EXTRATHYMIC T CELL DEVELOPMENT IN THE HUMAN TONSIL

DISSERTATION

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By

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Abstract

Human T cells are critical mediators of an adaptive immune response, and individuals with T cell deficiencies are prone to devastating infections and disease. It is well known that the thymus, an organ in the anterior mediastinum, is indispensible for the development of a normal T cell repertoire in mammals. Likewise, individuals with poor thymic function due to congenital abnormalities, chemotherapy, or thymectomy suffer from decreased peripheral T cell counts and a subsequent immune deficiency. In murine models, there is evidence that the mucosal lymphoid tissue of the intestine contributes to extrathymic T cell development during normal homeostatic conditions, as can other peripheral lymphoid organs during situations of poor thymic output or pharmacologic intervention. However, whether or not human extrathymic lymphoid tissue, such as the bone marrow, lymph nodes, spleen or tonsil can participate in T cell development has remained unknown and controversial. Indeed, the identification of an extrathymic pathway for T cell lymphopoiesis in humans may suggest alternative pathways for the development of specific T cell subsets or may suggest methods for augmenting T cell generation in the face of thymic injury, absence, or disease.

In the work summarized herein, we have identified a stepwise pathway of extrathymic T cell development in the human tonsil. This pathway begins with a CD34+ multipotent
progenitor cell and ends with a mature CD3$^+$ T cell. Importantly, we have identified every stage of thymic T cell development in the pediatric tonsil, suggesting that it supports a pathway of T cell lymphopoeisis as is similar to that in the thymus. Indeed, the phenotypic and gene expression profiles of each tonsillar subset were remarkably similar to the same subsets identified within the human thymus, although some differences were identified. Furthermore, each stage of T cell development within the tonsil displayed an ex vivo differentiation potential to develop into CD3$^+$ T cells. Finally, we provide immunohistochemical evidence that immature T cell precursors reside within a specific anatomical location of the tonsil, near regions of fibrous scaffolding. This finding suggests that specific microenvironments within the tonsil may be uniquely contributing to the extrathyamic differentiation of T cell developmental intermediates. This work provides an interesting and novel insight into the role that extrathyamic lymphoid tissue may contribute towards human T cell generation. Furthermore, it is our hope that these studies—when combined with the efforts of immunologists worldwide—will help us better understand how to combat the clinical manifestations of immune deficiency, malignancy, and autoimmune disorders.
This document is dedicated to Sean and Colin.
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Publications


**Fields of Study**

**Major Field:** Integrated Biomedical Science Program

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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCL2L1</td>
<td>BCL-2 like-1</td>
</tr>
<tr>
<td>BDCA-2</td>
<td>Blood dendritic cell antigen 2</td>
</tr>
<tr>
<td>C</td>
<td>Cortex</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>cDC</td>
<td>Conventional dendritic cell</td>
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<tr>
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<td>Dendritic cell</td>
</tr>
<tr>
<td>DL1</td>
<td>Delta-like 1</td>
</tr>
<tr>
<td>DL4</td>
<td>Delta-like 4</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FL</td>
<td>Flt3 ligand</td>
</tr>
<tr>
<td>FS</td>
<td>Fibrous scaffolding</td>
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<td>FSC</td>
<td>Forward scatter</td>
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<tr>
<td>GC</td>
<td>Germinal center</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<td>-------------</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human leukocyte antigen DR</td>
</tr>
<tr>
<td>HPC</td>
<td>Hematopoietic progenitor cell</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplant</td>
</tr>
<tr>
<td>hSLT</td>
<td>Human secondary lymphoid tissue</td>
</tr>
<tr>
<td>icCD3ε</td>
<td>Intracellular CD3 epsilon</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IFZ</td>
<td>Interfollicular zone</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
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<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional review board</td>
</tr>
<tr>
<td>ISP</td>
<td>Immature single positive</td>
</tr>
<tr>
<td>KL</td>
<td>Kit ligand</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage</td>
</tr>
<tr>
<td>M</td>
<td>Medulla</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa associated lymphoid tissue</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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</tr>
<tr>
<td>OM</td>
<td>Oncostatin-M</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PTCRA</td>
<td>Pre-T cell receptor alpha</td>
</tr>
<tr>
<td>Rag</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic epithelial cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>ThPOK</td>
<td>T helper-inducing POZ/Krueppel factor</td>
</tr>
<tr>
<td>TLP</td>
<td>T lymphoid progenitor</td>
</tr>
<tr>
<td>TSA</td>
<td>Tissue specific antigen</td>
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CHAPTER 1: BACKGROUND

1.1 T cell biology

The immune system is the body’s principal defense against microbial invasion as well as against malignantly transformed cells. A healthy immune response is characterized by an orchestrated cascade of events that can be broadly divided into innate immunity—generally a rapid and non-specific response to an altered environment—and adaptive immunity—a highly specific, but delayed response to non-self antigens [1]. At the core of an effective adaptive immune reaction is the body’s ability to correctly identify “self” versus “non-self”; that is, the immune system must be able to capably distinguish which antigens are normal, non-immunogenic self peptides and which are foreign or altered self particles worthy of mounting a response. An immune response produces powerful pro-inflammatory molecules and cytotoxic effector cells, capable of killing invaders as well as normal tissue. Thus, this ability to correctly identify “self” vs. “non-self” is crucial for maintaining a robust immune system that is effective, while not being self-destructive or autoimmune [2]. The human T cell is a well-characterized lymphocyte, serving a vital role in the generation and maintenance of such a specific adaptive immune response. Indeed, the ability to respond to virtually any foreign antigen or altered self antigen depends critically on the presence of a vast repertoire of T cells, each carrying a T cell receptor (TCR) with high binding avidity to a single antigen [2, 3].
During an immune reaction, foreign antigen is processed and presented to T cells via antigen presenting cells (APCs), which display the antigen on their cell surface in conjunction with a receptor termed a major histocompatibility complex (MHC). The cognate T cell recognizes this antigen via TCRs expressed on its surface [4]. Each TCR on a T cell surface is highly specific for one small portion of an antigen, termed an epitope, and each T cell expresses molecules of only a single TCR on its surface. Furthermore, a TCR should only strongly bind to antigens that are presented by an MHC complex. The ability for a TCR to recognize only one epitope of a given antigen with high sensitivity and avidity is dependent on somatic rearrangement of TCR genes during T cell development [4]. These unique characteristics of T cell biology ensure that the T cell response to any single antigen is highly specific and targeted.

Mature T cells can be subdivided based on the expression of one of two functional TCR classes: TCRαβ or TCRγδ [5]. Furthermore, mature TCRαβ T cells can be broadly characterized by the expression of either CD8 or CD4 on their surface, although newer advances in T cell immunology have suggested a role for a smaller subset that lacks CD4 and CD8 co-receptors [6-11]. Canonically, TCRαβ⁺CD8⁺ T cells are considered cytotoxic and are responsible for the direct killing of microbially infected cells or malignant tumor cells. CD4⁺ T cells, on the other hand, are responsible for the generation of cytokines in order to promote the expansion and activation of other immune cells, such as macrophages and B cells, giving this broad subset the moniker T helper cells (Th) [12]. Conversely, human TCRγδ⁺ cells may be CD4⁺CD8⁺, CD4⁺CD8⁻, or CD4⁻CD8⁺ [5].
and can produce abundant pro-inflammatory cytokines and also display cytotoxicity [13-16]. Whereas human TCRαβ+ T cell clones display a vast repertoire of individual antigen-specific TCR molecules, TCRγδ+ T cells display a limited number of individually rearranged TCR molecules, suggesting that unique signals drive the development and selection of TCRαβ+ or TCRγδ+ cells during T cell differentiation [5].

1.2 Thymic T cell development

Jacques Miller made the first formal recognition that the thymus has a fundamental role in immunological behavior in a 1961 Lancet report. Here, he noted that mice thymectomized at birth had drastic decreases in the numbers of circulating lymphocytes and were immuno-incompetent, while mice thymectomized in adulthood had no such deficiencies [17]. This led him to conclude that the thymus is important in generating long-lived lymphocytes, and would further explain why the thymus involutes with age. In 1967, he further concluded in a landmark Nature letter that, “The Thymus is important in providing an adequate pool of immunologically competent cells. Thymectomy does not reduce the number of precursor cells, but removes the influence necessary for their differentiation into antigen reactive cells [18].” Finally, in 1968 Miller detailed that of the two major lymphocyte subsets, T cells were derived from the thymus and B cells, which produce antibody, are derived from the bone marrow. Furthermore, this paper was the first to formally demonstrate that T cells do not produce antibody themselves, but that they are required for efficient antibody production from the marrow-derived B cells [19]. These seminal reports were the first to not only identify the thymus as the predominant
site of T cell development, but also found that hematopoietic progenitor cells from the bone marrow circulate through the peripheral blood to seed the thymus continuously. Thus, he was able to show that successful T cell development depended critically on a functional bone marrow as well as a thymic environment.

Today, our understanding of thymic T cell development is much more comprehensive. Thymocyte development has been characterized by discrete stages of differentiation. Each stage is defined by a combination of cell surface markers, many of which are called clusters of differentiation or CD markers. As thymocytes progress through each stage, the cell becomes sequentially more mature and sequentially more committed to the T cell lineage. Table 1 summarizes a phenotypic description of thymocyte development. At the most basic level, T cell lymphopoiesis in mice and humans can be broadly divided by tracking the expression of the co-receptors CD4 or CD8. Using these as markers for T cell differentiation, thymocytes are classified into the least mature double negative (DN) cells, which lack CD4 or CD8, immature single positive cells (ISP), which express CD4, double positive cells (DP), which express both CD4 and CD8, and finally mature single positive (SP) cells which express either CD4 or CD8 in conjunction with a mature TCR [20-22].

In humans, the DN fraction can be further divided into three main subsets: CD34⁺CD38<sup>dim</sup> hematopoietic progenitors, the slightly more mature CD34⁺CD38<sup>bright</sup> cells, and the CD34⁺CD1a⁺ pre-T cells [20-22]. All immune cells arise from CD34⁺
hematopoietic stem cells (HSCs) located in the human bone marrow. That the thymus is seeded by marrow-derived multipotent, but non-self-renewing, hematopoietic progenitor cells (HPCs) is well known. However, the use of several different combinations of cell surface antigens to characterize a probable thymic seeding progenitor by different researchers has made the exact identification of such a cell somewhat controversial [20, 23-25]. However, it is generally accepted that the earliest cells to enter the thymus are CD34$^+$CD38$^{\text{dim}}$ and lack the expression of markers associated with specific hematopoietic lineages (Lin$^-$). Furthermore, evidence suggests that these cells have the differentiation potential to become T cells, natural killer (NK) cells, or dendritic cells (DCs) [20, 25]. Upon differentiation, these early thymic progenitors increase the expression of CD38 to form a CD34$^+$CD38$^+$Lin$^-$ DN cell, which has been proposed to be a bipotent T/NK cell progenitor [20]. Next, the DN cells increase their expression of CD1a, a canonical marker for T cell commitment in the thymus [20-22, 26]. In 1998, Spits et al. demonstrated that while CD34$^+$CD1a$^-$ thymocytes gave rise to large numbers of DCs, NK cells, and T cells in an ex vivo differentiation assay, CD34$^+$CD1a$^+$ thymocytes primarily gave rise to T cells and only a small percentage of NK cells [26]. Since this review, the acquisition of CD1a has been cited as synonymous with T cell commitment [20, 26-28], despite the fact that Spits’ original data suggest that CD34$^+$CD1a$^+$ cells may contain some NK cell differentiation potential. Regardless, the appearance of CD1a on CD34$^+$ thymocytes distinctly characterizes one of the early stages of T cell development.
Following acquisition of CD1a, thymocytes differentiating along the TCRαβ pathway begin to increase expression of CD4, marking their transition to the ISP stage. Subsequently, the cells first acquire CD8α and then CD8β to become the well-characterized CD4+CD8+ DP thymocyte [20]. Within the DP stage thymocytes complete TCR gene rearrangement, acquire CD3 and begin to express TCRs on their surface. Furthermore, they undergo positive and negative selection, the processes by which the thymus ensures that a majority of T cells are highly sensitive and specific for foreign antigen, while not being self-reactive [20]. Following positive selection, αβ thymocytes stop expressing either CD4 and/or CD8, decrease expression of CD1a and become a naïve TCRαβ+ CD4+ or CD8+ T cell [20]. Whereas this process is very stereotypically defined for TCRαβ+ T cells, TCRγδ+ thymocytes seem able to diverge from this pathway at several steps along the way. Indeed, it appears as if a γδ thymocyte may increase expression of its functional TCR and CD3 following expression of CD1a, and before expression of CD4 or CD8, or it may diverge at any step along the way during the DP stages [29]. This may, in part, explain why a large fraction of TCRγδ+ T cells are CD4- CD8- [5, 29, 30].

Studies of TCR gene locus rearrangement support this model for early thymocyte differentiation. To generate a unique, functional TCR (either TCRγδ or TCRαβ), developing thymocytes rearrange segments within the TCRA, TCRB, TCRC, and TCRD (located within TCRA) genes [21]. This occurs through a process termed V(D)J recombination, wherein the cells recombine segments of the variable (V), joining (J), or
diversity (D) regions of each gene. This allows each T cell to generate a unique TCR and occurs in the thymus throughout life, enabling the continuous generation of new antigen-specific T cells. Abundant evidence suggests that V(D)J recombination of the TCR loci occurs in an ordered fashion, such that gene rearrangement occurs in the following sequence: \( TCRD > TCRG > TCRB > TCRA \) [21, 29, 31, 32].

In 2005, Dik et al. demonstrated that human TCR gene recombination begins at the earliest CD34\(^+\)CD38\(^{dim}\) stages of thymocyte development, where a fraction of cells contain a successfully rearranged TCRD locus [21]. They further demonstrated that TCRG rearrangement is first detected within the CD34\(^+\)CD38\(^{+}\)CD1a\(^-\) fraction of thymocytes, rearranged TCRB in the CD34\(^+\)CD1a\(^+\) stage, and rearranged TCRA within CD4\(^+\) ISP cells. Furthermore, they detected varying levels of successful rearrangement of all of the TCR gene segments within DP human thymocytes, as well as within SP mature T cells [21]. This ordered acquisition of TCR recombination events further suggests that thymocyte development progresses from a CD34\(^-\)CD38\(^{dim}\) multipotent progenitor to a CD34\(^+\)CD38\(^{bright}\) cell, a CD34\(^+\)CD1a\(^+\) pre-T cell, a CD4\(^+\) ISP, and then to a CD4\(^+\)CD8\(^+\) DP cell.

This sequential process of rearrangement helps explain why TCR\(\gamma\delta\)\(^+\) thymocytes may diverge from the typical TCR\(\alpha\beta\) T cell developmental scheme at several stages. Since the actual recombination events that are responsible for TCR rearrangement occur in such a way that each rearrangement involves a random re-association of gene segments, each
recombination may produce either a functional or non-functional TCR. There is evidence that an individual T cell may begin to rearrange each TCR gene so that if non-functional TCRD and TCRG recombination events are produced, the cell then progresses to rearrange TCRB [29]. If a successful TCRB recombination event occurs, the cell is signaled through a process termed β-selection, to continue rearranging TCRA. If TCRA rearrangement is also successful, the thymocyte begins to express a functional TCRαβ. However, if functional TCRD and TCRG rearrangements occur prior to β-selection, the cell diverges from the TCRαβ pathway to become a TCRγδ+ T cell [29]. In humans, β-selection can begin as early as the CD34+CD1a+ stage, and spans through the DP stages of development [21, 32-34], explaining why TCRγδ+ thymocytes may break off of this developmental pathway at any of these stages.

In both mice and humans, the thymus is organized to support different stages of T cell development in anatomically distinct microenvironments [35-41], ensuring that the proper environmental signals are present to promote normal development. Whereas developing thymocytes make up greater than 99% of cells within the thymus, the remaining 1% is made up of various types of stromal cells that serve a vital role in the generation of self-tolerant T cells [42]. Thymic epithelial cells (TECs) are one component of the complex stromal network within the thymus, and crosstalk between TECs and thymocytes regulates the differentiation, lineage specification, survival, apoptosis, proliferation, and maturation of developing T cells. The TEC network is also broadly divided histologically into two main anatomic regions: the outer thymic cortex and the
inner thymic medulla [42]. This anatomical organization is critical to maintaining proper thymopoiesis.

