTREC in sorted CD3<sup>hi</sup> CD27<sup>+</sup> CD4<sup>SP</sup> thymocytes subsets.

Freshly prepared thymocytes were enriched for CD27 expressing cells by positive selection using magnetic beads coupled to an anti-CD27 antibody and the RoboSep® system (Stemcell Research). CD27-enriched cells were then stained for FACS with antibodies against CD3, CD4, CD8, CD27, CD31 and CD45RA and sorted into 4 subsets of CD3<sup>hi</sup> CD27<sup>+</sup> CD4<sup>SP</sup> thymocytes based on their expression of CD31 and CD45RA. Genomic DNA was extracted immediately after sorting and stored at -80°C until sjTREC measurement by qPCR. sjTREC/10,000 cells values were compared by one-way ANOVA to determine whether the subsets were globally different (p<10<sup>-4</sup>) followed by multiple pair comparisons by Sidak’s tests with single pooled variance (*:p<0.05, ****:p<10<sup>-4</sup>) (n=10, mean ± SEM).
Figure 5: Expression of CD69 and CD62L as late markers of maturity on thymocyte subsets within subsets expressing CD31 and CD45RA. CD69 expression is downregulated while CD45RA and CD62L expression are upregulated immediately prior to egress of mature T cells from the thymus. Post-natal human thymocytes were stained for CD3, CD4, CD8, CD27, CD31, CD45RA, CD62L and CD69. Cells were gated on CD3<sup>hi</sup>CD27<sup>+</sup>CD4<sup>+</sup>SP (A.) or CD8<sup>+</sup>SP (B.) and then on subsets defined by CD31 and CD45RA. CD69 and CD62L expressions are displayed for each subset in lower panel dot-plots (results representative of 5 experiments).
Table S3: Percentages of the CD31 and CD45RA defined subsets in CD3hiCD27+ CD4 single positive (a) or CD8 single positive thymocytes (b); and expression of CD69 and CD62L in each of these subsets.

<table>
<thead>
<tr>
<th>a</th>
<th>... within 3hi 27+ 4SP</th>
<th>4SP</th>
<th>... within 31+RA-</th>
<th>... within 31-RA-</th>
<th>... within 31+RA</th>
<th>... within 31-RA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>% 31+RA-…</td>
<td>48.2 ± 3.5</td>
<td>% 69-62L-…</td>
<td>0.6 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>2.3 ± 0.4</td>
<td>5.1 ± 0.7</td>
</tr>
<tr>
<td>% 31-RA-…</td>
<td>26.5 ± 4.9</td>
<td>% 69+62L-…</td>
<td>37.5 ± 7.1</td>
<td>56.8 ± 5.5</td>
<td>19.5 ± 5.7</td>
<td>32.8 ± 5.7</td>
</tr>
<tr>
<td>% 31+RA+…</td>
<td>21.0 ± 2.6</td>
<td>% 69+62L+…</td>
<td>61.0 ± 7.0</td>
<td>40.5 ± 5.5</td>
<td>68.7 ± 3.8</td>
<td>53.2 ± 2.9</td>
</tr>
<tr>
<td>% 31-RA+…</td>
<td>4.3 ± 0.6</td>
<td>% 69-62L+…</td>
<td>1.0 ± 0.4</td>
<td>1.1 ± 0.5</td>
<td>9.4 ± 2.6</td>
<td>9.0 ± 3.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b</th>
<th>... within 3hi 27+ 8SP</th>
<th>8SP</th>
<th>... within 31+RA-</th>
<th>... within 31-RA-</th>
<th>... within 31+RA</th>
<th>... within 31-RA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>% 31+RA-…</td>
<td>62.5 ± 5.6</td>
<td>% 69-62L-…</td>
<td>2.0 ± 0.4</td>
<td>7.9 ± 2.0</td>
<td>3.7 ± 0.9</td>
<td>20.0 ± 20.0</td>
</tr>
<tr>
<td>% 31-RA-…</td>
<td>4.9 ± 2.0</td>
<td>% 69+62L-…</td>
<td>65.5 ± 6.0</td>
<td>86.5 ± 1.8</td>
<td>39.8 ± 5.4</td>
<td>50.0 ± 22.4</td>
</tr>
<tr>
<td>% 31+RA+…</td>
<td>32.5 ± 5.3</td>
<td>% 69+62L+…</td>
<td>31.4 ± 6.1</td>
<td>5.1 ± 1.8</td>
<td>50.2 ± 6.0</td>
<td>10.0 ± 10.0</td>
</tr>
<tr>
<td>% 31-RA+…</td>
<td>0.2 ± 0.1</td>
<td>% 69-62L+…</td>
<td>1.1 ± 0.4</td>
<td>0.5 ± 0.5</td>
<td>6.3 ± 1.3</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*n=5, mean ± SEM*
**Figure 6: CD31 expression level on FoxP3+ thymocytes.**

