Transcriptional Priming for Intrathymic Dendritic Cell Development

By

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A thesis submitted in conformity with the requirements for the

Doctor of Philosophy

Department of Immunology

University of Toronto

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ABSTRACT

It has been conclusively shown that conventional dendritic cell (DC)-restricted precursors are generated in the bone marrow and migrate through the blood to the spleen and lymph nodes. However, the origins and developmental programs of thymic DC subsets remain obscure. Here, I have established unique transcriptional profiles for splenic and thymic DC subsets and I have used this information to identify candidate precursors in the thymus that are transcriptionally primed towards the DC lineages. I have also shown that DCs can develop in the presence of moderate levels of Delta-like-4 (Dll4)-Notch signaling, consistent with development of these cells within the thymic medulla, and that these levels of Dll4 are restrictive for myeloid development. The addition of Fms-like tyrosine kinase 3 ligand (Flt3L), exclusively, to DC cultures enables greater DC development in the presence of medium or high levels of Delta-like-1 (Dll1) or Dll4 ligand. Similarly, transient overexpression of inhibitor of DNA binding 2 (Id2) can enhance DC development in vivo from thymocyte precursors without significantly
perturbing T cell development. Thymic DC populations are severely disrupted in recombination activation gene (RAG)-deficient mice, which lack medulla, and interleukin 7 receptor α (IL-7Rα)-deficient mice, which have reduced cellularity, indicating that a properly structured thymus and developing T cell pool are required for optimal thymic DC development or maintenance. Finally, I have demonstrated that early T cell progenitor (ETP), double negative 1 d (DN1d) and DN1e subsets have the capacity to generate thymic DCs in vivo in the context of a fully structured thymus. Our data supports a model in which the expression of chemokine (C-C motif) receptor 7 (CCR7) and chemokine (C-C motif) receptor 4 (CCR4) on DC-lineage primed ETP, DN1d and DN1e thymocyte subsets allow them to preferentially migrate to the medulla after entering the thymus, where they are exposed to a microenvironment that favors DC development.
Dedications

This thesis is dedicated to Mom and Dad for their unconditional love and support.
Acknowledgements

I would first like to thank my supervisor, Dr. Michele Anderson, for the years of scientific training and guidance. Our brainstorming sessions and your scientific vision has taught me to think critically and creatively. The endeavors in my project have allowed me to learn numerous scientific techniques and to teach these techniques to others. You have taught me the art of scientific writing and that everything becomes clearer when represented in a gene regulatory network. Overall, you have taught me how to run a laboratory and I thank you.

I would like to thank my committee members, Dr. Juan Carlos Zúñiga-Pflücker, Dr. Mark Cattral, Dr. Pam Ohashi and Dr. James Booth. Each of you has offered invaluable and unique insight into my project to turn the needle in a haystack into something I could see.

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To my family, thank you for providing unconditional love and support. Each of you has inspired me to do what I love and to do it well.

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List of Abbreviations

BM       Bone marrow
CAR      Coxsackie adenovirus receptor
CCR4     Chemokine (C-C motif) receptor 4
CCR7     Chemokine (C-C motif) receptor 7
CD       Cluster of differentiation
cDC      Conventional dendritic cell
CDP      Common dendritic cell precursor
CLP      Common lymphoid progenitor
CMJ      Cortico-medullary junction
CMP      Common myeloid progenitor
cTEC     Cortical thymic epithelial cell
CTP      Circulating T cell progenitor
DC       Dendritic cell
DII1/DL1 Delta-like-1
DII4/DL4 Delta-like-4
DN       Double negative (CD8^- CD4^-)
DP       Double positive (CD8^+ CD4^+)
ETP      Early T lineage progenitors
FACS     Fluorescence-activated cell sorting
FL       Fetal liver
Flt3     Fms-like tyrosine kinase 3
Flt3L    Fms-like tyrosine kinase 3 ligand
FTOC     Fetal thymic organ culture
GMP      Granulocyte/macrophage precursor
GSI      Gamma secretase inhibitor
HEB      HeLa E-box binding protein
HSC      Hematopoietic stem cell
IAD      IRF (interferon regulatory factor) association domain
ICSBP    Interferon consensus-binding protein
Id2      Inhibitor of DNA binding 2
IKDC     Interferon-producing killer dendritic cell
IL-3     Interleukin 3
IL-7     Interleukin 7
IL-7Rα   Interleukin 7 receptor alpha
IRF      Interferon regulatory factor
IPC      Interferon-producing cell
ISRE     Interferon-stimulated response element
LC       Langerhans cell
LMPP     Lymphoid-primed multipotent progenitor
LN       Lymph node
LSK      Lineage^- Sca1^+ cKit^+
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MDP</td>
<td>Macrophage/dendritic cell precursor</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent progenitor</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PDCA-1</td>
<td>Plasmacytoid dendritic cell antigen 1</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>S-F</td>
<td>Stroma-free</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>tDC</td>
<td>Thymic dendritic cell</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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Chapter I