During T cell development, DN thymic homing progenitors enter the thymus at the corticomedullary junction, a highly vascular region between the outer cortex and inner medulla [35, 41, 43]. These cells differentiate as they travel outward towards the subcapsular zone, which characterizes the outer-most portions of the cortex just beneath the fibrous thymic capsule [35, 41, 43]. The developing progenitors then travel inwards through the cortex as they differentiate into CD4⁺CD8⁺ DP cells, and undergo gene rearrangement and β selection. Finally, the cells enter the medulla where they are subjected to positive and negative selection, mediated in large part by interactions with TECs and thymic-resident APCs. During positive selection, TECs and APCs present an immense variety self-antigens complexed with MHC molecules on their surface to developing TCR⁺ thymocytes. Thymocytes that encounter and recognize these complexes with low avidity are selected to survive, whereas those that do not recognize the complexes at all die by apoptosis. This ensures that the developing thymocytes are capable of recognizing peptide-MHC complexes [2, 3, 44]. During negative selection, those thymocytes that survived positive selection again encounter self-antigens presented by TECs and APCs. This time, those with a sufficiently high avidity for the self-antigen-MHC complex are selected to die. Thus, negative selection favors T cells that do not have a strong avidity for self-peptides [2, 3, 44]. When this system works correctly, these two processes combine to generate thymocytes that are sufficiently capable of recognizing the
specific MHC complexes of an individual’s body but that do not bind self-peptides with sufficient strength to activate an immune response. This is one of the main mechanisms by which the thymus maintains the proper recognition of self vs. non-self in its developing T cell pool.

Once a T cell has passed these developmental milestones, it is able to leave the thymus via vessels within the thymic medulla [29, 35, 37, 41, 43]. The characteristic three-dimensional spatial organization of the thymus is thought to be critical for establishing and maintaining T cell development, and for ensuring that proper self vs. non-self discrimination is maintained within the developing T cell pool [35, 41]. Thus, the thymus is a highly specialized organ that has evolved specifically to support T cell generation, and it has long been presumed that it is unique among other lymphoid organs in its ability to do so.

1.3 Extrathymic T cell development

Without question, the thymus is the predominant site for mammalian T development. It is well known that no other organ in the body can compensate for a non-functioning thymus [45-47], and that individuals with poor thymic function are prone to debilitating immune deficiencies [1, 47-52]. While the role of the thymus in generating a majority of the body’s T cell repertoire has been well established, the ability of extrathymic lymphoid organs to also support T cell genesis remains controversial, especially in humans. Indeed, relatively recent advances within the last decade have shown that human and mouse
hematopoietic progenitor cells may be coaxed to differentiate into functional T cells in two-dimensional co-cultures with OP9-DL1 cells, a mouse bone marrow cell line expressing the Notch ligand Delta-Like 1 (DL1) [52-54]. This technologic innovation suggests that while the thymus is important in normal T cell repertoire development [47, 55, 56], the three-dimensional thymic microenvironment is not absolutely required for functional T cell differentiation [47, 52].

Substantial evidence has accumulated suggesting that certain T cell subsets can develop extrathymically in mice during normal T cell homeostasis [57-71], as well as under pharmacologic augmentation [46, 72-77]. Specifically, within the mouse intestinal mucosa a subset of specialized TCRγδ and αβ T cells, termed intraepithelial lymphocytes (IELs), appear to develop from hematopoietic progenitor cells within gut cryopatches (CPs) and mesenteric lymph nodes [57-71]. Using athymic mice that expressed GFP under the recombination-activating gene 2 (Rag-2) promoter, Guy-Grand et al. were able to track the development of lymphocytes undergoing somatic rearrangement of a TCR. They found that GFP+ cells were easily identified as precursors to IELs in mesenteric lymph nodes and Peyer’s Patches of mice with or without a thymus [71], suggesting that these cells were originating and undergoing TCR rearrangement in a thymic-independent manner. Furthermore, they were able to conclude that not all IEL are gut-derived, but that in euthymic mice some IEL are still generated through the canonical thymic-dependent pathway [71]. Interestingly, gut IEL’s display a limited TCR repertoire, with a bias for antigens related to MHC-class I-like molecules [78-80]. This suggests that these cells
undergo a different set of selection events than do non-IEL peripheral T lymphocytes, which express a much more broad TCR repertoire.

Furthermore, several reports have identified robust extrathymic T cell lymphopoiesis in the lymph nodes of mice that have been chronically exposed to the growth factors oncostatin M (OM) or leukemia inhibitory factor (LIF) [46, 72-77]. Interestingly, this induction of extrathymic T cell development was completely thymic independent, and all stages of T cell differentiation were identified within peripheral lymph nodes of OM-exposed mice. Furthermore, no induction of T cell development was noted in other peripheral lymphoid organs, such as the spleen or bone marrow [75, 76]. Although these studies in OM-induced T cell development represent an artificial mechanism for inducing extrathymic T cell lymphopoiesis, these findings nonetheless provide evidence that extrathymic T cell development can occur within secondary lymphoid organs, and that this phenomenon may be particularly relevant in situations of extreme immune system deregulation or pharmacologic intervention.

While animal models provide a useful tool for manipulating in vivo environments and studying real-time development of thymic and extrathymic T cells, investigating the production of human T cells as they develop in situ is much more technically challenging. For this reason, less is known about the relative contribution of extrathymic T cell development in humans under normal homeostatic circumstances or within the context of human disease. Several reports have provided intriguing evidence that humans,
like mice, can produce T cells extrathyrmically [81-86]. Specifically, in 2003 Klein et al. demonstrated that post-natal human bone marrow contains CD7^+CD10^+ cells that contain transcripts for *Rag-1*, *Rag-2*, *PTCRA*, and *TdT* mRNA, genes all known to be involved in TCR rearrangement [81]. Furthermore, these T lineage precursors (TLP), as they termed them, contained somatically rearranged TCRB DNA. However, this report did not extensively immunophenotype these CD7^+CD10^+ TLPs, and did not establish whether they belonged to the earliest CD34^+ DN stages of T cell development or if instead, they were more differentiated ISP or DP pre-T cells, all of which can express both CD7 and CD10 [20]. Thus, it is unclear if the TLPs identified by Klein et al. represent a single stage of T cell development that may have trafficked to the bone marrow from the thymus, or if instead they are indicative of multiple stages of T cell differentiation occurring within the bone marrow itself.

A separate study in 2004 provided evidence that neonatal intestine may support extrathymic T cell development in humans as well. This study identified CD5^+CD7^+CD3^- cells, which may represent CD3^- T cell precursors, within the intestinal mucosa of infants. Additionally, this report demonstrated that whole intestinal lysates contained transcripts for *Rag-1* and *TdT*, suggesting that TCR recombination may occur in this tissue as well [86]. However, this report did not further characterize the CD5^+CD7^+CD3^- cells for antigens specifically related to early T cell development—antigens such as CD34, CD1a, CD4, or CD8. Thus, it is unclear if these CD5^+CD7^+CD3^- cells represent a single stage of T cell development, or if instead it represents precursors in a complete
program of extrathymic T cell differentiation. Furthermore, since mRNA for recombination-associated genes was detected in whole lysates of intestinal tissue, rather than from specific cellular subsets, it cannot be determined if these genes were expressed within T cell precursors, B cell precursors, or multipotent early hematopoietic precursors, all of which have been shown to express Rag-1 or TdT in various tissues [20]. Thus, the entire implications for this study are unknown.

Several investigators have also identified TdT$^+$ or Rag-1$^+$ cells within the human tonsil. Specifically, these cells have been localized by immunohistochemistry (IHC) to extrafollicular regions of the tonsil near the fibrous scaffolding [82-85]. While limited analyses with two-color immunohistochemistry has suggested that some of these TdT$^+$ cells co-express CD10 or CD34, but lack markers for mature T or B cells, little is known about the lineage-association or origin of these cells. Thus, it was previously unknown if these cells belong to a program of T or B cell development within the human tonsil. In summary, while several reports have provided interesting but incomplete evidence suggesting extrathymic T cell development may occur in human lymphoid tissues, no comprehensive model for human extrathymic T cell lymphopoiesis had been described prior to our 2012 Journal of Clinical Investigation report [87].

1.4 Human secondary lymphoid tissue as a site for hematopoiesis

Within the last decade, it has become apparent that human secondary lymphoid tissues (hSLT), such as the tonsils and lymph nodes, may participate in varying levels of
hematopoiesis. The ability of these tissues to do so has been largely studied in the context of natural killer cell development. Initially, a 2005 study demonstrated that the human tonsil and lymph nodes contain resident CD34^+ cells that give rise to mature NK cells [88]. This finding was further expanded upon by a follow-up publication, wherein Freud et al. described a comprehensive model for NK cell development in hSLT [89], beginning with a CD34^+ multipotent population and ending with a CD56^+ mature NK cell. This model describes human NK cell development as a progression of five discrete developmental stages, each of which is easily identifiable in the tonsil and lymph nodes. The first two stages are of particular interest to this discussion, as both of these populations are CD34^+, and have ex vivo differentiation potentials to develop into NK cells, DCs, and T cells [89]. In brief, stage 1 pro-NK cells are minimally defined as CD34^+CD117^- and are not IL-15 responsive, but have the ability to develop into NK cells in the presence of additional cytokines. Stage 2 pre-NK cells, on the other hand, are defined as CD34^+CD117^+, and have the ability to develop into mature CD56^+CD3^- NK cells in the presence of IL-15 alone [89].

However, the minimal definitions of stage 1 pro- and stage 2 pre-NK cells do not account for the immense phenotypic heterogeneity found within each population, as discussed in a 2006 review [90]. Indeed, Freud and Caligiuri found that stage 1 or 2 NK cells express variable levels of the lymphocyte-associated markers CD7, CD2, CD38, and CD127 [90]. The phenotypic heterogeneity within stages 1 and 2, along with the ability of each of these stages to differentiate into non-NK lineages, raises the question of whether the bulk
CD34$^+$ population of hSLT contains distinct subpopulations each capable of giving rise to different hematopoietic lineages.

1.5 Summary and significance

The importance of the human thymus in normal T cell development has been clearly documented. However, whether or not extrathymic lymphoid tissue can participate in this process remains controversial and unclear. Based on the description of NK cell development within the human lymph node and tonsil, we sought out to investigate if hSLT had any role in the extrathymic differentiation of T cells. Hypothetically, the identification of T lymphopoiesis within an extrathymic tissue could have substantial implications for health and disease. In Chapter 2, we provide evidence that the human tonsil contains a complete program of extrathymic T cell differentiation.
### Table 1: T cell development in the human thymus

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<th>DN1</th>
<th>DN2</th>
<th>DN3 (Pre-T)</th>
<th>ISP</th>
<th>Early DP</th>
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**TCR gene rearrangement**

| TCR δ      | +   | +   | +   | +   | +   | +   | +  | [21]       |
| TCR γ      | -   | +   | +   | +   | +   | +   | +  | [21]       |
| TCR β      | -   | -   | +   | +   | +   | +   | +  | [21]       |
| TCR α      | -   | -   | -   | +   | +   | +   | +  | [21]       |

1. **Key:**
   - - indicates no expression of the indicated marker
   - + indicates expression of the indicated marker
   - +/- indicates variable expression on the indicated marker

2. **Information is adapted from material that is reviewed in references [20, 21, 26]**

3. **Indicates where the rearrangement of the corresponding TCR can be first detected by PCR. Not all cells of any subset will contain rearranged DNA of any specific TCR gene.**
2.1 Introduction

It is well established that a healthy, functional thymus is required for the development of a robust T cell repertoire. Individuals with poor thymic function due to disease, anatomical malformation, or medical therapies can suffer from debilitating immune deficiencies because of an inadequate T cell response [1, 48-51, 91]. However, whether or not human T cells can develop in extrathymic tissues is currently unknown and controversial. Several investigators have published important data suggesting that extrathymic lymphoid organs produce cells expressing genes involved in TCR rearrangement [81-86], and some of these reports have further characterized these cells as pre-T cells immunophenotypically [81, 82, 86]. However, to our knowledge, there has not been a complete description of extrathymic T cell development that is comparable to the model described in the human thymus. That is, if an extrathymic organ can support complete T cell differentiation from a bone marrow-derived hematopoietic progenitor, every step of thymic T cell development should be identifiable within this tissue. Yet prior to 2012, a comprehensive model for extrathymic T cell development, beginning with a multipotent hematopoietic progenitor cell and ending with a naïve T cell, had not been reported.
The ability of human secondary lymphoid tissue hSLT to support NK cell development has been extensively studied within the past several years [88-90, 92-96], and has laid the foundation that the human tonsil, lymph nodes, and uterine mucosa are not only sites of peripheral immune function, but also sites for hematopoiesis. Thus, we sought to determine if the human tonsil could support the development of non-NK cell lineages as well. Specifically, we were interested in investigating this tissue as a site for extrathymic T cell development.

In our 2012 *Journal of Clinical Investigation (JCI)* report, we provide evidence that the human pediatric tonsil contains a complete program of extrathymic T cell development, beginning with a multipotent progenitor and progressing through CD3+ mature T cells. This study identified five populations of putative T cell developmental intermediates in the tonsil: 1) CD34+CD38dimLin- multipotent progenitor cells; 2) CD34+CD38brightLin- cells, 3) CD34+CD1a+CD11c- pre-T cells; 4) CD34-CD1a+CD3- cells resembling CD4+CD8+ double positive (DP) thymocytes; and 5) CD34-CD1a+CD3+ cells resembling near-mature T cells. Importantly, the immunophenotype, as characterized by flow cytometry, was remarkably similar between these five populations from the tonsil and their well-characterized counterparts within the human thymus. Furthermore, each population gave rise to CD3+ T cells in *ex vivo* differentiation assays, and the first four populations retained NK cell developmental potential as well. Finally, we have localized TdT+CD1a+ T cell precursors to the fibrous scaffolding regions of the tonsil, identifying
what may be a critical microenvironment for extrathymic T cell differentiation in this tissue. Together, these data provide the first comprehensive description of a model for extrathymic T cell development in the postnatal human [87].

2.2 Results

Identification of putative early thymic T cell precursors

Low expression of CD38 on CD34+ hematopoietic cells is associated with an immature phenotype, and characterizes the earliest progenitors within the bone marrow, umbilical cord blood, and thymus. Furthermore, increased expression of CD38 on CD34+ cells is associated with differentiation [20, 21, 25, 97, 98]. Freud and Caligiuri reported that tonsillar stage 1 pro-NK cells, which are minimally defined as CD34+CD117−, express variable levels of CD38, whereas stage 2 pre-NK cells, which are presumably more mature NK cell precursors, express uniformly high levels of CD38 [90]. This phenotypic heterogeneity within stage 1 suggested that perhaps a CD34+CD38dim multipotent progenitor might reside within the human tonsil. However, at the time, it was not known if this population expressed antigens specific to a particular hematopoietic lineage.

To investigate the possibility that the tonsil contains a multipotent HPC, we magnetically enriched CD3−CD19− tonsillar mononuclear cells for those which express CD34, and then analyzed the expression of CD34, CD38 and the lineage (Lin) antigens CD11c, BDCA-2, CD1a, CD117, CD161, CD3, and CD19 by flow cytometry (Figure 1a). We found that the CD34+ compartment of the human tonsil contained a CD34+CD38dim subset, which
lacks the indicated lineage antigens (Lin\(^-\)). Furthermore, we identified a CD34\(^+\)CD38\(^{bright}\)Lin\(^-\) subset, which demonstrated dimmer expression of CD34 in addition to increased CD38 staining (Figure 1a) \([87]\). These findings suggest that the tonsil contains a subset of CD34\(^+\)CD38\(^{dim}\)Lin\(^-\) hematopoietic cells, as well as an increasingly mature CD34\(^+\)CD38\(^{bright}\)Lin\(^-\) subset. As mentioned, human thymocyte development also begins with a CD34\(^+\)CD38\(^{dim}\)Lin\(^-\) cell, and then proceeds to a CD34\(^+\)CD38\(^{bright}\)Lin\(^-\) stage \([20]\). Thus, these findings suggested that the human tonsil contains the earliest steps of human T cell development.