A. Phenotype of FoxP3+ thymocytes using the principal thymocyte markers (CD3, CD4, CD8, CD1a, CD27, CD25, CD127, CD69, CD62L). B. Expression of CD31 and CD45RA on FoxP3- vs. FoxP3+ CD4 SP thymocytes. C. Gating scheme for measuring the expression of FoxP3 in CD3hi CD4 SP thymocytes subsets based on the expression of CD31 and CD45RA. D. Frequency of FoxP3+ cells within the CD31/CD45RA defined subsets as described above (n=15, in red: mean ± SEM, Student's t-test, ****:p<10^-4).
Phenotype of ICOS\textsuperscript{\textgreater} thymocytes using the following thymocyte markers: CD3, CD4, CD8, FoxP3, CD31, CD45RA, CD25, CD127. A. Phenotype of ICOS\textsuperscript{\textgreater} thymocytes using the following thymocyte markers: CD3, CD4, CD8, FoxP3, CD31, CD45RA, CD25, CD127. B. Expression of ICOS, CD25 and CD127 on FoxP3\textsuperscript{\textgreater} CD3\textsuperscript{\textless} thymocytes divided in the 4 subsets based on the expression of CD31 and CD45RA.

Figure 7: FoxP3\textsuperscript{\textgreater} thymocytes in the CD45RA\textsuperscript{\textless} CD31\textsuperscript{\textless} subset display activation markers ICOS, CD25, CD127.
CHAPTER 5: CONCLUSION AND DISCUSSION
**Salient Findings of the thesis work**

Cell-mediated peripheral immunity fundamentally depends upon thymopoiesis, or the healthy development of thymocytes through various maturation stages; selection within the thymus of functional and diverse T cell receptors (TCRs) with a broad breadth of recognition and proper avidity for antigens; and finally, successful egress of mature thymocytes from the thymus to the periphery. This final stage, while crucial to the population of the periphery of the human body with the various cell populations that will partake in CD4 and CD8 T cell mediated immunity, is not yet fully understood. Substantial progress has been made in murine models toward a greater understanding of the thymopoiesis and thymic egress processes \(^{193,265}\); however, several situations in which the mouse does not accurately represent the human highlight the need for a greater number of human studies in this field (see introduction to murine models.) In the work detailed in the preceding three chapters, I examined *Sphingosine-1-phosphate* (S1P), a signaling chemotactic molecule found ubiquitously in the human body enacting a myriad of roles, and its receptors S1P-R1-5, and found that S1P/ S1P-R1 ligation is essential for the response of mature human thymocytes to S1P, functional downstream signaling via the MEK/Erk/Akt pathway, and egress toward the S1P gradient. Moreover, surprisingly, this egress receptor is increased on mature thymocyte populations during HIV infection of the thymus. This is highly interesting taken in the context of the need for adequate T cell output for HIV patients in order to reconstitute depleted CD4+ cells, a task which is often a tall order.

The major findings of **Chapter 2** of the dissertation are: 1) S1P-R1 is expressed within the thymus and is mainly expressed by mature CD3hiCD27+CD45RA+CD69- human thymocytes (the population of thymocytes that has completed the maturation and selection process and is prepared to egress the thymus for the periphery); 2) S1P/ S1P-R1 ligation is required for mature
thymocytes to respond to S1P, measured by migration across a transwell plate system simulation of the in vivo S1P gradient; and 3) Finally, not only do mature thymocytes migrate across a concentration gradient and membrane in response to S1P, but also increase downstream signaling via the MEK/Erk/Akt pathway, measured by phosphorylated Akt (pAkt), which has been shown by Mudd et al to be a veritable proxy measurement of functional S1P-R1 downstream activity\textsuperscript{185}.

In Chapter 2 I also characterized S1P-R1 expression and function in the fetal thymus and, interestingly, I found that expression is quite similar to the postnatal thymus, with the greatest mRNA and protein found on CD3hiCD27+CD45RA+CD69- thymocytes. Mature S1P-R1+ fetal thymocytes, like postnatal thymocytes, also increase pAkt signaling in response to S1P/ S1P-R1 ligation. Due to limited cells obtained from fetal thymi, which are naturally smaller than postnatal samples, I was unable to examine migration of mature fetal thymocytes toward S1P, although I anticipate that fetal thymocytes would likely migrate in a similar manner to postnatal cells, having comparable expression of this requisite egress-mediating receptor on the same population of cells as observed in the postnatal thymus. Supporting this hypothesis, I performed phosphoflow-based analysis of pAkt in postnatal and fetal thymocytes and observed that fetal thymocytes respond in a similar manner (increasing pAkt expression) to postnatal thymocytes to exposure to S1P (see Chapter 4.)