These early steps in thymocyte differentiation are easily identified within the bone marrow and umbilical cord blood as well, and the minimal definitions of CD34\(^+\)CD38\(^{dim}\) and CD34\(^+\)CD38\(^{bright}\) HPCs are not unique to the T cell lineage. Indeed, many if not all, lymphoid lineages begin with cells sharing these characteristics \([20]\). Thus, the mere presence of CD34\(^+\)CD38\(^{dim}\)Lin\(^-\) and CD34\(^+\)CD38\(^{bright}\)Lin\(^-\) cells did not immediately indicate whether or not the tonsil could participate in extrathymic T cell development. To establish this possibility, we first had to investigate the tonsil for the presence of downstream T cell developmental intermediates.

To investigate this point further, we next examined magnetically-enriched CD34\(^+\) tonsillar cells for the presence of CD1a\(^+\) cells. We found that within the tonsil, there was a small subset of CD34\(^+\)CD1a\(^+\) cells, which lacked expression of the lineage antigens CD161, BDCA-2, CD117, CD19, and CD14 (Figure 1b and unpublished observations).
However, CD1a is a marker that is canonically associated with two separate lineages—within the thymus CD1a expression defines several stages of T cell precursors [20, 21], whereas outside of the thymus CD1a is typically associated with antigen presenting cells [99, 100]. Thus, we examined tonsillar CD34+CD1a+ cells for expression of CD10, a marker found on immature thymocytes [20], and CD11c, a marker found on most APCs. Interestingly, we found that CD34+CD1a+ tonsillar cells can be divided into cells which express either CD10 or CD11c (Figure 1b) [87]. Based on these data, we hypothesized that the human tonsil contains a resident CD34+CD1a+CD10+CD11c- pre-T cell (defined as CD34+CD1a+CD11c- for the remainder of this thesis).

**Phenotypic characterization of putative early tonsillar T cell precursors**

In order to better understand the nature of the tonsillar CD34+CD38dimLin-, CD34+CD38brightLin-, and CD34+CD1a+CD11c- subsets so far identified, we extensively phenotyped each subset by flow cytometry for the presence or absence of antigens canonically associated with the T cell, NK cell, or DC lineages. For comparison, we similarly analyzed these same three populations from the human thymus. Between the two tissues, we found the most substantial differences while comparing the CD34+CD38dimLin- subsets. Most notably, the tonsillar subset largely lacked expression of CD2, CD5, and CD7, whereas a majority of thymic CD34+CD38dimLin- cells expressed each of these antigens. However, these cells within both tissues shared many features as well. For example, most CD34+CD38dimLin- cells from the tonsil and thymus expressed CD10, CD33, CD45RA, and HLA-DR, as well as variable levels of CD25 and
intracellular TdT. Similarly, this subset from both tissues lacked expression of CD4, CD8, CD56, and CD116 (Figure 2) [87].

We also found notable differences in the antigen expression patterns of CD34+CD38\textsuperscript{bright}Lin\textsuperscript{−} cells within the tonsil versus those in the thymus. Specifically, whereas these cells from the thymus uniformly expressed CD5, CD7, and intracellular CD3\varepsilon (icCD3\varepsilon), CD34+CD38\textsuperscript{bright}Lin\textsuperscript{−} tonsillar cells expressed only variable or dim expression of these antigens. However, CD34+CD38\textsuperscript{bright}Lin\textsuperscript{−} cells of both tissues expressed CD2, CD10, and low levels of CD45RA, but lacked expression of CD4, CD8, CD56 or CD116. Interestingly, the expression of several antigens on tonsillar CD34+CD38\textsuperscript{bright}Lin\textsuperscript{−} cells suggested a phenotype that was intermediate to that of CD34+CD38\textsuperscript{dim}Lin\textsuperscript{−} and CD34+CD1\textsubscript{a}+CD11c\textsuperscript{−} cells of this same tissue. For example, intermediate expression of CD5, CD7, CD25, CD127, HLA-DR, and TdT on CD34+CD38\textsuperscript{bright}Lin\textsuperscript{−} cells exemplify this (Figure 2) [87].

In our comparisons of thymic CD34+CD1\textsubscript{a}+CD11c\textsuperscript{−} cells to the same subset in the tonsil, we found that cells from both tissues were remarkably similar. Both thymic and tonsillar CD34+CD1\textsubscript{a}+CD11c\textsuperscript{−} cells expressed CD2, icCD3\varepsilon, CD5, CD7, and CD10, and both expressed moderate levels of CD127 and TdT. On a similar note, this subset from both tissues lacked substantial expression of CD8, CD25, CD56, CD116, and HLA-DR. Importantly, the lack of CD116 (the GM-CSF receptor) and HLA-DR further supported our hypothesis that tonsillar CD34+CD1\textsubscript{a}+CD11c\textsuperscript{−} cells do not belong to an APC subset.
Furthermore, a portion of the CD34⁺CD1a⁺CD11c⁻ cells in both the tonsil and thymus expressed CD4 (Figure 2) [87], an indicator that some cells may be progressing to the CD4⁺ immature single positive (iSP) stage of thymocyte development. Together, these data suggest that the pediatric tonsil contains the earliest stages of T cell development.

**Evidence for a resident tonsillar double positive pre-T cell**

Our data suggesting that the first three stages of human T cell development reside within the tonsil led us to examine the possibility that this tissue also contains CD34⁻ T cell developmental intermediates. One of the canonical hallmarks of thymic T cell development is the CD4⁺CD8⁺ DP pre-T cell [20], and the minimal definition as CD4⁺CD8⁺ is sufficient to identify a thymocyte as such. Within the tonsil, we consistently found a small population of CD4⁺CD8⁺ DP cells (Figure 3a, top right) [87]. However, a growing body of literature suggests that CD4⁺CD8⁺ cells found within adult peripheral blood actually represent mature memory T cells [101-103]. Since CD1a expression is very typical of DP pre-T cells in the thymus [20], but is not expressed by normal adult peripheral blood DP memory cells [101], we used expression of CD1a and CD3 to further characterize CD4⁺CD8⁺ cells in the tonsil, thymus, and peripheral blood. As can be seen in Figure 3a, thymic DP cells uniformly express CD1a, and express varying levels of CD3. Peripheral blood DP cells, on the other hand, lack CD1a expression and are uniformly CD3⁺. Within the tonsil, we found that DP cells can be divided into those which are CD3⁺CD1a⁻, as well as those that are CD1a⁺CD3⁻/+ [87]. These findings suggest that the minimal definition as CD4⁺CD8⁺ may not be sufficient to
identify a putative tonsillar DP pre-T cell that is similar to the DP pre-T cells found within the thymus, and that CD1a expression may be important for defining tonsillar T cell precursors.

CD1a is expressed throughout thymic T cell development, until its loss on CD3⁻ naïve T cells [20]. To further explore the CD1a-expressing cells of the tonsil, we magnetically enriched CD19-depleted cells for CD1a, and then analyzed the expression of CD1a with CD11c. As shown in Figure 3b, tonsillar CD34⁻CD1a⁺ cells can be divided based on their expression of CD11c. In flow cytometry, the forward scatter (FSC) and side scatter (SSC) properties of events gives an approximation of cellular size and granularity respectively. Interestingly, the CD34⁻CD1a⁺CD11c⁻ tonsillar subset displayed low FSC and SSC properties, suggesting these cells are small, agranular lymphocytes. On the other hand, CD34⁻CD1a⁺CD11c⁺ tonsillar cells displayed substantially higher forward and side scatter properties, suggesting these cells are canonical granular APCs. Within the thymus, the CD34⁺CD1a⁺ compartment is primarily CD11c⁻ and displays low forward and side scatter [87]. These data suggest that CD11c expression on CD34⁺CD1a⁺ tonsillar cells is useful for distinguishing large CD11c⁺ APCs from a novel population of CD34⁻CD1a⁺CD11c⁻ lymphocytes.

In order to better understand the relationship between CD34, CD1a, CD3 and CD11c, tonsillar CD19-depleted cells were next simultaneously enriched for CD34 and CD1a-expressing cells. This method allowed us to examine the small CD34⁺ and CD1a⁺
compartment of the tonsil concomitantly, so that we could more easily analyze cells that expressed any combination of CD34 or CD1a. Thymic CD34+ and CD34− cells were similarly analyzed. Phenotypically, it appears as if CD11c− cells from both the tonsil and the thymus lose CD34 expression as they gain CD1a on their cell surface (Figure 3c, left). Furthermore, it appears as if the CD34−CD1a+ cells increase their expression of CD3 before losing CD1a (Figure 3c, right) [87]. As CD1a surface expression is seen on thymocytes beginning with CD34+ pre-T cells and spans all the way to CD3+ near-mature T cells [20], our finding that a similar pattern of CD1a expression is seen on a fraction of human tonsillar cells suggests that this tissue may be a site of extrathymic T cell development.

**CD34−CD1a+CD11c− tonsillar cells phenotypically resemble human thymocyte progenitors**

The identification of CD4+CD8−CD1a+ cells within the tonsil, as well as the relationship between CD34, CD1a, CD3, and CD11c suggested to us that the expression of CD1a on CD11c− tonsillar cells might identify a program of extrathymic T cell differentiation within this tissue. To further investigate these putative extrathymic T cell precursors, three CD34−CD11c− populations were identified in the tonsil and thymus as shown in Figure 4a: 1) CD1a+CD3−; 2) CD1a+CD3+; 3) CD1a−CD3+ cells. We then thoroughly phenotyped each of these subsets for its expression of markers associated with thymocyte development. As can be seen in Figure 4b-c, the expression of the early T cell markers CD10 and intracellular TdT decreased as cells progressed through these subsets, whereas
the expression of CD5 and CD7 increased [87]. The CD34\(^-\)CD1a\(^+\)CD3\(^-\) cells from both
the tonsil and thymus were uniformly CD4\(^+\), and many co-expressed CD8 (Figure 4b-c).
However, within the tonsil there was substantial donor-to-donor variation in the
percentage of CD1a\(^+\)CD3\(^-\) cells that expressed CD8 (a range of ~65-97%, data not
shown). Nonetheless, this data indicates that CD4\(^+\)CD8\(^+\)CD3\(^-\) DP pre-T cells reside
within this tonsillar subset. Expression of surface T cell receptors (TCR) began in the
CD1a\(^+\)CD3\(^+\) cells of both tissues, and whereas TCR\(\gamma\delta\) expression peaked at the
CD1a\(^+\)CD3\(^+\) stage, TCR\(\alpha\beta\) levels were highest at the CD1a\(^+\)CD3\(^-\) stage. All subsets
expressed icCD3\(\varepsilon\) (Figure 4b-c) [87]. Finally, the total CD34\(^-\)CD1a\(^-\)CD11c\(^-\) subset
within the human tonsil and thymus lacked the expression of the lineage antigens CD56,
CD117, CD94, CD16, CD19, CD14, CD116, BDCA-2, and only a small subset expressed
low levels of CD161 (Figure 5a-b) [87]. Together, these data suggest that the CD34\(^-\)
CD1a\(^+\)CD11c\(^-\) compartment of the human tonsil highly resembles the well-characterized
CD34\(^-\)CD1a\(^+\) cells of the thymus. Furthermore, the gradual loss of early T cell antigens
and the increased expression of mature T cell markers suggest that the putative
extrathymic T cell precursors of the tonsil progress from a CD34\(^-\)CD11c\(^-\)CD1a\(^+\)CD3\(^-\) cell
to a CD34\(^-\)CD11c\(^-\)CD1a\(^+\)CD3\(^+\) cell, and finally to a CD1a\(^-\)CD3\(^+\) mature T cell.

**Expression of T cell development-associated genes within putative tonsillar T cell
precursors**

Thus far we have relied heavily on flow cytometry to carefully phenotype and
characterize a program of extrathymic T cell development within the pediatric tonsil.
However, just as cell surface proteins can help track the stages of lymphocytes within a tissue, expression of key genes associated with developmental milestones can also help identify whether or not specific populations belong to a particular lineage. Within human T and B cell development, some of the most crucial and lineage-defining proteins are those that are involved in the rearrangement of the T and B cell receptor genes. Within the thymus, Rag-1 and pre-T cell receptor α (PTCRA) are both required for successful TCR gene rearrangement, and they are expressed at highest levels in CD34+CD1a+, CD4+ ISP, and CD4+CD8+ DP thymocytes [21, 23, 104, 105]. Similarly, genes associated with other functions crucial to thymocyte biology may help track T cell development within the tonsil. Specifically, BCL-X has been identified in both mouse and human thymocytes, and the BCL-XL (also known as BCL2L1) isoform plays a unique role in inhibiting apoptosis in murine DP cells [106, 107]. The gene ThPOK, on the other hand, is involved in the terminal differentiation of CD4+ T cells and is expressed primarily after the acquisition of CD3 [108].

To explore if gene expression can similarly identify an extrathymic program of T cell development within the human tonsil, we used fluorescence activated cell sorting (FACS) to isolate six populations of human tonsillar and thymic cells (Figure 6): 1) CD34+CD38dimLin−, 2) CD34−CD38brightLin−, 3) CD34−CD1a+CD3−, 4) CD34−CD1a+CD3−, 5) CD34−CD1a+CD3−, and 6) CD34−CD1a−CD3+ cells. We then performed Real-time PCR on these subsets to compare the relative amounts mRNA for the genes Rag1, PTCRA, BCL2L1, and ThPOK in the six subsets. In duplicate
experiments, *Rag1*, *PTCRA*, and *BCL2L1* mRNA was highest in populations 3 or 4 of the tonsil, whereas *ThPOK* was expressed primarily in populations 5 and 6. In analysis of two thymic donors, similar findings were obtained (Figure 7) [87]. These findings suggest that within the tonsil, putative extrathymic T cell precursors do express the genes *Rag1* and *PTCRA*, and this may indicate that successful TCR gene rearrangement occurs in this tissue as it does in the thymus.

**Ex viv o T cell differentiation potential of putative tonsillar T cell precursors**

Within the last decade, significant advances in immune cell differentiation assays have allowed researchers to recapitulate the complex biological signals needed to drive T cell development from early human HSCs [53, 89, 104, 109-111], allowing us to examine the *ex vivo* T cell differentiation potential of putative T cell precursors. One such assay utilizes the OP9-DL1 cell line, which expresses an active form of the Notch ligand Delta-like 1 (DL1), along with the supplemental cytokines Flt3 ligand (FL) and IL-7 [53, 111]. To test the hypothesis that the tonsil contains extrathymic T cell developmental intermediates, we sorted the six populations shown in Figure 6, and cultured them on the OP9-DL1 cell line with FL and IL-7, conditions known to support T cell differentiation. After 26 days, the cells were harvested and analyzed for the presence of CD3+ T cells by flow cytometry. To exclude the OP9-DL1 cells, which are GFP+, from analysis we gated on GFP CD45+ events (Figure 8). Whereas tonsillar and thymic populations 1-3 from expanded on average 93-270 fold, populations 4-6 only expanded 1-6 fold (Table 2) [87], suggesting that the earlier populations are more proliferative in these conditions. All six
populations gave rise to CD3\(^+\) cells within these cultures (Figure 8a-b). The percentage of GFP\(\text{CD45}^+\) cells that were CD3\(^+\) steadily increased in both tissues as we progressed from population 1 through population 6 (Figure 8c), as did the percentage of GFP\(\text{CD45}^+\) cells that co-expressed a TCR (either TCR\(\alpha\beta\) or TCR\(\gamma\delta\)) (Figure 8d).