The major findings of Chapter 3 of the dissertation are: 1) HIV-1 infection of the thymus induces increased S1P-R1 expression on mature CD3hiCD27+CD45RA+CD69- thymocytes and, surprisingly, also increases expression of S1P-R1 on the less mature CD3+CD27+CD45RA+CD69+ thymocyte subset, a population which in healthy individuals does not yet express S1P-R1, as these cells are not fully mature and are normally retained in the
healthy thymus until completion of maturation and selection\textsuperscript{266}. 2) Additionally, S1P-R1 appears to retain functionality, as measured by pAkt staining, for at least 5-9 weeks of HIV infection (acute infection). This result is intriguing in light of work by Mudd et al which demonstrated that in chronic infection of older HIV-infected individuals on HAART, peripheral T cells display an impaired response to S1P\textsuperscript{185} (discussed further below.) I performed the work comprising the third chapter of the dissertation utilizing humanized NSG thymus/liver implant mice to directly inject the thymic implant with HIV and perform all analyses \textit{ex vivo}. This allowed me to generate data that I consider to be reasonably representative of human HIV infection.

Increased S1P-R1 expression during HIV infection may be attributed in part to the cytokine changes within the infected thymus. I observed increased expression of Interferon-alpha (IFN-α) and Tumor Necrosis Factor-alpha (TNF-α) in the infected implants of humanized mice. Exposure to IFN-α alone at 24 and 48 hours downregulated S1P-R1, in keeping with previous reports of work in cell lines\textsuperscript{49} but exposure to TNF-α alone for 24 hours upregulated S1P-R1. Dual exposure for 5 days in culture to TNF-α and IL-7, a key cytokine for promotion of survival of thymocytes\textsuperscript{267,268} that is also increased during HIV infection, increased S1P-R1 expression to a greater extent than no treatment or TNF-α alone.

\textit{Expression and Function of S1P receptor 1 in the human thymus (Chapter 2)}

The greatest expression of the chemotaxis-mediating receptor S1P-R1 was found within the CD3hiCD27+CD45RA+CD69- subset of mature human thymocytes; this is the expected phenotype of thymocytes prepared to egress the thymus for the periphery. This population of mature thymocytes additionally responded to S1P by migration toward 100nM S1P in transwell
plate assays and enhanced pAkt signaling following S1P exposure (Chapters 2-3.) I further demonstrated that in vitro inhibition of S1P signaling in human thymocytes utilizing a specific agonist to S1P-R1 results in internalization of the receptor and impedes migration in transwell plate migration assays, thereby bringing to light the requirement for S1P-R1/ S1P signaling in the egress of mature thymocytes. This is, to my knowledge, the first demonstration of expression and function of S1P receptors in the human thymus. The sole work previously published that examined whether the S1P system functions in thymic egress has been in traditional (non-humanized) mouse models, in which it was discovered that S1P/ S1P-R1 ligation was required for the egress of murine CD4 and CD8 single positive (SP) thymocytes from the thymus. In humans, much of what we take as truth regarding thymocyte egress is in fact extrapolated from murine studies, such as the length of time that developing cells spend within the thymus and various aspects of the selection process. As there was a need for a greater number of studies in human tissue, I utilized human postnatal and fetal thymus specimens to perform many of the assays in this thesis work.

Interestingly, I found S1P-R1 expression and function to be similar in human fetal thymocytes as to postnatal cells. As the developing fetus has a “primordial thymus” by 7 weeks of gestation and begins to produce T cells that have undergone TCR rearrangement in the thymus at approximately 8.5-9.5 weeks of gestation it follows that the specimens I received, which were between 14 and 20 weeks of gestation, would likely have a need for high thymic output to populate the peripheral compartment of CD4 and CD8 cells. Unfortunately, the specimen ages fell within quite a narrow window, so I am unable to extend my observations to the third trimester, during which thymic output may be highest during gestation, as it is known that neonates have a strikingly high proportion of mature CD3+ cells (and probably thymic output) for the first three months of life. Regardless, the fact that the S1P-dependent thymic egress
system is already present and functioning by 14 weeks of gestation, and is likely required at an even earlier time point, perhaps by 8.5-9.5 weeks when TCR-rearranged T cells can be found in the thymus, is highly interesting in and of itself. The limited research on thymic output in the fetus has not yet tackled examination of the requirement for various egress receptors in great detail, and this is the first report of the requirement for the S1P system in the immune system of the developing fetus.