Next, we used these data to compare the relative ability of each tonsillar population to generate mature T cells with that of the same population from the thymus. While we found no statistically significant differences when comparing tonsillar vs. thymic populations 1, 4, 5, or 6, we did find that population 2 and 3 cells from the thymus generated a significantly higher proportion of CD3\(^+\) cells than did populations 2 and 3 from the human tonsil (population 2, 51.9\(\pm\)7.0\% from the thymus vs. 4.1\(\pm\)1.12\% from the tonsil, \(P < 0.0001\); population 3, 57.23\(\pm\)4.8\% from the thymus vs. 32.36\(\pm\)8.1\% from the tonsil, \(P = 0.0044\); Figure 8c). Similarly, we found that thymic populations 2 and 3 generated significantly more CD3\(^+\) cells that co-expressed a TCR (either TCR\(\alpha\beta\) or TCR\(\gamma\delta\)) than did the same populations in the tonsil (population 2, 39.3\(\pm\)8.5\% from the thymus vs. 1.41\(\pm\)0.6\% from the tonsil, \(P < 0.0001\); population 3, 44.1\(\pm\)7.2\% from the thymus vs. 20.3\(\pm\)5.7\% from the tonsil, \(P = 0.0041\); Figure 8d). To rule out potential contamination of mature CD3\(^+\) T cells within our culture system, CD19\(^+\) B cells were similarly sorted and cultured on the OP9-DL1 cell line, but no GFP CD45\(^+\) cells survived in these culture conditions [87].
To gain further insight into the kinetics of T cell development from tonsillar T cell precursors, we repeated the OP9-DL1 T cell differentiation assays, but harvested the progeny after 7 or 14 days. In triplicate experiments, populations 1-4 failed to produce substantial numbers of CD3⁺TCR⁺ cells after 7 or 14 days (Figure 9). Populations 5 and 6, on the other hand maintained TCR expression. Importantly, this indicates two things: 1) That the acquisition of TCRs is a late development in T cell differentiation and 2) that the appearance of CD3⁺TCR⁺ cells in our 26 day culture was not merely a result of contaminating T cells within our sorted populations [87]. Finally, we performed 26-day cultures in the presence of FL and IL-7 but on the OP9 cell line, which lacks notch ligand expression. In these conditions, we saw no T cell differentiation from populations 1-4, whereas populations 5 and 6 did maintain a population of CD3⁺TCR⁺ cells. Again, this provides evidence that the outgrowth of CD3⁺TCR⁺ cells from our 26 day OP9-DL1 experiments was not merely a result of contaminating population 5 or 6 cells [87].

**Ex vivo NK cell developmental potential of putative tonsillar T cell precursors**

The human tonsil has been extensively characterized as a site for NK cell development [88-90, 92-95]. To better understand the nature of the putative extrathymic T cell precursors we have thus far identified in the tonsil, the six tonsillar or thymic populations identified in Figure 6 were cultured in NK cell-promoting conditions. After 18-19 days of culture on the OP9 cell line with FL, c-Kit ligand (KL), IL-3, IL-7, and IL-15, the cells were harvested and analyzed for GFP⁺CD45⁺ progeny. Whereas cultures of population 5 and 6 from either tissue remained exclusively CD3⁺ (Figure 10a), populations 1-3 from
both the tonsil and thymus gave rise to CD56<sup>+</sup>CD3<sup>-</sup> NK cells. Furthermore, population 4 cells from the tonsil gave rise to NK cells in 3 of 3 experiments, whereas the same population from the thymus gave rise to NK cells in 1 out of 5 experiments (Figure 10b-e). In three of these experiments with thymus population 4, no GFP<sup>+</sup>CD45<sup>+</sup> cells survived. The resulting CD56<sup>+</sup>CD3<sup>-</sup> cells from all cultures expressed CD161 and low levels of NKp46 (Figure 10b-c) [87]. Interestingly, we also saw variable expression of CD5 on many of the NK cells derived from populations 1-4 of both tissues. Expression of CD5 was lowest on population 1 and 2 and increased in populations 3 and 4, where greater than 50% of the CD56<sup>+</sup>CD3<sup>-</sup> cells from these latter two populations were also CD5<sup>+</sup> (Figure 10b-c) [87].

To further investigate the T and NK cell differentiation potential of the putative tonsillar T cell precursors, single cells were sorted from tonsillar populations 1–4 into wells containing OP9-DL1 cells and the cytokines FL, IL-7, and IL-15. The individual cells were cultured in this system for 14 days. After culture, each well was harvested and examined for the presence of CD5<sup>+</sup>CD3<sup>-</sup> T cell precursors, CD3<sup>+</sup> T cells, or CD56<sup>+</sup>CD3<sup>-</sup> NK cells (Table 3). On average, between 16% and 67% of the wells plated sustained CD45<sup>+</sup> cells at the time of harvest. All 4 populations had potential to differentiate into CD5<sup>+</sup>CD3<sup>-</sup> T cell precursors and CD56<sup>+</sup>CD3<sup>-</sup> NK cells. Furthermore, there was an increasing tendency for a single cell to generate CD3<sup>+</sup> cells as we progressed from population 1 to 4 [87].
CD1a<sup>+</sup>TdT<sup>+</sup> cells reside near and within the fibrous scaffolding of the human tonsil

Several reports have identified TdT<sup>+</sup> cells within the human tonsil near the fibrous scaffolding [82-85]. However, little is known about which hematopoietic lineage these cells belong to and why they reside within this specific geographical location of the tonsil. We have thus far shown that varying levels of TdT expression are seen on CD34<sup>+</sup> and CD1a<sup>+</sup> populations of the human tonsil (Figures 2 and 4) [87]. Specifically, our identification of CD1a<sup>+</sup>CD11c<sup>-</sup>TdT<sup>+</sup> cells in the tonsil led us to hypothesize that the previously identified TdT<sup>+</sup> cells may be extrathymic T cell precursors. To test this hypothesis, we used IHC to investigate the TdT<sup>+</sup> cells within paraffin-embedded tonsils. First, we confirmed that TdT<sup>+</sup> cells are largely restricted to the extrafollicular regions of the tonsil, near the fibrous scaffolding (Figure 11a). While we did not detect CD11c staining on these cells, they did variably co-express CD1a and CD34 (Figure 11b). Furthermore, CD34<sup>+</sup>CD1a<sup>+</sup> cells were identified in the same region of the tonsil fibrous scaffold (Figure 11b). Interestingly, TdT<sup>+</sup> cells were identified in both pediatric and adult tonsils, although they appeared less frequent in adult tissue. [87].

To further examine the microenvironment of the tonsil, we used IHC to investigate the expression of the notch ligands delta-like 1 (DL1) and delta-like 4 (DL4) within the tonsillar fibrous regions, as notch ligands are essential for normal thymic T cell development [53, 104, 109, 110]. We found that DL1 and DL4 were both easily identified in the tonsil, frequently near the TdT<sup>+</sup> cells of the fibrous scaffolding (Figure 11c) [87].
Finally, we examined the expression of Rag1 protein together with TdT in both the tonsil and thymus. As Rag1 and TdT are both integrally involved in TCR gene rearrangement [112-114], we were interested to see if they were co-expressed within the fibrous scaffold. Furthermore, we examined whether or not both proteins were located within the nucleus, as would be expected if they were participating in TCR recombination. We found that within serial sections of the tonsil, cells expressing nuclear Rag1 or TdT were found within the fibrous scaffolding (Figure 12a). When this same region was examined for simultaneous expression of Rag1 and TdT, we found that cells expressing only Rag1 or TdT could be identified, but cells co-expressing both proteins were also found (Figure 12b). For comparison, we performed identical analysis on paraffin-embedded thymic samples as well. In Figure 12c-d, we show that expression of nuclear TdT and Rag1 are geographically concentrated in the outer cortex of the thymus, and that the subcapsular zone of this tissue contains cells that co-express both proteins. Taken together, these data suggest that the TdT+ cells previously identified in the tonsil belong to a program of extrathymic T cell differentiation and localize to a specific microenvironment near the tonsillar fibrous scaffold.

**Putative tonsillar pre-T cells represent a small population with substantial donor-to-donor variability in size**

To better understand the nature of the putative T cell developmental intermediates of the human tonsil, we analyzed the frequency of populations 1-6 (Figure 6) within ten
independent tonsil donors. As shown in Table 4, population 6, which represents the bulk of CD3⁺ T cells within the tonsil, not surprisingly makes up a large majority of the otherwise Lin⁻ (CD19, CD161, CD56, CD117, CD11c, BDCA2) cells of this tissue. On the other hand, the first five populations make up less than 3% of the Lin⁻ cells (Table 4). Indeed, populations 1-4 make up from 0.004-.083% of Lin⁻ cells on average. The smallest of these subsets, population 3, comprised only 0.0003-0.015% of Lin⁻ cells, or approximately 200-15,000 cells per tonsil tissue segment (Table 4). To illustrate the relative donor-to-donor variability of this particular subset, we analyzed 24 pediatric donors for the relative frequency of CD34⁺CD1a⁺CD11c⁻ cells (population 3) in the total CD34⁺ compartment, and have shown flow cytometric analysis of four representative donors in Figure 13. We found that within just the CD34⁺ fraction, which itself makes up less than 1% of total tonsillar cells [88], only 0.15-19.4% were further defined as CD34⁺CD1a⁺CD11c⁻ (mean, 3.82% ± 1.06%; median, 1.47%). Interestingly, there was no correlation between the percentage of CD34⁺CD1a⁺CD11c⁻ cells and the age (1-16 years) or gender of the donor. This data demonstrates that the putative extrathymic T cell developmental intermediates are a small subset of the total tonsillar mononuclear cell fraction, and that their relative frequency exhibits substantial donor-to-donor variability. Regardless, they are a consistent finding within pediatric tonsils and their significance should not be understated by their relative rarity.

2.3 Discussion and summary
In this chapter we have provided evidence that the human tonsil contains resident extrathymic T cell precursors, and that this tissue may host a complete program for extrathymic T cell differentiation. This model is divided into five subsets of T cell developmental intermediates, which can be minimally defined as: 1) CD34^+CD38^{dim} Lin^-; 2) CD34^-CD38^{bright} Lin^-; 3) CD34^-CD1a^-CD11c^-; 4) CD34^-CD1a^-CD3^-CD11c^-; and 5) CD34^-CD1a^-CD3^-CD11c^-.

We have provided phenotypic, gene expression and functional evidence that these subsets represent stages in a program of tonsillar T cell development. CD34^+CD38^{dim}Lin^- cells phenotypically resemble the multipotent hematopoietic progenitors that make up the earliest stages of thymocyte development, although differences were noted in these two populations as well. Furthermore, these cells retain the ability to develop into both NK cells and CD3^+ T cells. The CD34^-CD38^{bright}Lin^- cells of the tonsil share many phenotypic properties as the same subset identified in the human thymus, although again some differences were found. Similarly, these cells can develop into both NK cells and T cells ex vivo. Tonsillar CD34^-CD1a^-CD11c^- and CD34^-CD1a^-CD3^-CD11c^- cells respectively resemble pre-T cells and early DP thymocytes in terms of phenotype, gene expression, and differentiation capabilities. Finally, CD34^-CD1a^-CD3^-CD11c^- tonsillar cells resemble near mature T cells that are beginning to express TCRs on their surface.

Furthermore, in identifying CD34^+/^-CD1a^-CD11c^- cells that express variable levels of TdT, CD4, CD8, and additionally the mRNA for *Rag-1* and *PTCRA*, we have provided evidence that the tonsil does contain extrathymic T cell precursors that are capable of
rearranging TCR genes. Expression of TdT, Rag-1 and PTCRA are required for successful TCR rearrangement in TCRαβ lineage cells, and they have been shown to be highest in CD34+CD1a+, CD4+ ISP, and CD4+CD8+ DP thymocytes [21, 23, 104, 105]. Similarly, we have found that tonsillar populations 3 and 4, express TdT protein, Rag-1 mRNA and PTCRA mRNA. As these two populations are remarkably similar to thymic CD34+CD1a+ pre-T cells and DP cells respectively, this suggests that these extrathymic T cell precursors may undergo similar recombination events as seen in canonical thymic T cell development.

We have also confirmed earlier reports suggesting that TdT+ cells reside near the fibrous scaffolding of the tonsil [82, 84-86]. We have extended this work by also demonstrating that these TdT+ cells variably express CD1a, CD34, and Rag1, suggesting that the CD34+/−CD1a+TdT+ cells (populations 3 and 4) reside within a unique microenvironment of this tissue. The geographical distribution of these TdT+ lymphoid precursors to a specific anatomical location suggests that the development or trafficking of T cell progenitors within the tonsil is not a random event. In theory, if T cell precursors were merely prematurely leaving the thymus to circulate peripherally as mature T cells do, one would expect them to aggregate in the lymphoid-rich interfollicular zones where most tonsillar T cells reside. Instead, we (and others) have localized these TdT+ cells to a distinct region, which is otherwise sparse in lymphocytes. Future investigations should explore the role of the fibrous zone of the tonsil and its ability to either support T cell development completely or to support the trafficking of T cell progenitors here.
Finally, we have shown that the earliest tonsillar T cell developmental intermediates retain NK cell differentiation potential beyond what we hypothesized based on previous reports of thymic CD34\(^+\)CD1a\(^+\) pre-T cells [26]. It is often reported that acquisition of CD1a on the surface of CD34\(^+\) thymocytes marks commitment to the T cell lineage [20, 26, 27, 34]. Much of this assumption has been based on the fact that CD34\(^+\)CD1a\(^+\) cells begin to undergo TCR\(\beta\) rearrangement, a developmental milestone thought to signal irreversible commitment to the T cell lineage [20, 34], and on a 1998 review article wherein it was reported that CD34\(^+\)CD1a\(^+\) thymocytes have weak NK cell potential, but strong T cell potential [26]. Interestingly, we found that in addition to populations 1 and 2, population 3 cells (CD34\(^+\)CD1a\(^+\)CD3\(^-\)CD11c\(^-\)) from the tonsil or the thymus retained robust NK cell differentiation potential. Furthermore, we found that population 4 (CD34\(^-\)CD1a\(^+\)CD3\(^-\)CD11c\(^-\)) tonsillar cells similarly displayed NK cell potential in our \textit{ex vivo} assays. These data suggest that T cell commitment may not occur as early as previously assumed, or if it does that it may not be as rigidly irreversible.

In summary, we have provided a comprehensive model for extrathymic T cell development within the human tonsil. To our knowledge, this is the first description of a stepwise program of T cell differentiation in an extrathymic tissue that begins with a multipotent CD34\(^+\)CD38\(^{\text{dim}}\) progenitor and progresses through stages similar to what has been well characterized within the human thymus. Currently, the implications of this phenomenon for human health and disease are unknown. Future studies should
investigate the role of tonsillar T cell development in the context of malignancy, autoimmunity, inflammation and HIV.

2.4 Experimental procedures

The following experimental procedures have been published in our recent 2012 *JCI* report [87]:

**Human samples**

Human tonsils and thymuses were obtained from Nationwide Children’s Hospital (Columbus, OH). Tonsils were obtained from pediatric patients undergoing tonsillectomies, whereas thymuses were obtained from children undergoing thoracic surgery. Paraffin-embedded adult tonsils and reactive lymph nodes were obtained from the Biospecimen Shared Resource at the Ohio State University, and adult peripheral blood was obtained from the American Red Cross (Columbus, OH). Mononuclear cells were obtained from tonsils and thymuses following manual disaggregation and Ficoll-Paque centrifugation. For experiments wherein tonsillar cells were enriched for CD34-expressing cells only, total cells were depleted of CD19+ and CD3+ cells by magnetic depletion, and were enriched for CD34+ cells using an indirect CD34 selection kit (Miltenyi). For experiments where tonsillar cells were enriched for CD1a+ cells only, total cells were depleted of CD19+ cells and then magnetically enriched for CD1a-expressing cells (Miltenyi). To simultaneously enrich for CD34 and CD1a-expressing tonsillar cells, CD19-depleted mononuclear cells were subjected to positive selection
using the indirect CD34 selection kit (Miltenyi) and CD1a magnetic beads (Miltenyi) concurrently. To isolate thymic T cell progenitors, total thymic cells were subjected to positive CD34 selection using the indirect CD34-isolation kit.

**Flow cytometry and cell sorting**

Antibodies used for flow cytometry were purchased from BD Biosciences, except those for CD56, CD1a, CD127, Nkp46, CD16, CD161-PE (Beckman Coulter); and those for CD161-APC, CD161-FITC, CD11c-FITC, and BDCA-2 (Miltenyi). Intracellular staining for TdT and icCD3ε was performed after surface staining using the BD Cytofix/Cytoperm Plus kit (BD). Flow cytometry was performed on a FACS Vantage (BD) or LSR II (BD), and data was analyzed using FlowJo software (Treestar, Inc). Cell sorting was performed on a FACS Aria (BD). The complete gating strategy and a representative purity assessment is shown in Figure 14. Purities of sorted populations were routinely >95% pure.

**Immunohistochemistry**

Immunohistochemistry was performed as described [88, 94, 115] using the UltraView Universal system (Ventana Medical). Anti-TdT (1:100), α-CD1a (1:10), α-CD34 (1:100), α-CD11c (1:100), α-DLL1 (1:300), and α-DLL4 (1:300) were purchased from Abcam, and α-Rag1 (1:10) was purchased from Santa Cruz. For immunohistochemistry involving α-DLL1 and α-DLL4 antigen retrieval was performed for 30 minutes prior to staining. For immunohistochemistry involving α-Rag1 antigen retrieval was performed for 30
minutes along with digestion in Ventana Protease 1 (1:100) for 4 minutes. Images were obtained using a DP 12 camera, a BX50 microscope, and UPLANF1 objectives (Olympus). DAB and fast-red stained sections were digitally converted to fluorescent green and red, respectively, using the Nuance FX system (Cambridge Research & Instrumentation).

**Differentiation Assays**

Cultures were performed in α-MEM plus L-glutamine (Gibco) supplemented with 20% fetal bovine serum, penicillin G plus streptomycin (100 μg/ml, Gibco) with 500-5,000 cells per well. Medium was supplemented with the following cytokines as indicated: Human recombinant flt3 ligand (FL) (100 ng/ml, Miltenyi), c-kit ligand (KL,) (100 ng/ml, Amgen), IL-3 and IL-7 (10 ng/ml, Miltenyi), and IL-15 (1 nM, Miltenyi). T cell differentiation assays were performed as described [53, 89] on OP9-DL1 cells (a gift from J.C. Zúñiga-Pflücker, University of Toronto, Toronto, Canada) with IL-7 and FL. NK cell differentiation assays were performed on OP9 cells (a gift from J.C. Zúñiga-Pflücker) with FL, KL, IL-7, IL-3, and IL-15. For single cell cultures, individual cells from populations 1-4 of the human tonsil were sorted directly into 59-60 wells containing OP9-DL1 cells, FL, IL-7, and IL-15 for 14 days. Medium was changed every 4-5 days.

**Real-time RT-PCR**

Tonsillar populations 1-6 were sorted from eight tonsil donors as shown in Figure 6 and Figure 14. From these, we generated two pools for each population, with each pool
containing cells from four of the eight donors and approximately 3,500 cells. 5,000 cells from Populations 1-6 were sorted from two thymus donors for comparison. RNA was extracted using the RNeasy Micro Kit (Qiagen), and real-time RT-PCR was performed as previously described [94], using ABI Taqman primer/probe sets for *PTCRA* (probe Hs00300125_m1), *Rag1* (probe Hs00172121_m1), *BCL2L1* (probe Hs00236329_m1), and *ThPOK* (probe_Hs01035470_m1). Expression levels were normalized to *18S* expression and were analyzed using the comparative ΔΔCT method [116].

**Statistical Considerations**

To investigate whether age or gender correlates with the percentage of CD34+CD1a+CD11c- cells, a spearman correlation and a two-sample t-test were used. In reporting the percentages of CD3+ T cells generated in T cell differentiation assays the mean percentage ± SEM of GFP'CD45' cells was listed. For comparison of CD3+ cells generated from populations in the human thymus vs. tonsil, the linear mixed effects models were used to take account of the correlation among observations from the same donor. After Holm’s adjustment for multiple comparisons, p values ≤0.005 were considered significant.

**Study Approval**

Tonsillar, thymic, and peripheral blood samples were obtained with approval from the Ohio State University Comprehensive Cancer Center IRB. As all human samples were obtained as de-identified tissue, informed consent was not required.
2.5 Figures and Tables

The data in the following figures and tables have been published in our recent 2012 JCI report [87]:

**Figure 1:** The human tonsil contains CD34\(^+\)CD38\(^{\text{dim}}\)Lin\(^-\), CD34\(^+\)CD38\(^{\text{bright}}\)Lin\(^-\), and CD34\(^+\)CD1a\(^+\)CD11c\(^-\) cells: (A) Expression of CD34 and lineage (Lin) markers (CD11c, BDCA-2, CD117, CD161, CD19, CD3, CD1a) on CD34-enriched tonsillar cells (left). Total lymphocytes were gated on Lin\(^-\) events and analyzed for their expression of CD34 and CD38 (right). The number above each gate indicates the mean percentage of CD34\(^+\)Lin\(^-\) cells falling within that gate (B) Expression of CD1a on CD34-enriched tonsillar cells (left). Events were gated on CD34\(^+\)CD1a\(^+\) cells and analyzed for their co-expression of CD10 and CD11c (right). The number within the upper left and lower right quadrants represents the mean percentage of CD34\(^+\)CD1a\(^+\) cells that are defined as either CD11c\(^+\) or CD10\(^+\) respectively. All histograms are from a representative tonsil where n\(\geq\)3.
Figure 1: The human tonsil contains $\text{CD34}^+\text{CD38}^{\text{dim}}\text{Lin}^-$, $\text{CD34}^+\text{CD38}^{\text{bright}}\text{Lin}^-$, and $\text{CD34}^+\text{CD1a}^+\text{CD11c}^-$ cells
Figure 2: Phenotypic comparison of CD34^+CD38^{dim}Lin^-, CD34^+CD38^{bright}Lin^-, and CD34^+CD1a^+CD11c^- cells in human tonsil and thymus: (A) Tonsillar cells were magnetically depleted of CD3 and CD19-expressing cells, and were then enriched for CD34^+ cells. Enriched cells are gated on CD34^+CD38^{dim}Lin^- (left), CD34^+CD38^{bright}Lin^- (middle), or CD34^+CD1a^+CD11c^- (right) events. (B) Thymic cells were magnetically enriched for CD34^+ cells and then gated on the same three populations as in (A). Filled lines indicate staining with the indicated antibody, whereas open lines indicate staining with an isotype-matched control antibody. The number above each gate indicates the percentage of events falling within that gate. Data in each histogram is from a representative donor where n≥3 for tonsil and n≥2 for thymic data.
Figure 2: Phenotypic comparison of CD34\(^+\)CD38\(^{\text{dim}}\)Lin⁻, CD34\(^+\)CD38\(^{\text{bright}}\)Lin⁻, and CD34\(^+\)CD1a⁺CD11c⁻ cells in human tonsil and thymus
Figure 3: CD4^+CD8^+CD1a^+ cells reside within the human tonsil: (A) CD34-depleted cells from the human tonsil, thymus, and total peripheral blood mononuclear cells were analyzed for expression of double positive (DP) cells (left). DP events were then analyzed for expression of CD1a and CD3 (right) (B) CD19-depleted tonsillar cells were magnetically enriched for CD1a-expressing cells. CD34^+CD1a^-CD11c^- and CD34^-CD1a^+CD11c^+ tonsillar cells were analyzed for their forward and side scatter properties. CD34^- thymic cells were analyzed for expression of CD1a and CD11c, and CD1a^+ thymic cells were analyzed for forward and side scatter properties. (C) CD19-depleted tonsillar cells were simultaneously enriched for both CD34 and CD1a-expressing cells. Enriched cells were gated on CD11c^- (left) or CD34^-CD11c^- (right) events and analyzed for expression of CD34 and CD1a (left), and CD3 and CD1a (right). Thymic CD34^+ and CD34^- cells were similarly analyzed after CD34 enrichment. Data in A-C are from a representative donor where n≥3. Numbers within each gate represent the percentage of events falling within that gate for the representative donor shown.
Figure 3: CD4⁺CD8⁺CD1a⁺ cells reside within the human tonsil
Figure 4: Putative T cell precursors in the human tonsil acquire T-associated antigens in a fashion similar to those in the thymus: (A) CD34⁺CD11c⁻CD1a⁺CD3⁻, CD34⁺CD11c⁻CD1a⁺CD3⁺, and CD34⁺CD11c⁻CD1a⁻CD3⁺ cells were identified in the human tonsil and thymus. Tonsils were depleted of CD19⁺ cells and were then enriched for CD1a-expressing cells (top), whereas CD1a⁺ cells were readily apparent in CD34-depleted thymic mononuclear cells without enrichment (bottom). (B) CD1a-enriched tonsillar cells were gated on CD34⁻11c⁻ events and analyzed for the expression of antigens on the three populations shown in A. (C) CD34-depleted thymic cells was gated on the same three populations. Filled lines indicate staining with the indicated antibody, whereas open lines indicate staining with an isotype-matched control antibody. Data in each histogram is from a representative donor where n≥3 for tonsil and n≥2 for thymic data.
Figure 4: Putative T cell precursors in the human tonsil acquire T-associated antigens in a fashion similar to those in the thymus
Figure 5: CD34−CD11c−CD1a+ tonsillar cells lack antigens associated with non-T cell lineages: (A-B) Total CD34−CD11c−CD1a+ cells from the tonsil (A) and thymus (B) were analyzed for expression of the lineage markers shown. In each histogram, the filled line represents staining with the indicated antibody, whereas the open line indicates staining with an isotype-matched control. Each histogram is representative of 3 tonsil (A) or 2 thymic (B) donors.
Figure 5: CD34^-CD11c^-CD1a^+ tonsillar cells lack antigens associated with non-T cell lineages
Figure 6: Identification of six T cell developmental intermediates in the human tonsil for cell sorting: Tonsillar cells were depleted of CD19$^+$ cells, enriched for CD34$^+$ and CD1a$^+$ cells, and then gated on the six populations shown. Thymic cells were enriched for CD34$^+$ cells, and populations 1-3 were sorted from this CD34-enriched fraction, whereas populations 4-6 were sorted from the CD34$^-$ fraction. A complete gating strategy and representative sort purities is shown in Figure 14.
Figure 6: Identification of six T cell developmental intermediates in the human tonsil for cell sorting
**Figure 7: Quantification of gene expression in tonsillar precursor cells by Real-time RT-PCR:** Expression of the genes *Rag1, PTCRA, BCL2L1, and ThPOK* in six populations of human tonsillar (left) and thymic (right) cells. Cells were sorted from eight tonsil donors as shown in **Figure 6**, and two pools were generated for each population consisting of cells from four of the eight donors (see **Experimental Procedures**). Cells were sorted from two thymic donors. For analysis of *Rag1, PCTRA*, and *BCL2L1* (A-C) expression is displayed relative to that of population 2, which was arbitrarily set at 1. For ThPOK (D) expression is displayed relative to that of population 5 for the tonsil and population 6 for the thymus, each of which was arbitrarily set at 1.
Figure 7: Quantification of gene expression in tonsillar precursor cells by Real-time RT-PCR
**Figure 8: T cell differentiation potential of putative extrathymic T cell precursors in the human tonsil:** (A-B) Cells were sorted from the human tonsil (A) or thymus (B) as indicated in Figure 6. All six populations were cultured independently on OP9-DL1 cells with FL and IL-7 for 26 days. After harvest, cells were analyzed for the expression of CD3, CD4, CD8, TCRαβ, and TCRγδ. Data are gated on GFP^CD45^+ events to exclude OP9-DL1 stromal cells from analysis. Numbers represent the mean percentages of GFP^- CD45^+ cells that stained positive for both antigens indicated on the dot plot. (C-D) The progeny of each population from the human tonsil (white bars) was compared to the same population sorted from the human thymus (black bars) (C) Comparison of the mean percentage of GFP^CD45^+ cells from each tissue that stained positive for CD3 after 26 days of culture. (D) Comparison of the mean percentage of GFP^CD45^+ cells from each tissue that stained positive for both CD3 and a TCR (either TCRγδ or TCRαβ) after 26 days of culture. Data shown is representative of experiments performed with 7 individual tonsils or 4 thymus donors.
Figure 8: T cell differentiation potential of putative extrathymic T cell precursors in the human tonsil
Figure 9: Short-term T cell differentiation assays on putative extrathymic T cell precursors: Tonsillar populations 1-6 were sorted as shown in Figure 6, and were then cultured on the OP9-DL1 cell line with the cytokines FL and IL-7 for either 7 (A) or 14 (B) days. Data are from a representative donor; n=3 tonsils. The number in the upper right hand corner of each plot represents the mean percentage of events staining double positive for the antigens shown.
Figure 9: Short-term T cell differentiation assays on putative extrathymic T cell precursors.
Figure 10: NK cell differentiation potential of putative extrathymic T cell precursors in the human tonsil (A) Tonsil and thymus cells were sorted as described in Figure 6. Sorted cells were cultured on OP9-GFP cells and cultured in FL, KL, IL-3, IL-7, and IL-15 for 18-19 days. Harvested cells were gated on GFP^CD45^+ events and analyzed for expression of CD3 and CD56. (B-C) GFP^CD45^+ progeny from populations 1-4 were gated on CD3^- events and analyzed for expression of CD56, CD161, NKp46, and CD5. CD3, CD56, CD161, and CD5 data are representative of independent experiments performed with 3 tonsil or 5 thymus donors. NKp46 data are representative of 2 experiments performed with tonsil donors or 3 experiments performed with thymic donors. No data (ND) are available for NKp46 or CD5 expression on progeny of thymic population 4 cells due to the low numbers of harvested cells. All dot plots and gate frequencies are from a representative experiment.
Figure 10: NK cell differentiation potential of putative extrathymic T cell precursors in the human tonsil
**Figure 11: CD1a<sup>+</sup>TdT<sup>+</sup> cells reside near the fibrous scaffold of the human tonsil.** (A) Immunohistochemical staining of paraffin-embedded tonsillar sections localizes TdT<sup>+</sup> cells (dark brown staining) to the fibrous scaffold region. The image on the left (50x) is of a representative section including a germinal center (GC), the surrounding interfollicular zone (IFZ), and an adjacent fibrous scaffold region (FS). The image on the right is of the same tonsil section, but at a power of 100x. (B) Cells co-expressing TdT, CD1a, and CD34 are found within the scaffold region of the tonsil. TdT (green) and CD1a (red) co-expression is shown on the left, TdT (green) and CD34 (red) co-expression is shown in the middle, and CD34 (red) and CD1a (green) are shown on the right. Note the yellow cells in each section, which represent cells co-expressing both proteins of interest (yellow arrows). Blue staining indicates hematoxylin counterstain. (C) TdT (green), DL1 (red, left) and DL4 (red, right) proteins are expressed in the same geographical region of the human tonsil. Images in B-C are at a power of 400x.
Figure 11: CD1a$^+$ TdT$^+$ cells reside near the fibrous scaffold of the human tonsil
Figure 12: TdT\(^+\)Rag1\(^+\) cells reside near the fibrous scaffold of the human tonsil

(A) Immunohistochemical staining of paraffin-embedded tonsillar sections localizes TdT\(^+\) cells to the fibrous (FS) scaffold region (left image). White signal indicates co-localization of TdT protein with the nuclear stain hematoxylin. A serial section of the same tonsillar region localizes Rag1\(^+\) cells to the fibrous scaffold and germinal center (GC) regions (right image). Again, co-localization of nuclear Rag1 and hematoxylin is indicated by white signal. (B) A serial section of the same tonsillar region at a magnification of 400X that has been co-stained with antibodies for both TdT (green) and Rag1 (red) demonstrates that within the FS region of the tonsil, there are cells that express only Rag1 or TdT, as well as cells that co-express both proteins (yellow signal and arrows). (C) Immunohistochemical staining of paraffin-embedded thymic sections localizes TdT\(^+\) and Rag1\(^+\) cells to the cortex (c). In both images, white signal indicates co-localization of the protein of interest with nuclear hematoxylin. The thymic medulla (M) is indicated for reference. (D) A serial section of the same thymic region at a magnification of 400X that has been co-stained for TdT (green) and Rag1 (red) proteins demonstrates the presence of Rag1\(^+\)TdT\(^+\) (yellow) cells within the thymic cortex. Images in A and C were taken at a magnification of 200X.
Figure 12: TdT$^{+}$Rag1$^{+}$ cells reside near the fibrous scaffold of the human tonsil
Figure 13: Frequency of CD34⁺ CD1a⁺ CD11c⁻ cells in four representative tonsils
CD34-enriched tonsillar cells were analyzed for the presence of CD34⁺ CD1a⁺ CD11c⁻ cells in 24 donors, four of which are shown here. Numbers in the bottom right quadrant of each dot plot indicate the percentage of CD34⁺ cells that are CD1a⁺ CD11c⁻ in that particular donor.
Figure 13: Frequency of CD34+ CD1a+ CD11c- cells in four representative tonsils
**Figure 14: Sorting strategy for isolating 6 populations from tonsillar and thymic cells** (A) Tonsillar mononuclear cells were magnetically depleted of CD19+ cells, and were simultaneously enriched for CD34 and CD1a-expressing cells. Cells were gated and sorted into the six populations as shown. First, we gated on lymphocytes and excluded doublets by gating on SSC-W and FSC-W low events. Next all populations were gated on CD11c, BDCA-2, CD56, CD161, CD117, and CD19 negative events (Lin−), and populations 1 and 2 were additionally gated on CD3 and CD1a negative events. For sorting populations 4, 5, and 6, cells were gated on Lin−CD34− events. (B) Thymic mononuclear cells were magnetically enriched for CD34-expressing cells. Populations 1-3 were sorted from the CD34-enriched fraction, and populations 4-6 were sorted from the CD34-depleted fraction. (C-D) Representative sort purities from the tonsil (C) and thymus (D). Sort purity for a representative donor is shown for each gate, and overall purity was routinely greater than 95%, and often in excess of 98%.
Figure 14: Sorting strategy for isolating 6 populations from tonsillar and thymic cells
Table 2: Expansion of populations 1-6 in T cell differentiation assays