_HIV-1 induced changes in S1P receptor 1 expression and function- implications for T cell reconstitution in HIV patients (Chapter 3)_

T cell depletion and persistent immune activation are major factors leading to HIV-1 pathogenesis. Sphingosine-1-phosphate (S1P) and one of its receptors, S1P1 (the murine analogue of S1P-R1) are essential in the mouse for egress of naïve T lymphocytes from the thymus to the periphery. In addition, it has been shown that IFN-α, as it is induced following immune activation or viral infection, interferes with the egress of mature murine thymocytes. In HIV-induced immune activation, increased levels of IFN-α and CD69 are present, which are likely to influence S1P receptor expression and thereby exit of naïve T cell subsets to the periphery. S1P/ S1P-R1 mediated emigration of human mature thymocytes had not been reported prior to this work. I have shown in Chapter 2 that only mature CD3hiCD27+CD45RA+ human thymocytes that have lost CD69 expression can respond to S1P and thus have the potential to emigrate from the thymus. Importantly, the identification of such factors that promote egress of mature single positive thymocytes to the periphery is critical not only for the study of basic T cell immunology but also for the field of T cell reconstitution in HIV infected patients and patients of other immune disorders. Several reports have indicated a transient
increase in thymic output following acute HIV infection, which is most likely beneficial\cite{160-162}, but whether this is maintained over the long term and the receptors and chemokines responsible for egress remain unknown. The role of S1P and its receptors in T cell reconstitution during HIV infection had not been investigated prior to my work presented in Chapter 3.

For the work presented in Chapter 3 I used a humanized mouse model well established at the UCLA AIDS Institute/Center for AIDS Research (CFAR) core facilities, the innovative NSG thy/liv mouse (“BLT” upon CD34+ hematopoietic progenitor injection at 5 weeks.) Importantly, this advanced in vivo model involves extensive reconstitution of the immune system with primary human cells that reside in several anatomic locations and thus closely mimics the situation in HIV infected humans. I have demonstrated that humanized mice infected intrathymically with CCR5 or CXCR4 tropic HIV-1 for 5 or 9 weeks display increased S1P-R1 expression within the CD3hiCD27+CD45RA+CD69- mature thymocytes subset. Perhaps more intriguingly, I also observed upregulated S1P-R1 within the CD3hiCD27+CD45RA+CD69+ (“second most mature”) population. Observation of heightened S1P-R1 levels in HIV infection raises the critical new question of whether upregulated S1P-R1 translates to enhanced functionality of thymocyte egress, and, if so, whether this mechanism may be modulated as a means to new therapeutics for HIV patients that, even with HAART, are unable to recover optimal CD4 levels. Moreover, my observation begs the question, “Why is this egress receptor increased in a population of thymocytes that are not yet fully mature?” Proposed mechanisms for this increased expression, and data thus far demonstrating the role of cytokines, will be discussed below. Regarding the implications of this finding, I have observed that S1P-R1 on mature CD4 and CD8 SP thymocytes analyzed ex vivo from HIV-infected thymi appears functional as assayed by pAkt; therefore I propose that this increase in S1P-R1 expression most likely contributes to regeneration of functional naïve T cells following the acute phase of HIV-1
infection and may therefore be a target for patients otherwise unresponsive to other immunotherapies.

I discovered that Kruppel-Like Factor 2 (KLF2), which is known to control the transcription of S1P-R1, CCR5, and chemotaxis-associated receptors including CD62L, is additionally upregulated on total thymocytes (mRNA). This concomitant increase in KLF2 and S1P-R1 is in keeping with the established role of KLF2 as a modulator of S1P-R1 in mice\(^\text{26}\) and with its purported analogous role in the human thymus. KLF2 is itself regulated in part by FOXO1, another transcriptional regulator expressed throughout various stages of thymocyte maturation\(^\text{193,272,273}\). My preliminary investigations revealed that FOXO1 mRNA in samples from HIV-infected thymi of humanized mice, like KLF2 and S1P-R1, is upregulated in these thymi. Moreover I plan to utilize reporter assays to determine whether HIV directly turns on FOXO1 transcription, in this way affecting KLF2 and, subsequently, S1P-R1 transcription (see “Future Directions” below.) If HIV accessory proteins do not directly upregulate FOXO1, how might it be increased during HIV infection?