<table>
<thead>
<tr>
<th></th>
<th>Pop 1</th>
<th>Pop 2</th>
<th>Pop 3</th>
<th>Pop 4</th>
<th>Pop 5</th>
<th>Pop 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tonsil</strong></td>
<td>93.6±39.7</td>
<td>107.2±32.8</td>
<td>96.8±69.4</td>
<td>2.8±1.7</td>
<td>1.8±0.66</td>
<td>2.8±0.66</td>
</tr>
<tr>
<td><strong>Thymus</strong></td>
<td>270.5±150.3</td>
<td>83.0±20.7</td>
<td>61.0±30.2</td>
<td>0.75±0.25</td>
<td>3.0±2.7</td>
<td>5.8±2.4</td>
</tr>
</tbody>
</table>

*The number of human cells obtained from each culture was determined by counting total harvested cells (using trypan blue exclusion), and multiplying this number by the fraction of live cells defined as GFP(-)CD45(+). Data is represented as mean fold increase ± SEM.*
Table 3: Single cell differentiation potential of populations 1-4

<table>
<thead>
<tr>
<th>Average</th>
<th>% Wells Analyzed</th>
<th>% CD5+ CD3-CD56-Pre-T cells</th>
<th>% CD3+ T cells</th>
<th>% CD56+CD3-NK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population 1</td>
<td>24.58</td>
<td>88.10</td>
<td>0.00</td>
<td>81.25</td>
</tr>
<tr>
<td>Population 2</td>
<td>22.50</td>
<td>60.51</td>
<td>13.92</td>
<td>62.50</td>
</tr>
<tr>
<td>Population 3</td>
<td>67.50</td>
<td>98.72</td>
<td>51.74</td>
<td>28.38</td>
</tr>
<tr>
<td>Population 4</td>
<td>15.83</td>
<td>100.00</td>
<td>57.78</td>
<td>11.11</td>
</tr>
</tbody>
</table>

^A All numbers represent the mean percentage from two independent experiments.
Table 4: Frequency of extrathymic T cell precursors in the tonsil

<table>
<thead>
<tr>
<th>Frequency of putative extrathymic T cell precursors in the human tonsil</th>
<th>Pop 1</th>
<th>Pop 2</th>
<th>Pop 3</th>
<th>Pop 4</th>
<th>Pop 5</th>
<th>Pop 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Frequency (%)</strong>&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.016</td>
<td>0.016</td>
<td>0.004</td>
<td>0.083</td>
<td>2.42</td>
<td>94.38</td>
</tr>
<tr>
<td><strong>Range (%)</strong></td>
<td>0.006-0.032</td>
<td>0.005-0.031</td>
<td>0.0003-0.015</td>
<td>0.006-0.242</td>
<td>0.083-11.24</td>
<td>80.60-98.30</td>
</tr>
<tr>
<td><strong>Mean Number per Tonsil</strong>&lt;sup&gt;B&lt;/sup&gt;</td>
<td>21,052</td>
<td>19,323</td>
<td>3,915</td>
<td>81,954</td>
<td>2.1x10^6</td>
<td>1.1x10^8</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>3,575-57,750</td>
<td>6,340-50,989</td>
<td>216-15,338</td>
<td>3,331-245,909</td>
<td>77,571-1.15x10^6</td>
<td>4.6x10^7-2.16x10^8</td>
</tr>
</tbody>
</table>

<sup>A</sup> Mean frequency is expressed as a percentage of Lin<sup>-</sup> (CD19, CD117, CD56, CD161, CD11c, BDCA-2) cells as determined by flow cytometry.

<sup>B</sup> The absolute number of precursors in each population was calculated by multiplying the number of CD19-depleted mononuclear cells in each tonsil (determined by trypan blue exclusion) by the proportion of cells making up each subset (determined by flow cytometry).
3.1 hSLT as a site for divergent pathways of hematopoiesis

In their 2006 review article, Freud and Caligiuri hypothesized that the CD34$^+$ cells within the human tonsil were not only composed of pre-NK cells (stage 1 and 2), but also of CD34$^+$CD123$^{\text{bright}}$ pre-plasmacytoid dendritic cells (pDC). This was based on their unpublished findings that tonsillar CD34$^+$CD123$^{\text{bright}}$ cells, which were previously included within the CD34$^+$CD117$^-$ stage 1 pro-NK cell definition, did not contain any NK cell differentiation potential \textit{ex vivo}, but did express high levels of IL-3R$\alpha$ (CD123), a characteristic marker for pDCs [90]. This, along with their reports of significant heterogeneity within the tonsillar CD34$^+$ compartment, led us to further investigate the possibility that the CD34$^+$ subset of the tonsil is the starting point for several divergent pathways of hematopoiesis. We have known for some time that the tonsil supports NK cell development [88-90], and in Chapter 2 we outlined evidence suggesting that the tonsillar CD34$^+$ compartment may also contribute to extrathymic T cell development [87]. Next, we investigated the CD34$^+$ fraction of pediatric tonsils for the presence of cells expressing markers for APCs.

Antigen presenting cells are key components of the innate immune system. Collectively, they are responsible for surveying the tissue microenvironment and processing antigens, which they express on their cell surface via MHC molecules. T cells can then recognize these peptides and respond accordingly. Importantly, APCs make up a diverse subset of cells within hSLT, including the tonsils. Furthermore, like all cells of the immune system,
they develop from CD34+ hematopoietic progenitor cells [32]. When we investigated the tonsil for the presence of CD34+ cells that could be contributing to the development of APCs in this tissue, we found that there were consistently populations of CD34+ cells that variably expressed the markers CD11c, BDCA-2, CD123, CD33, and the GM-CSF receptor (CD116) (Figure 15a). CD11c is a marker that is commonly expressed on many APCs, including conventional dendritic cells (cDCs), monocytes, and CD1a+ Lanherhan’s cells [117-119]. Expression of BDCA-2 and high levels of CD123 characterize pDCs, and BDCA-2 is highly specific for this cell type [120]. CD33 is an antigen found on monocytes, macrophages and cDCs, although CD33 may also be expressed at lower levels on CD34+ HPCs of non-APC lineages [87, 90]. CD116 is a receptor that is found on many APC types as well [121], and is responsible for enabling these cells to differentiate and proliferate in response to the cytokine GM-CSF.

Significantly, mature APCs do not express CD34 on their cell surface. Thus, the identification of tonsillar CD34+ cells that express these markers is indicative that perhaps this organ has a role in the generation of DCs in situ.

To further explore this possibility, we analyzed CD34+ tonsillar cells for the expression of CD11c and BDCA-2. We found that there are CD34+ cells that are BDCA-2’CD11c+ and those that are BDCA-2’CD11c-. Furthermore, there was also a population of CD34+ cells that co-expressed both DC markers (Figure 15b). Conventional DCs express the markers CD11c, CD116, and CD33, and lack the pDC marker BDCA-2. Consistent with this phenotype, the CD34+CD11c+BDCA-2- cells of the human tonsil expressed CD116
and CD33, and expressed low levels of CD123. Furthermore, to establish whether or not these cells would have fallen within the previously defined stage 1 pro-NK cell subset or the stage 2 pre-NK cell subset, we further analyzed them for the expression of CD117. Interestingly, there are CD34⁺CD11c⁺BDCA-2⁻ cells that lack CD117 and those that express it (Figure 5c). This suggests that this population spans both stage 1 and 2 NK cells. The finding that these cells express classical DC markers (CD116, CD33) further supports the hypothesis that they belong to a DC lineage, but whether or not they may contribute to some NK cell differentiation as well is unknown. Alternatively, it is possible that the presence of CD34⁺CD11c⁺CD117⁺ cells within the broad definition of stage 2 may be the reason why Freud et al. found that bulk stage 2 cells gave rise to cDCs in culture [89].

Plasmacytoid DCs are specialized dendritic cells that respond to activation by producing large amounts of type-1 interferons [120]. Typically, human pDCs are defined as CD11c⁻ BDCA-2⁺CD123bright cells. We were able to confirm and extend Freud and Caliguri’s description of a putative pre-pDC within the tonsil by demonstrating that the CD34⁺CD123bright cells mentioned in the 2006 review fall within the CD34⁺BDCA-2⁺CD11c⁻ fraction of tonsillar cells. Furthermore, they co-express CD116 and low levels of CD33 (Figure 15d), as would be expected for a pDC [121]. Finally, as suggested by Freud and Caliguri, these cells are unlikely to contribute to NK cell differentiation, as they lack expression of the stage 2 antigen CD117 and further lack expression of the NK cell marker CD161 (Figure 15d).
These findings suggest that the human tonsil contains CD34\(^+\) precursors that can generate not only NK and T cells, but also cDCs and pDCs \textit{in situ}. The finding that there are mutually exclusive populations of cells that are likely parts of specific hematopoietic lineages is an important corollary to Freud et al.’s published finding that both Stage 1 and Stage 2 cells can differentiate into T cells, NK cells, and DCs in culture [88]. As stage 1 and 2 were minimally defined by the expression of only CD34 and CD117, one question remaining from their work was whether or not stage 1 and 2 cells were composed of multipotent cells that could individually give rise to T cells, NK cells, and DCs at the single cell level, or if instead there were multiple populations of lineage committed cells that fell within these minimal definitions. The findings reported in Chapter 2, as well as the unpublished data presented in Figure 15, suggests that it is likely a combination of both of these possibilities. The clonal assays presented in Table 3 suggest that CD34\(^+\)CD38\(^{\text{dim}}\)Lin\(^-\), CD34\(^+\)CD38\(^{\text{bright}}\)Lin\(^-\), and CD34\(^+\)CD1\(^a\)CD11c\(^-\) cells of the tonsil, all of which fall into the minimal definition of CD34\(^+\)CD117\(^-\) stage 1 cells, are multipotent for the T and NK cell lineages. Furthermore, Freud and Caligiuri’s finding that tonsillar CD34\(^+\)CD123\(^{\text{bright}}\) pre-pDCs cannot differentiate into NK cells suggests that some truly committed cell types reside within the CD34\(^+\) compartment of the human tonsil as well. Our finding of CD34\(^+\)CD11c\(^+\) cells that express markers specific for APCs, but lack common NK cell markers, further supports this. Combining the phenotypic evidence we have gathered in this thesis as well as that provided by the previously published findings of Freud and Caligiuri, we propose that CD34\(^+\) tonsillar cells give rise to several
divergent pathways of hematopoietic lineages. Future investigations should explore the single cell differentiation potential of each CD34\(^+\) subset defined here in order to confirm or deny this hypothesis.

Furthermore, most of our studies performed on CD34\(^+\) cells of the human tonsil were completed on CD34-enriched cells that were obtained from CD19-depleted tonsillar tissue. Between 50-75\% of the mononuclear cells of the human tonsil are CD19\(^+\) B cells (unpublished observation), whereas CD34\(^+\) cells make up approximately 1\% of the tissue [89]. We found that de-bulking the total mononuclear cell fraction of B cells allowed for a much more efficient and successful CD34 magnetic enrichment. Thus, we were unable to thoroughly assess the tonsillar tissue for the presence of CD34\(^+\)CD19\(^+\) pre-B cells.

While B cell development was not a primary focus of this thesis, whether or not the CD34\(^+\) compartment of the human tonsil can give rise to this specific lymphocyte subset is an important question. First, the tonsil is a robust producer of secretory IgA antibodies, which are produced by germinal center B cells. Furthermore, B cell activation is an integral part of the tonsillar immune response [122], making the role of the tonsil in the development of specific B cell subsets a relevant question. Secondly, there is evidence that the thymus specifically inhibits B cell development from the thymic seeding progenitors, but promotes the development of T cells, NK cells, and DCs [32]. So an interesting question regarding the role of the tonsil as a source of extrathymic T cell differentiation is whether or not the early HPCs identified in the tonsil are similarly
biased against B cell development, or if instead they contribute to B cell lymphopoiesis. Future investigations should explore the role of the tonsil in human B cell differentiation.

3.2 The identification of a true tonsil-seeding progenitor

In the study presented in Chapter 2, we provide evidence that the human tonsil contains a CD34^+CD38^{dim}Lin^- hematopoietic progenitor cell. As shown in Figure 2, this cell uniformly expressed CD45RA, CD10, HLA-DR, and low levels of CD33 [87], similar to lymphoid-biased HPCs reported in the bone marrow, umbilical cord blood, and thymus. In ex vivo cultures we demonstrated that this cell could develop into CD3^+ T cells as well as NK cells (Figures 8 and 10). Even at the clonal level, individual CD34^+CD38^{dim}Lin^- cells from the tonsil were able to differentiate into CD56^-CD3^- NK cells as well as CD5^+ T cell precursors (Table 3) [87]. Taken together, the phenotype and differentiation potential of these cells suggests that this cell may represent an early hematopoietic progenitor capable of giving rise to multiple hematopoietic lineages. The established knowledge of the role of the tonsil in human NK cell development [88-90, 94, 95], the evidence we have presented in Chapter 2, and the unpublished data presented in Figures 15 suggests that the human tonsil can support multiple lineages of hematopoiesis. Thus, a pertinent question is whether or not the CD34^+CD38^{dim} population identified in Chapter 2 is capable of giving rise to each of these cell types simultaneously and if it is a true early tonsil-seeding progenitor similar to the thymus-seeding progenitor.
That all hematopoietic lineages arise from CD34$^+$ cells within the human bone marrow is well known [32]. These cells, which have been extensively characterized and subdivided into many different subsets based on phenotype and differentiation capability [32], may either remain within the bone marrow or traffic to peripheral sites, such as the thymus, to further differentiate into the various cells of the immune system. Our description of a tonsillar CD34$^+$CD38$^{\text{dim}}$ cell, that is in many ways similar to the CD34$^+$CD38$^{\text{dim}}$ cells of the human thymus, suggests that perhaps the same bone marrow-derived progenitor cell seeds these two tissues. However, in Chapter 2, we noted several differences between thymic CD34$^+$CD38$^{\text{dim}}$Lin$^{-}$ cells and those from the tonsil. Specifically, a majority of the thymic subset expressed CD2, CD7, and CD5, whereas most tonsillar CD34$^+$CD38$^{\text{dim}}$Lin$^{-}$ cells did not. These three antigens are classically associated with the T cell lineage and are often used to characterize pre-T cells in the human thymus [32]. Thus, the absence of CD2, CD7, and CD5 on tonsillar CD34$^+$CD38$^{\text{dim}}$Lin$^{-}$ cells, but not on those of the thymus suggests two alternative possibilities. First, it is possible that the human tonsil and thymus are seeded by different progenitors, each capable of giving rise to NK and T cells in ex vivo differentiation assays. Alternatively, it is possible that the human tonsil and thymus are seeded by the same CD34$^+$CD38$^{\text{dim}}$CD2$^-$CD7$^-$CD5$^-$ progenitor that lacks CD2, CD7, and CD5.

Evidence suggests that murine thymic seeding progenitors rapidly differentiate upon entry to the thymus, presumably in response to the specialized thymic microenvironment [123, 124]. Thus, perhaps CD34$^+$CD38$^{\text{dim}}$CD2$^-$CD7$^-$CD5$^-$ cells within the thymus rapidly
differentiate into CD2⁺CD7⁺CD5⁺ early thymic progenitors upon entering the thymic microenvironment, whereas those in the tonsil largely do not. Several investigators have asserted that the thymic seeding progenitor is a CD34⁺CD38dimCD45RA⁺CD10⁺CD7⁺ cell [20, 26, 34, 125, 126]. Much of this is based on the identification of lymphoid progenitor cells that co-express CD7 and CD10 within umbilical cord blood [20, 126, 127], and on the fact that a large portion of the CD34⁺CD38dim fraction of the human thymus co-expresses CD7 and CD10. However, several groups have failed to identify a CD34⁺CD38dimCD45RA⁺ lymphoid progenitor that is also CD10⁺CD7⁺ within the post-natal bone marrow or peripheral blood [23, 126, 128], suggesting that it is not a CD10⁺CD7⁺ cell that serves as the thymic seeding progenitor throughout life. Indeed, more recent evidence has suggested a CD34⁺CD10⁺CD7⁻ cell seeds the post-natal thymus. In their 2007 Journal of Experimental Medicine report, Six et al. identified CD34⁺CD10⁺CD7⁻ cells with B, T and NK cell differentiation capabilities in the post-natal human bone marrow, peripheral blood, and thymus [23]. Furthermore, we demonstrate in Figure 2b that a majority of the thymic CD34⁺CD38dimLin⁻ cells that we characterized by flow cytometry are CD7⁺. However, a small percentage (4-17%) lack CD7 expression. Thus, it is entirely conceivable that the human thymus is seeded by a CD34⁺CD45RA⁺CD10⁺CD7⁻ cell that rapidly increases the expression of CD7, CD5, and CD2 upon thymic entry. If this is the case, it is also similarly possible that the CD34⁺CD38dimLin⁻CD2⁻CD5⁻CD7⁻ cells identified within the human tonsil represent these same bone marrow derived HPCs.
3.3 The role of the thymus in extrathymic T cell development

One very important question regarding the potential for extrathymic T cell development in the tonsil or other hSLT, is whether or not it is dependent on a functional, intact thymus. Perhaps extrathymic T cell differentiation in the tonsil occurs in the absence of a thymus as can IEL development in the mouse. Alternatively, tonsillar T lymphopoiesis could be thymus dependent. Conceivably, there are two ways that the thymus may promote T cell differentiation in extrathymic locations: 1) it may export extrathymic T cell precursors that are capable of seeding non-thymic lymphoid tissue; or 2) it may provide critical cytokines or environmental cues that can promote T cell differentiation in peripheral locations. As just discussed, it is possible that both the tonsil and thymus are seeded by bone marrow-derived HPCs that then give rise to T and/or NK cells and DCs within each tissue. However, it is also possible that the extrathymic T cell precursors identified within the tonsil traffic from the thymus rather than the bone marrow. If this second scenario were true, several unanswered questions arise. First, if the program of extrathymic T cell development identified in Chapter 2 is truly thymus-dependent, why is it that the TdT\(^+\) extrathymic T cell precursors aggregate specifically within the regions surrounding the fibrous capsule of the tonsil, rather than within the T-cell rich interfollicular zones? What factors drive the migration of extrathymic T cell precursors to this region, and why do mature thymic-derived T cells not similarly aggregate here?

Furthermore, which extrathymic T cell precursors traffic to the tonsil? Do the CD34\(^+\)CD38\(^{dim}\)Lin\(^-\) early thymic progenitors leave the thymus relatively early in their development, or do later stages of T cells traffic here? The presence of every stage of T
cell differentiation within the human tonsil suggests that either these cells enter the tonsil at their earliest stage of differentiation (i.e., at the CD34⁺CD38⁻Lin⁻), or that the thymus exports every single stage of T cell development at some basal level.

However, there is some evidence that the latter scenario is unlikely in adults at least. If the thymus was continually exporting every stage of T cell differentiation to the periphery, these cells should not only be identifiable within peripheral lymphoid organs, but should also be found within the blood. As discussed in Chapter 2, we identified CD1a⁺TdT⁺ cells in both adult and pediatric tonsil, and we further provided evidence that within the CD11c⁻CD1a⁺ fraction of human pediatric tonsils resides a CD4⁺CD8⁺ DP pre-T cell. However, we did not identify CD4⁺CD8⁺CD1a⁺ DP pre-T cells in adult peripheral blood [87]. This is consistent with the findings of Nascimbeni et al. who reported that adult peripheral blood CD4⁺CD8⁺ DP cells largely lacked CD1a expression [101]. Thus, it appears as if CD4⁺CD8⁺CD1a⁺ DP pre-T cells do not traffic within adult blood. Therefore, if these cells do reside within the adult tonsil, as suggested by our immunohistochemical identification of TdT⁺CD1a⁺ cells, they must be derived from an earlier T cell precursor within the tonsil itself. Unfortunately, we were unable to obtain peripheral blood from pediatric donors for these studies. Future investigations should establish if children export early T cell precursors from the thymus, and if so which ones they do.
Alternatively, the thymus may not export T cell precursors themselves, but may produce a critical environmental signal or growth factor that promotes extrathymic T cell development peripherally. Interestingly, the cytokines known to be critical for T cell development also have known extrathymic roles as well. IL-7, for example, is the only known cytokine that is indispensable for early T cell development in the thymus. Blocking IL-7 signaling in T cell differentiation assays prevents the generation of CD4⁺ ISP pre-T cells [129], and individuals with defects in IL-7 signaling account for a majority of patients with severe combined immunodeficiency disorders (SCID), wherein individuals have severe defects in T cell development [20]. However, IL-7 is not only necessary for T cell development. It is likely an important contributor to NK cell differentiation [130], and very importantly, it is involved in the homeostatic proliferation of mature T cells in peripheral lymphoid organs [131-133]. Interestingly, in individuals that undergo complete thymectomy, plasma IL-7 levels increase dramatically as a homeostatic mechanism to induce the peripheral expansion of T cells in the absence of thymic T cell output [134]. This underscores the fact that the thymus is not required for robust IL-7 production peripherally. Thus, even in the absence of a thymus, and perhaps even more so, IL-7 should be available to promote extrathymic T cell differentiation in hSLT. However, this does not rule out the possibility that the thymus provides some other environmental cue that is necessary for extrathymic T cell production in the tonsil.

Is there evidence that humans can undergo extrathymic T cell differentiation in the absence of a thymus? Complete thymectomy during early infancy is commonly
performed as a necessary part of cardiac surgery, as the large neonatal thymus resides within the mediastinum and obstructs access to the heart and great vessels [134]. In the years immediately following thymectomy, individuals undergo drastic decreases in the number of circulating CD4$^+$ and CD8$^+$ naïve T cells [135-137]. However, in some adults that underwent childhood thymectomy, total peripheral T cell counts are normal compared to non-thymectomized individuals [51, 138]. It is unclear if peripheral expansion of mature T cells is entirely responsible for this regeneration of the T cell pool, or if extrathymic T cell differentiation may contribute to T cell recovery in thymectomized patients. At least one study has shown that some thymectomized individuals spontaneously begin producing naïve T cells post-surgery. However, this group also demonstrated that many of these patients had residual or regenerated thymic tissue on MRI investigation [134]. Interestingly, in some thymectomized children, specific subsets of T cells predominate years after surgery. A 2006 study by Torfadottir et al. found that whereas most CD3$^+$ T cell subsets were drastically reduced following complete thymectomy, the proportion of TCRγδ T cells was significantly increased and the relative proportion of CD4$^+$CD25$^+$ T regulatory cells was unchanged [137]. At the time, they hypothesized that extrathymic maturation was responsible for the generation of these particular subsets.

Another clinical scenario involving an absolute lack of thymic tissue is seen in the presentation of complete DiGeorge’s syndrome. DiGeorge’s syndrome is a congenital malformation that occurs during the development of the third and fourth pharyngeal
pouches, which give rise to the thymus, parathyroid gland, and the musculature and cartilage of the larynx. The result of this malformation is a varying degree of cardiac anomalies, parathyroid dysfunction, and thymic aplasia [139, 140]. The T cell deficiency in patients with DiGeorge’s syndrome is highly varied, and many patients experience no clinical recourse. This is typically referred to as partial DiGeorge’s syndrome and is characterized by the presence of reduced thymic volume, but not a complete absence of a thymus. In individuals with complete DiGeorge’s Syndrome, CD3+ cells make up less than 1% of the lymphocyte population, and it is generally assumed that these patients have zero thymic tissue [139-141].

Studies from patients with complete DiGeorge’s syndrome underscore the extreme importance of thymic tissue in the generation of a normal immune response. It appears as if many patients with complete thymic aplasia do not have any circulating T cells in their peripheral blood [141]. This would suggest that extrathymic T cell development is not capable of generating a large repertoire of functional T cells in the absence of a thymus. However, it is possible that T cells are generated in the tonsils or lymph nodes of these individuals at some level, and that they do not proliferate or circulate extensively. Furthermore, at least one report has suggested that a patient with complete DiGeorge’s syndrome did spontaneously generate peripheral blood CD3+ cells, but that these cells failed to proliferate in response to cytokines as do normal, healthy naïve T cells [142]. This patient was observed to be completely athymic by medical imaging during life as well as upon autopsy. The origin of the circulating T cells in this case is unknown, as is
why they were not normal, proliferating cells [142]. The authors speculate that the absence of a thymus may allow for a low level of extrathymic T cell generation, but that a thymus is still required for the complete development of differentiating T cells.

Thus, in the clinical scenarios of surgical thymectomy and complete DiGeorge’s syndrome, there appears to be small pieces of evidence suggesting that there may be a role for extrathymic T cell differentiation, at least of certain T cell subsets. Importantly, in both cases, the measurement for T cell development always relies on analyzing the peripheral blood for new, naïve CD3+ T cells. It is entirely possible that extrathymic T cell development in the human tonsil is a local process, wherein mature T cells do not leave the tonsil in large numbers. Without having analyzed the tonsil or other hSLT for the presence of T cells or their precursors in the setting of complete thymectomy or DiGeorge’s syndrome, it is unknown to what extent the tonsil may be producing local T cell subsets in these situations. In summary, at the current time we do not fully understand the role of the thymus in extrathymic T cell differentiation.

3.4 The tonsil microenvironment as a site for T lymphopoiesis

The human palatine tonsil is considered a mucosa-associated lymphoid tissue (MALT), similar to intestinal Peyer’s Patches. Furthermore, as the predominant lymphoid tissue of the oropharynx, the tonsil is the immunologic gatekeeper of both the respiratory and digestive system in that it is the first port of entry for pathogenic antigens [122]. The stratified squamous epithelial surface of the tonsil is highly invaginated into a network of
deep crypts. These crypts create a large surface area through which antigens from the oropharynx enter the body. Surrounding these crypts are fibrous regions containing stromal cells, nerves, blood vessels, and lymphatics [122]. As shown in Chapter 2, CD1a⁺TdT⁺ cells within the human tonsil aggregate within and near these areas of fibrous scaffolding [87]. This anatomical description of the tonsil and the location of CD1a⁺TdT⁺ cells within it, suggests that perhaps these fibrous regions support extrathymic T cell development for the purpose of generating T cells specifically involved in mucosal immunity.

This is intriguing given that so many investigators have reported that specific murine T cells develop within the gut mucosa itself [57-71]. Perhaps, the pathway of extrathymic T cell development outlined in Chapter 2, is analogous to that identified in these studies of murine mucosal immunity. Immunologically, the intestinal mucosa is an extension of the more concentrated and histologically organized tonsil epithelium. Thus, it is possible that the complete program of extrathymic T cell differentiation we described in Chapter 2 is present in the intestinal mucosa as well. This is further supported by Williams et al.’s 2004 description of CD5⁺CD7⁺CD3⁻ cells in the neonatal human intestine [86]. While this study did not completely immunophenotype these cells, nor did it provide evidence that these cells do in fact become mature T cells, it was remarkable in laying the foundation for the possibility that human MALT may participate in extrathymic T cell development. Thus, our 2012 JCI report helps to confirm their initial findings and further describes a possible complete program of extrathymic T cell lymphopoiesis in MALT.
tissue. In order to better understand the extent and nature of this phenomenon, it would be intriguing to investigate the presence of a similar pathway of T cell genesis in the intestinal mucosa of children, adults, and individuals with inflammatory bowel disease.

One of the leading assumptions in T cell biology is that the thymus provides a specific microenvironment that is uniquely suitable for generating normal, functional T cells. If complete T cell differentiation does occur extrathymically, it is important to establish whether or not it occurs in a microenvironment that is identical to that which is provided by the thymus, or if instead a different environmental milieu shapes the development of extrathymically-derived T cells. In Chapter 2, we did provide evidence that the Notch ligands DL1 and DL4 are present in the same microenvironment as are tonsillar TdT+ cells. Developing thymocytes express the Notch1 receptor on their surface, and interactions between Notch and its ligands in the thymic microenvironment are essential for normal T cell differentiation [53, 104, 109, 110]. The presence of DL1 and DL4 in the same anatomical location as the tonsillar CD1a+TdT+ pre-T cells suggests that at minimum, this critical developmental factor is available within the tonsil microenvironment. Furthermore, it is known that IL-7, a critical cytokine for T cell development is also present in the peripheral lymphoid organs, as it plays an important role in promoting the peripheral expansion of mature T cells [20, 129].

Is there evidence that the tonsil microenvironment could provide the necessary stromal-pre-T cell interactions necessary for maintaining self-tolerance? The tonsil does contain
an abundance of APCs that present antigens to peripheral T cells, but whether or not they are capable of mediating a process similar to thymic positive and negative selection is unclear. Furthermore, it is unclear if the repertoire of antigens presented in the tonsil, which is likely much more biased towards foreign antigen than is the antigen pool of the secluded, non-mucosal thymic environment, would have a significant impact on the development of TCR\textsuperscript{+} extrathymic pre-T cells. During negative selection, thymic stromal cells ectopically express tissue specific antigens (TSA) from every tissue of the body [143-146]. Thus, developing thymocytes are selected based on their inability to bind these TSAs with high avidity. The ability to express TSAs with such breadth in an ectopic environment is thought to be unique to the thymus [143-146]. Thus, whether or not the tonsil is capable of doing so would be an important question in determining if it is also capable of driving normal T cell development. Interestingly, in murine studies of extrathymic T cell development, it has been shown that gut-derived IEL’s have a limited TCR repertoire, suggesting that the environmental pressures they experience during development is different than those experienced by thymic-derived T cells [78-80]. Future studies should thoroughly compare the microenvironment of the human tonsil, specifically the regions near the fibrous scaffolding, to that of the human thymus. These investigations should seek to answer if the tonsil supports the differentiation of a T cell pool with a limited TCR repertoire, and if so, is this for the purpose of generating a specific subset of T cells with a role in tonsillar or mucosal immunity.

3.5 The role of extrathymic T cell development in health and disease
In Chapter 2, we provided substantial evidence for a comprehensive program of extrathymic T cell development within the human tonsil. These data are novel and, indeed, the existence of human extrathymic T lymphopoiesis has long been controversial. Yet, to date, it is unknown what the physiological significance of tonsillar T cell development may be. The finding that early CD3− extrathymic T cell precursors make up an incredibly small fraction of the total mononuclear cell pool within the average pediatric tonsil (Figure 13 and Table 4) suggests that extrathymic T cell development likely does not give rise to the majority of tonsillar CD3⁺ T cells. Rather, we believe that, like most peripheral T cells, a large majority of the tonsillar-resident CD3⁺ cells are thymus-derived. However, this does not preclude extrathymic T cell development from contributing in a significant way to the generation of specific subsets of T cells within normal individuals, or perhaps playing a much larger role in the setting of disease or otherwise poor thymic function. It is our hope that this initial study lays the foundation for future investigations exploring the specific physiological or non-physiological conditions where this process may be amplified, inhibited, or exploited. We have already discussed the possible role of extrathymic T cell development in the setting of infant thymectomy and DiGeorge’s syndrome. Below we discuss some of the other possible clinical scenarios wherein extrathymic T cell differentiation may be important. However, at the present time it is impossible to speculate about the complete spectrum of conditions where this phenomenon may be acting.

**Chronic inflammation and tonsillar hypertrophy**
One particularly relevant question raised by our work is whether or not chronic inflammation is associated with extrathymic T cell development in this tissue. The studies performed in Chapter 2 were completed with tissue obtained from pediatric patients undergoing routine tonsillectomy. While it can be assumed that the patients were in relative good health at the time of the elective surgery, it must also be assumed that they were having their tonsils removed for a clinically significant reason. Currently, we do not have IRB approval to obtain data regarding the diagnosis, prognosis, or treatment of any of the patients from which the tonsils were taken. Thus, we do not know the primary reason for tonsillectomy, or what underlying disease process may be occurring in each individual case. As shown in Figure 13 and Table 4, there appears to be substantial donor-to-donor variability in the frequency of tonsillar extrathymic T cell precursors. This suggests that there may be underlying mechanisms accounting for such variability.