FOXO1 is constitutively regulated by phosphorylation, with its phosphorylated state being nonpermissive to KLF2 binding. In a non-phosphorylated state, FOXO1 is able to translocate to the nucleus and activate KLF2 transcription. This field is still developing, but it is currently thought that TCR signaling during T cell development ties into phosphorylation pathways that maintain FOXO1 in an inactive state until the final stages of development, when it needs to be dephosphorylated (active) to initiate KLF2 transcription. This dephosphorylation is accomplished by the reduction in TCR signaling and subsequent reduction in phosphorylation\(^\text{274}\). If HIV infection alters TCR signaling in the thymus, which appears likely, based on work by Abraham, et al which demonstrates that HIV-1 Nef “reprograms TCR signaling output from a broad
response to selective activation of the RAS-Erk pathway as well as work by others (reviewed in), this may provide an alternate mechanism for the dysregulated KLF2 and S1P-R1 translation I have observed. Alternately, HIV infection may interfere with the normal signaling of IL-7, another candidate for regulation of FOXO1 during thymocyte development, which may provide another mechanism for the dysregulated KLF2 and S1P-R1 translation I have observed. IL-7/IL-7R ligation induces various signaling pathways (reviewed in) and I also recently found that treatment with exogenous IL-7 decreases pAkt in mature thymocytes (Resop, R. unpublished data), thus it follows that disrupted IL-7 signaling during HIV infection may potentially alter FOXO1, KLF2 and S1P-R1 expression. Future experiments (see below) will address these various permutations of FOXO1 behavior disruptions.

KLF2 is, in addition to a regulator of thymocyte chemotaxis receptor expression, an established quiescence-promoting, anti-proliferative factor in T cells. For this reason, a valid alternative to the above scenario is that KLF2 may be upregulated by HIV infected thymocytes and possibly additionally by neighboring cells as a survival mechanism. Quiescent cells, which are non-proliferating, are less permissive to HIV infection than more activated cell populations and are also less susceptible to apoptosis (T cell quiescence as relates to HIV infection is reviewed in.) Future work (see below) will examine whether S1P-R1/KLF2+ cells display a quiescent phenotype and whether KLF2 and S1P-R1 are protective against HIV infection or death during HIV infection (due to infection itself or bystander effect.) My preliminary results (data not shown) indicate that S1P-R1+ thymocytes may indeed have a quiescent, non-dividing phenotype.

Finally, cytokine perturbations during HIV infection of the thymus most likely contribute to changes in S1P-R1 expression. This is discussed extensively in Chapter 3, but briefly, I observed that TNF-α and IFN-α are both upregulated at both 5 and 9 weeks of infection in the
thymus. Cytokines can change expression of cell surface receptors and thus I propose that cytokine changes during HIV infection may affect the cell surface expression of S1P-R1 on human thymocytes. This possibility will be the subject of future experiments. [See Current Working Model Figure below.]

Alternative Markers for Developing Thymocytes and CD31 (PECAM) Expression in the thymus (Chapter 4)

In tandem with my characterization of S1P receptors in the human thymus and changes in the S1P/ S1P receptor system induced by HIV infection, I participated in work in collaboration with M. Douaisi to further characterize recent thymic emigrants in the periphery. Together we found that CD31 (PECAM-1) has variable expression on developing subsets within the human thymus. For this reason, we propose that CD31 may be utilized in conjunction with other established
markers to further delineate exact populations of developing thymocytes in the human thymus. Intriguingly, CD45RA+CD31- (CD4 SP) human thymocytes are enriched in FoxP3+ and ICOS+ activated natural Treg. CD31 has been shown to influence TCR signaling (see Chapter 4 discussion for extensive review); therefore CD31 may maintain a similar role in the human thymus.

CD31 may also be used as a marker of CD4 SP RTE in HIV infected patients; in this way our findings in Chapter 4 relate well to my work in Chapter 3 to characterize S1P-R1 (egress receptor) changes in the human thymus during HIV infection. CD31 may need to be expressed on all mature thymocytes prepared to egress and thus its expression should correlate with S1P-R1 expression. Future work will examine the question of whether CD31 expression may change in parallel with S1P-R1 expression during HIV infection. We will also examine the question of whether CD31 expression is a definite requirement for T cells to egress the thymus.

**Future Directions and Therapeutic Potential**

Future work in the field of S1P-R1 expression and function in the thymus during HIV-1 infection will focus on two sub-areas: 1) Primarily, fully characterizing the molecular mechanisms of the observed increase in S1P-R1 during HIV infection. This characterization is the aim of ongoing work in which I am using various genetic constructs as well as primary thymus tissue from corrective cardiac surgery to elucidate the potential roles of HIV accessory proteins on FOXO1, KLF2 and S1P-R1 transcriptional activity as well as possible changes in IL-7 signaling. Additionally I plan to further test the effects of cytokines on these transcription factors. 2) Secondly, once the molecular mechanisms of S1P-R1 increase have been elucidated, the
modulation of S1P/S1P-R1 signaling should be considered for examination as a potential local (intrathymic) therapeutic possibility for HIV patients that do not recover optimal levels of mature peripheral T cells (reviewed by Aiuti et al.\textsuperscript{165}) There are several drawbacks to consider that accompany this otherwise promising therapy, which will be discussed in detail below.

\textit{In vitro future work}

In order to determine the molecular mechanism of HIV-1 accessory protein-induced increase in S1P-R1, I am applying a two-tiered overall approach, first utilizing several reporter constructs and primary tissues for \textit{in vitro} assays and later extending this work to the humanized NSG thy/liv mouse model. My current ongoing \textit{in vitro} work consists of three main strategies: First, I plan to determine whether HIV-1 accessory proteins may directly turn on transcription of S1P-R1 and/or its regulatory elements by using reporter constructs generated as previously described\textsuperscript{283,284} with GFP fused to the promoter of \textbf{S1P-R1}, \textbf{KLF2}, or \textbf{FOXO1}, a regulator of KLF2\textsuperscript{193}. I will expose transduced CEM cells to multiple titrations of purified HIV-Tat, Vpr, Vif, Nef and Vpu\textsuperscript{285-288} and analyze by flow cytometry for GFP for virus-mediated increase of promoter activity\textsuperscript{284,289,290}. In order to examine a direct link between KLF2 or FOXO1 upregulation and subsequent S1P-R1 induction, I am additionally planning to use two versions of \textbf{S1P-R1 reporter constructs}: a lentiviral vector expressing GFP under control of the wild type S1P-R1 promoter and a vector expressing GFP under control of a mutant S1P-R1 promoter nonpermissive to KLF2 binding (created by site-directed mutagenesis of a consensus KLF2 binding site [S1P1mt\textsuperscript{291}].)

In the second part of my \textit{in vitro} approach to examine the mechanism of S1P-R1 increase on human thymocytes during HIV infection, I plan to determine whether HIV-1 accessory proteins directly bind to S1P-R1, KLF2 or FOXO1 by using the following two complementary methods to
differentiate between activation via direct binding and other bystander effects of HIV accessory proteins to the promoters of the above genes: 1) Chromatin Immunoprecipitation (ChIP) on predicted putative binding sites to examine the binding of Tat, Nef, Vif, Vpr and Vpu to the promoter regions of the S1P-R1, KLF2 and FOXO1 genes. I will design primers to the regions of interest and perform pull-down assays in order to determine whether Tat, Vpr, Vpu, Vif or Nef directly bind to putative sites in the S1P-R1, KLF2 and/or FOXO1 genes. 2) Having identified binding sites I will use mutated HIV-Tat, Vpr, Vpu, Vif or Nef (described in\textsuperscript{292}) in addition to wildtype viral proteins to compare the effect on S1P-R1, KLF2 and FOXO1 protein and mRNA expression in primary human thymocytes as measured by flow cytometry and qPCR. If I identify binding sites and observe upregulation of S1P-R1 and/or its regulatory elements with wildtype-only viral proteins, I may infer that direct binding of viral proteins to S1P-R1 or its regulatory elements is required for upregulation.

To further investigate the potential connection between altered IL-7 signaling during HIV infection and subsequent immune activation, I will additionally investigate whether IL-7 signaling, as measured by pSTAT5 (phosphoflow) changes during HIV infection of the thymus. As described above, changes in IL-7 signaling could potentially alter FOXO1 expression and thus effect KLF2 and S1P-R1 expression. If I observe changes in IL-7 signaling I will then consider incorporation of reporter assays similar to those described above to examine a connection between IL-7 and FOXO1.

The third part of my future \textit{in vitro} work to examine HIV-induced S1P-R1 upregulation will focus on the potential that dysregulation of cytokines during HIV infection in the thymus may contribute to increased S1P-R1 expression. This question has been treated briefly in \textbf{Chapter 3}. To further probe this question, I will use the FOXO1, KLF2 and S1P-R1 reporter assays
described above in conjunction with IFN-α or IFN-γ, employing an IFN-α agonist or antibody to the IFN-α receptor as a negative control\textsuperscript{293} and an IFN-γ-specific neutralizing antibody to abrogate potential cytokine-induced upregulation of KLF2/ S1P-R1\textsuperscript{294}. I will analyze for FOXO1/KLF2/S1P-R1 upregulation by qPCR and flow cytometry. Next, KLF2 and S1P-R1 reporter assays will be performed in the presence of IFN-α and/or IFN-γ together at varying concentrations and will be used in order to investigate the implications of a disturbed balance between IFN-α and IFN-γ in immune activation during HIV infection. Based on the literature and my cytokine data described in Chapter 3, I expect that IFN-α will not upregulate S1P-R1 but that IFN-γ expression should augment S1P-R1 transcription and protein expression. Thus, I expect to observe an effect, using the aforementioned reporter constructs, by maintaining IFN-α at constant levels while titrating IFN-γ to higher concentrations. Finally, I will use human postnatal thymocytes to examine \textit{in vitro} the effect of HIV and interferon exposure on human thymocytes. The treatments for postnatal thymocytes will include infection with CCR5 and CXCR4 tropic isolates of HIV-1 or exposure to purified IFN-α or IFN-γ followed by analysis for KLF2 and S1P-R1 upregulation as detailed above; protein expression will be quantified by flow cytometry. Expression of KLF2 and S1P-R1 as measured by RT-qPCR will be compared between the interferon-exposed and HIV-infected cultures of thymocytes.