As of 2002, routine tonsillectomy was still the most common surgical procedure performed on children [147]. The most common reason for pediatric tonsillectomy is obstructive sleep apnea, but many patients undergo tonsillectomy for recurrent infectious tonsillitis as well [148]. In obstructive sleep apnea, the tonsillar tissue undergoes generalized hypertrophy that may be related to fat deposition, inflammation, or of unknown cause [149]. In the case of recurrent acute tonsillitis due to *Streptococcus pyogenes* (Group A strep), tonsillar hypertrophy appears to be strictly associated with increased inflammation. In these cases, there is an overall increase in the pro-inflammatory cytokine profile of the pediatric tonsil as well as an increase in the
activation of IL-17-producing T cells [148]. At present, it is unclear how either benign hypertrophy or chronic inflammation may affect tonsillar extrathymic T cell development. Yet, the fact that the tonsils studied in Chapter 2 were removed from patients for a particular—if unknown—reason remains an important corollary to our work. It would of significant interest to investigate further the correlation of extrathymic T cell precursor frequency to the reason for tonsillectomy, and to investigate the overall role of chronic inflammation on the differentiation of tonsillar pre-T cells.

**Autoimmunity**

As discussed previously, another question raised by our demonstration of T cell development within the human tonsil is whether or not this tissue provides a microenvironment that is similar to that which is found within the thymus. If it does not, it is entirely possible that the identified pathway of tonsillar T cell development represents an aberrant process that operates outside of the safeguards of the thymic niche. If tonsillar T cell development does take place without the same environmental pressures ensuring central tolerance, it is possible that the tonsil may actually support the generation of autoreactive T lymphocytes. Indeed, tonsillar T cells have been associated with several autoimmune processes, including IgA nephropathy [150, 151] and psoriasis [152].

IgA nephropathy is an autoimmune syndrome wherein IgA-immune complexes are deposited on the renal glomerulus, leading to renal dysfunction and occasionally failure
Interestingly, it appears as if some of the IgA that is deposited within the kidneys originates in the patient’s tonsil, where there is a disproportionate increase of IgA-producing B cells. Furthermore, recurrent streptococcal and haemophilus-associated tonsillitis exacerbates renal symptoms, and surprisingly, tonsillectomy can improve the clinical scores of patients with IgA nephropathy [151]. While IgA nephropathy is an autoimmune disorder largely mediated by self-reactive B cells, there are also notable differences found in the T cell compartment of tonsils from individuals with and without IgA nephropathy. For example, the repertoire of TCRs expressed in the tonsils of patients with IgA nephropathy seems to be significantly altered and limited compared to those expressed in a healthy tonsil [150], and the number of CD4⁺CD25⁺ regulatory T cells is decreased in the tonsils of patients with IgA nephropathy [153].

Furthermore, a similar connection between tonsillar inflammation and autoimmune processes is seen during the clinical manifestations of psoriasis [152]. Psoriasis is an inflammatory skin disease characterized by the infiltration of lymphocytes into the dermis and epidermis of affected patients, and the disease appears to be largely mediated by auto-reactive T cells [152]. Interestingly, multiple forms of psoriatic skin lesions are characteristically associated with or are preceded by streptococcal infections of the tonsil [152, 154, 155]. Within the skin lesions of individuals with psoriasis, there is an oligoclonal expansion of antigen-specific T cells with a very limited TCR repertoire, and similar oligoclonal T cells are identifiable within the tonsils of these patients as well [152]. Furthermore, at least one report has demonstrated evidence that the oligoclonal
self-reactive T cells found within psoriatic lesions originate specifically within the tonsil, and that the tonsil may be at least partially responsible for maintaining the autoimmune disorder [152, 156]. In the report by Diluvio et al., three patients experienced multi-year remission of their psoriasis symptoms following tonsillectomy [156]. Furthermore, the clinical connection between the streptococcal infection, the human tonsil, and psoriatic skin lesions appears to be driven by the fact that the specific oligoclonal T cells found within individuals with psoriasis are cross-reactive with antigens produced by *Streptococcal pyogenes* and with self-antigens found within human keratin [152].

Thus, while the mechanisms of disease seen in IgA nephropathy and psoriasis are different, both disorders involve an oligoclonal expansion of T cells with specific TCR rearrangements within the human tonsil, and both diseases seem to be partially remedied by tonsillectomy. Furthermore, there appears to be a connection between common tonsil infections and the flare up of both autoimmune processes. Of interest to this dissertation, is whether or not the oligoclonal T cells found within the tonsils of these patients are thymic derived, or instead if the tonsil is more likely to harbor self-reactive T lymphocytes because of an underlying pathway of extrathymic T cell development that is not subjected to the same environmental pressures found within the human thymus.

Future studies should investigate the role of this tonsillar T cell development in the pathogenesis of such autoimmune diseases.

**Malignancy**
Another clinical scenario where extrathymic T cell development within hSLT should be further explored is in the setting of human malignancy. One of the incredibly important tasks of a mature T cell is to survey the body for cancerous cells. When our immune system is working optimally, T cells recognize transformed cells before a tumor develops and clear them from the body. In the setting of established malignancy, mature T cells frequently invade a tumor in order to help the body fight the invading cells. Interestingly, sporadic case studies of follicular dendritic cell sarcomas with CD1a⁺ pre-T cell infiltration have been made in recent years. These tumors originate within the germinal centers of the lymph nodes. While not a T cell cancer, they frequently contain activated T cells that infiltrate and try to fight the tumor. Accordingly, the identified CD1a⁺CD3⁺/- pre-T cells within the affected lymph nodes were not cancerous. Rather, they were infiltrating the cancerous sarcoma. Interestingly, both of these cases occurred in patients with sarcoma and concurrent myasthenia gravis, an autoimmune disorder of the neuromuscular system [157, 158]. Myasthenia gravis is caused by the formation of auto-antibodies and auto-reactive T cells which are specific for the human acetylcholine receptor. In many patients, thymectomy is curative, suggesting that the original formation of auto-reactive T cells occurs within the thymus. However, some patients have no clinical response to thymectomy, suggesting that myasthenia gravis also critically depends on the expansion of auto-reactive T cells peripherally [159]. It is not known whether or not the particular patients in the two reports involving follicular sarcoma would benefit from thymectomy for the treatment of their myasthenia gravis, or if the pre-T cells identified in these studies were thymus-derived. Furthermore, it is unclear if
these individuals suffered from myasthenia gravis before the onset of the lymph node sarcomas, or if instead the myasthenia gravis developed after the onset of malignancy, perhaps due to the expansion and development of extrathymic pre-T cells during the course of tumor formation. Thus, whether or not aberrant extrathymic T cell differentiation may contribute to pre-T cell infiltration in the lymph nodes or tonsils of patients with follicular sarcoma should be further explored.

**Physiologic thymic atrophy associated with aging**

The physiological atrophy of the human thymus with age has been well documented and studied since before Jacques Miller identified the role of the thymus as an immunological organ [160]. Indeed, it is well known that thymic volume is highest during the fetal and perinatal periods of human life, and declines thereafter [161]. The human immune system undergoes significant functional changes throughout life that can result in a dampened immune response, a process termed immunosenescence [162]. These physiological changes within the immune system can account for much of the increased susceptibility to infection, inflammation, and malignancy seen in elderly populations [163, 164]. One particularly notable age-related change in the immune system is the decreased production of early lymphocytes in the bone marrow and thymus. This results in an overall decrease in new, naïve T cells that emerge from the thymus, and in a concomitant clonal expansion of existing memory and effector T cells in the secondary lymphoid organs [165]. The clonal expansion of peripheral T cells further leads to a skewing of the TCR repertoire towards T cells specific for antigens already encountered by the host previously in life.
Thus, the ability to mount an immune response to new pathogens is substantially decreased in elderly individuals [162].

The production of new, antigen-specific T cells in aged individuals seems to be affected at multiple stages along the thymic T cell developmental pathway. In 2004, Min et al. demonstrated that there is a decreased influx of early thymic progenitors into the thymus of aged mice [166]. However, decreased immigration of HPCs from the bone marrow could not account for the total decrease in T cell production, as early thymic progenitors were significantly less proliferative in old mice than those in young mice, and their intrinsic ability to differentiate into mature T cells was dampened as well [166]. Furthermore, others have demonstrated significant changes in the thymic stromal compartment during normal physiologic aging, notably an age-related change in the organization of TECs and an increase in adipocytes within the stromal network [161]. Thus, a variety of age-related changes seem to account for the overall decreased thymic output of new, naïve T cells throughout life.

How this process may involve or effect extrathymic T cell production is unclear. From our knowledge of age-related immune system changes, two hypothesis could be generated. First, it is possible that in the face of significant thymic atrophy, extrathymic T cell development in the tonsil and other peripheral lymphoid organs increases as a compensatory mechanism in aged individuals. Sempowski et al. found that transcript levels of Oncostatin M (OM) and leukemia inhibitory factor (LIF) increase in the
thymuses of aged humans, and they have been shown to directly cause thymic atrophy in normal mice [167]. This is interesting given that mice exposed to OM and LIF also exhibit robust extrathymic T cell development in their peripheral lymphoid organs [46, 72-77]. Together, these findings suggest that perhaps as OM and LIF-induced thymic atrophy occurs in aging individuals, the extrathymic peripheral lymphoid organs begin to play a larger role in T cell genesis.

Second, it is possible that extrathymic T cell development is most robust early in life and that it experiences an age-related decrease just as thymic T cell production does. Most of our studies were performed on tonsil segments obtained from pediatric donors, aged 1-16. As mentioned in Chapter 2, we saw no statistical correlation between the age and the frequency of CD34+CD1a+CD11c− pre-T cells within the total CD34+ compartment of the tissue. However, the frequency of these precursors in individuals older than 16 was not directly measured. Furthermore, we were measuring the frequency of pre-T cells as a fraction of total CD34+ cells, and it is possible that the CD34+ subset of the tonsil is proportionally altered throughout childhood and adolescence. As also mentioned in Chapter 2, we did analyze adult tonsils for the presence of CD1a+TdT+ cells by immunohistochemistry. Empirically, we did see fewer TdT+ cells in the fibrous scaffolding regions of adult tonsils when compared to pediatric tonsils. However, a small sample size (n≥3 for pediatric tonsil; n=2 for adult tonsil) may have affected this observation. Additionally, the reasons and implications for adult tonsillectomy vs. childhood tonsillectomy may have biased this finding. Thus, it is unclear if extrathymic T
cell development within the adult tonsil is absolutely decreased compared to that in pediatric tonsil. An interesting follow-up study to the work presented here would be to statistically analyze the presence of extrathymic T cell precursors from the adult vs. pediatric tonsil and to correlate this with age, thymic output, and T cell repertoire narrowing.

**Hematopoietic cell transplant**

One particularly challenging clinical scenario wherein T cell development plays a critical role in determining patient outcome is in the setting of allogeneic hematopoietic stem cell transplant (HSCT). HSCT is characterized by intravenous injection of donor-obtained hematopoietic stem cells, typically harvested from the bone marrow, peripheral blood, or umbilical cord blood. It is currently used to treat a wide variety of hematologic disorders, which may be either benign or malignant in nature [168-171], and advances in medical knowledge and practice have increased the median post-transplant survival of recipients dramatically in recent years [170, 172, 173]. However, the efficient regeneration of adaptive immune responses following HSCT remains a difficult clinical challenge for transplantation [1, 170]. Specifically, HSCT is typically preceded by either chemotherapy to treat an underlying malignancy or radioablative therapy to ameliorate the immune system of the recipient prior to transplant [1]. These preconditioning regimens destroy not only the peripheral T cell pool but also decimate the thymus. Thus, the regeneration of T cells following transplant depends not only on successful engraftment of HSCs, but also depends on the recovery of the thymus as an organ that can support robust T cell
lymphogenesis [1]. Post-transplant infection remains one of the major clinical recourses and a difficult obstacle to overcome following HSCT [1, 170]. Perhaps the pathway of T cell genesis identified in the human tonsil may serve as an important reservoir for either early T cell developmental intermediates or as a thymopoietic microenvironment to encourage quicker T cell recovery post-transplant. Thus, whether or not extrathymic T cell differentiation could contribute to a quicker recovery of the peripheral T cell pool in HSCT recipients is unknown and should be explored.

**Human immunodeficiency virus**

Individuals with a high viral load of the human immunodeficiency virus (HIV) experience a drastic decrease in the number of CD4+ cells throughout their body. This infection affects not only mature CD4+ T cells and CD4+ DCs, but also dramatically decreases thymic output of new, naïve T cells [174, 175]. As such, the clinical progression of HIV, and its progression to acquired immune deficiency syndrome (AIDS), is defined by a decreased ability to fight infection and malignancy. The presence of abundant thymic tissue in an HIV infected individual is associated with better clinical outcome and an increase in total CD4+ cells [176], and methods for improving thymic output in the scenario of HIV infection are of extreme interest in the study of HIV pathogenesis [1, 176]. Unfortunately, the T cells of the human tonsil are similarly impacted by HIV infection [177]. We would hypothesize that any pathway of extrathymic T cell differentiation in this tissue would be just as susceptible to HIV-mediated destruction, especially given the anatomical location of the tonsil at a major site
for pathogen entry into the body. However, it may be of some interest to better understand whether or not tonsillar T cell differentiation is more or less susceptible to the effects of HIV infection, and if there may be a pharmacologic method that might boost this pathway of extrathymic T lymphopoiesis in the setting of extremely low CD4 counts.

**Augmentation of extrathymic T cell differentiation and T cell regeneration**

As discussed, the efficient generation of functional naïve T cells in the face of thymic atrophy, disease, or malformation is a challenging clinical obstacle. One speculative implication for the identification of extrathymic T cell differentiation in the human tonsil is that it could eventually provide a reservoir for T cell genesis in patients with poor thymic function, congenital abnormality, or thymic atrophy. The findings that OM and LIF convert peripheral lymphoid organs into T lymphopoietic organs in athymic mice [46, 72-77], suggests that it may be possible to pharmacologically augment extrathymic T cell differentiation in humans as well. In humans, several agents have been identified as potential thymus-boosting growth factors. Specifically, growth hormone (GH) and flt3 ligand (FL) have both been shown to increase human thymic output in HIV and HSCT respectively [176, 178]. It would be intriguing to investigate the effects these thymus-boosting agents have on extrathymic T cell development as well. If tonsillar T cell development responds to either GH or FL by increasing total naïve T cell generation, perhaps these agents would be further beneficial in situations where the thymus is unable to recover, such as in individuals that are athymic due to DiGeorge’s syndrome or those that have undergone complete thymectomy. While these possibilities are speculative at
the current time, the identification of a program of extrathymic T cell development in the human tonsil suggests that perhaps this tissue may serve as an important reservoir for new T cell genesis in the face of poor thymic function.

3.6 Concluding remarks

In this thesis, we have provided evidence for a complete program of extrathymic T cell development within the human tonsil. To our knowledge, these studies are the first to describe a comprehensive model of human T cell lymphopoiesis outside of the thymus. The existence of such a pathway has been controversial in the field of T cell development for some time, and it is our hope that these findings provide novel insight into the capabilities of hSLT to participate in T cell generation. As we have discussed in this chapter, the extrathymic T cell developmental program of the tonsil could have significant implications for human health and disease. It will no doubt be very exciting to see what future studies reveal regarding the role of extrathymic T cell differentiation in human secondary lymphoid tissue.
3.7 Figures

Figure 15: Evidence for dendritic cell precursors within the human tonsil: (A) Human tonsillar mononuclear cells were depleted of CD3 and CD19-expressing cells, and were then magnetically enriched for CD34$^+$ cells. CD34-enriched cells were analyzed for the expression of CD34, CD11c, BDCA-2, CD116, CD123 and CD33 (B) Expression of CD11c and BDCA-2 on CD34$^+$ tonsillar cells. (C) The relationship of CD11c and CD116, CD117, CD123, and CD33 on CD34$^-$BDCA-2$^-$ tonsillar cells suggests that the pediatric tonsil contains CD34$^+$ precursors for conventional dendritic cells. (D) The relationship of BDCA-2 and CD116, CD117, CD123, CD33 on CD34$^+$CD11c$^-$ tonsillar cells suggests that the pediatric tonsil contains precursor plasmacytoid dendritic cells. Each dot plot is from a representative donor where n≥2 independent tonsils analyzed for each combination of antigens.
Figure 15: Evidence for dendritic cell precursors within the human tonsil
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