\textit{In vivo future work}

In the second branch of future experiments the \textit{in vitro} work will be extended to an \textit{in vivo} model of HIV infection, the HIS (NSG thy/liv implant) mouse (described in the introduction and Chapter 3) implanted with fetal thymus/liver tissue\textsuperscript{209} and injected with CD34+ fetal hematopoietic progenitors, allowing for the generation of a human-like immune system in mice including the development of T, B, and NK cells\textsuperscript{188} and see above.) I will focus on HIV accessory proteins that are discovered to result in S1P-R1 upregulation (Tat, Vpr, Vpu, Vif and/or Nef) and will
generate constructs comprised of lentiviral vectors expressing the appropriate proteins with deletions in the domains found to bind to S1P-R1, KLF2 and/or FOXO1 in in vitro ChIP experiments. I will test constructs for replication competence utilizing primary human thymocytes and p24 assay to quantify infectious viral particles produced\textsuperscript{29} corroborated by qPCR\textsuperscript{295}. In the event that hindered replication competence is observed (I do not expect this for Vpu, Vpr or Nef mutants, but possibly for Vif based on previous work\textsuperscript{296,297}) I will pursue this question utilizing neutralizing antibodies to various regions of the virus as described\textsuperscript{298-300} in lieu of the deletion constructs. For control HIV infection of mice (to obtain a baseline for the S1P-R1 increase previously observed) I will utilize JR-CSF (CCR5-tropic), and NL4-3 (CXCR4-tropic) HIV clones. Mice will be sacrificed 5 or 9 weeks post infection as I have previously described(\textsuperscript{301} and see Chapter 3) and total human thymocytes isolated to profile surviving cells and assess S1P-R1 expression. I will perform the following analyses: 1) Flow cytometry to quantify S1P-R1 expression on human thymocytes, utilizing an array of human thymocyte markers to elucidate the exact populations with altered S1P-R1. 2) qPCR to quantify S1P-R1, KLF2 and FOXO1 gene expression as well as to examine whether S1P-R1+ thymocytes express higher levels of pro- or anti-apoptotic genes such as \textit{BCL2} and \textit{BCL6} and to profile general immune activation markers, including interferon-stimulated genes. 3) Western blot to corroborate qPCR/flow cytometry results if sufficient thymocytes are obtained.

\textit{Potential for therapeutics}

Following full characterization of the molecular mechanisms of HIV-1 induced increase in S1P-R1 this project should shift to focus on potential therapeutic possibilities. If S1P-R1 expression on mature human thymocytes can be modulated, this may represent a potential avenue for extension of this work to therapy for HIV patients, aimed mainly to assist with repopulation of the peripheral T cell pool and re-diversification of the TCR repertoire, which is often inefficient even
with HAART treatment. As a potential therapy, modulation of the S1P/S1P-R1 axis would ideally enhance S1P-R1 on mature CD3hiCD69- thymocytes for a sustainable period of time. I have reported herein that S1P-R1 remains elevated on specific thymocyte populations through at least 9 weeks of infection in humanized mice (Chapter 4), but it is as of yet unclear how this time point would correspond to human infection in terms of how advanced infection would be in a human at just over two months. Moreover, it is still debatable as to whether the 5-9 week time frame in murine models represents a true acute or (by 9 weeks) a more chronic HIV infection. As Dion et al and others have reported a transient increase in thymic output during acute human infection but exhausted thymic function has been reported HIV infected individuals, such a therapy would likely aim to maintain this burst in thymopoiesis well into the chronic infection stage. Potential means by which this could be accomplished may include localized administration of lentiviral vectors that would confer cell-type specific effects. As this potential therapy pertains to lymphoproliferative diseases, such as certain lymphomas, dampening, rather than enhancing, S1P/S1P-R1 ligation, signaling or response may prove helpful in certain cases (see Chapter 2 Discussion.)

Several issues could arise from either enhancing or dampening S1P-R1 in the thymus. Primarily, and perhaps most seriously, if such a therapy were not limited to CD3hiCD69- mature thymocytes, disastrous consequences including the premature egress of immature T cells and the “flooding” of the periphery with immature T cells that have not yet completed positive and/or negative selection and TCR rearrangement (which would consequently be either useless, at best, or self-reactive, at worst) could ensue. This would indeed have dire consequences, especially in an immunocompromised individual such as an HIV infected person. In order to reduce the risk of this possibility, therapies that could target specific populations, or specific regions of the thymus, specifically the corticomedullary junction where thymocytes egress the
thymus upon completion of maturation, would need to be examined. Additional possible undesirable consequences of administration of such a therapy, were measures not taken to prevent it, might include “over activation” of S1P-R1, resulting in the egress of thymocytes at too rapid of a rate and the potential for feedback mechanisms within the thymus to push development to a pace quicker than is sustainable or healthy for thymocytes and or the organ itself. Also interesting to consider is the idea of the balance between repopulation of the periphery versus re-diversification of the TCR repertoire- will the latter be sacrificed to achieve the former?

Recent work by Dutrieux et al using Rhesus Macaques acutely infected with Simian Immunodeficiency Virus (SIV, also discussed in Chapter 3 discussion) revealed that the thymi of these animals displayed an “enhanced output” phenotype in which an altered cytokine profile within the thymus spurred quicker progression though the thymus microarchitecture, resulting in reduced proliferation concomitant with speedier development and egress. It follows that such an effect in the thymus, if maintained over the long term, would result in production of fewer T cells (due to the reduced proliferation reported) and ultimately thymic exhaustion. For this reason, adaptation of S1P/ S1P-R1 axis modulation for therapy would need to consider a mechanism by which the “dosage” could be controlled and S1P-R1 upregulated to a sufficient but not excessive extent. A possible course of action for achieving this aim may be a vector system with an inducible negative feedback loop. An alternate possibility would be to simultaneously block aberrant IFNα expression within the HIV-infected thymus, as this appears to be responsible for the altered cytokine profile (which includes reduced expression of CXCL12, normally responsible for maintaining thymocytes at the corticomedullary junction until they are prepared to egress, working in conjunction with S1P expression and other regulatory
mechanisms such as Dynamin-2 signaling at this anatomical location\textsuperscript{192,305,306}) reported in the abovementioned SIV study\textsuperscript{157}.

Alternately, dampening the S1P/S1P-R1 axis may have equally dire consequences including reduced potential for response to pathogens in the periphery due to hindered thymic output. This consequence may be considered acceptable in the face of certain alternatives, such as lymphomas. Assuming that S1P-R1 expression could be theoretically restricted to the CD3hiCD69-CD4/8SP population of mature medullary thymocytes and could be enhanced to an acceptable level without rampant overexpression, which could pose the risk of an imbalance in thymic and peripheral populations or CD3hiCD69- thymocytes egressing at too great of a rate, modulation of the S1P-S1P receptor system as a potential therapy to enhance the immunity and quality of life of HIV infected individuals as well as patients with other immune system disorders is an intriguing possibility for future investigations.
References


Thudichum, J. L. W. A treatise on the chemical constitution of the brain. (1884).


Carr, J. M. *et al.* Reduced sphingosine kinase 1 activity in dengue virus type-2 infected cells can be mediated by the 3’ untranslated region of dengue virus type-2 RNA. *The Journal of general virology* 94, 2437-2448, doi:10.1099/vir.0.055616-0 (2013).


Stacey, A. R. *et al.* Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more


Kohno, T. & Igarashi, Y. Attenuation of cell motility observed with high doses of sphingosine 1-phosphate or phosphorylated FTY720 involves RGS2 through its interactions with the receptor S1P. *Genes to cells : devoted to molecular & cellular mechanisms* **13**, 747-757, doi:10.1111/j.1365-2443.2008.01202.x (2008).


201  Piali, L. et al. The selective sphingosine 1-phosphate receptor 1 agonist ponesimod protects against lymphocyte-mediated tissue inflammation. *J Pharmacol Exp Ther* 337, 547-556, doi:jpet.110.176487 [pii]

10.1124/jpet.110.176487 (2011).


205  Hunt, P. W. et al. T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy. *J Infect Dis* 187, 1534-1543, doi:JID30217 [pii]

10.1086/374786 (2003).


Resop, R. S. Douaisi, M.; Craft, J.; Jachimowski, L.; Blom, B.; Uittenbogaart, C. Egress of naïve T-cells from the human thymus depends on S1P/S1P-R1 signaling. (In submission).


Demeure, C. E., Byun, D. G., Yang, L. P., Vezzio, N. & Delespesse, G. CD31 (PECAM-1) is a differentiation antigen lost during human CD4 T-cell maturation into Th1 or Th2 effector cells. *Immunology* **88**, 110-115 (1996).


284 Huddleson, J. P., Ahmad, N., Srinivasan, S. & Lingrel, J. B. Induction of KLF2 by fluid shear stress requires a novel promoter element activated by a phosphatidylinositol 3-


Sakai, K., Dimas, J. & Lenardo, M. J. The Vif and Vpr accessory proteins independently cause HIV-1-induced T cell cytopathicity and cell cycle arrest. *Proceedings of the*