Introduction

Amanda J. Moore\textsuperscript{1,2} and Michele K. Anderson\textsuperscript{1,2}

\textsuperscript{1}Sunnybrook Research Institute, Division of Molecular and Cellular Biology, Toronto, ON

\textsuperscript{2}University of Toronto, Department of Immunology, Toronto, ON

Table 1 and Figure 2 have been published in Advances in Hematology. 2013. Feb 18. Volume 2013. Article ID 949513. 1-16. PMID: 23476654.
1. **Innate Immunity and Dendritic Cells**

The cells of the immune system continually monitor the body to provide comprehensive immune responses to invading pathogens. The initiation of an immune response begins with the activation of the innate immune cells, which express a collection of pathogen recognition receptors (PRRs) that recognize molecules broadly expressed by infectious agents. The innate immune cells alert and activate the adaptive immune system to provide a more specific immune response to the pathogen, which will then contribute to immunological memory. Dendritic cells (DCs) are a specialized and heterogeneous group of innate immune cells, each possessing unique and complementary functions that bridge the gap between the innate and adaptive immune systems. The hallmark function of all DCs is to detect, process and present antigens to modulate adaptive immune responses mediated by T cells. The expression of PRRs by all DC populations allows the recognition of pathogen associated molecular patterns, which stimulates the upregulation of costimulatory molecules and promotes the activation of antigen-specific T cells. DCs are also important in mediating tolerance by sampling self antigen molecules, which is coupled with a lack of costimulatory molecules, thus rendering any self antigen-specific T cell inactive. DCs also excel in tailoring the adaptive immune response to the type of infection by activating certain T cell subsets. However, inflammatory and autoimmune disorders often result from hyperactivated immune cells, and DCs appear to initiate these misguided immune responses.
1.1 Dendritic Cell Subsets

Scientific exploration of DCs has become increasingly complex with the recognition that DCs exist as a multiple distinct populations. Although named for their cellular size and morphology\(^1\), these DC populations have primarily been defined by their combinatorial cell surface marker expression. However, they also differ in their developmental origins, transcriptional regulation, patterns of migration or residence, and anatomical and microenvironmental localization. DCs can be broadly classified as two major subsets: the inflammatory or infection-derived DCs, which develop from monocytes in response to stimulation, and the steady-state DCs, which are present at all times. The DCs present under steady state conditions include CD8\(^+\) and CD8\(^-\) classical or conventional DCs (cDCs), plasmacytoid DCs (pDCs), and migratory CD103\(^+\) CD11b\(^-\) DCs, CD103\(^-\) CD11b\(^+\) DCs and Langerhans cells (LCs) (Table 1). The CD8\(^-\) cDCs can be further classified as CD4\(^+\) or CD4\(^-\) DCs, both of which express high levels of CD11b\(^2\). However, the majority of gene perturbation analyses that have examined CD8\(^+\) cDCs, CD8\(^-\) cDC and pDCs as well as global gene analysis have shown mostly congruent gene expression between the CD4\(^+\) and CD4\(^-\) subsets\(^3\); thus, we will classify CD8\(^-\)CD4\(^+\) and CD8\(^-\)CD4\(^-\) DCs as CD8\(^-\) DCs for simplicity.
Table 1. Surface molecule expression of steady state dendritic cell subsets.
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<th>CD8⁻ cDC</th>
<th>pDC</th>
<th>CD103⁺ DC</th>
<th>CD11b⁺ DC</th>
<th>CD103⁺ CD11b⁺ DC</th>
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<td>PU.1, RelB, Flt3</td>
<td>E2-2, PU. 1, Ikaros, IRF-8, Flt3</td>
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<td>CD11c⁺ CD11b⁺ CD1c⁺</td>
<td>CD123⁺ CD303⁺ CD304⁺</td>
<td>−</td>
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Legend: int = integral, int = intestine, + = present, − = absent, +/− = present/absent, −* = thymic expression, +/− = present/absent
1.1.1 Lymphoid Tissue-Resident Dendritic Cells

The cDCs and pDCs are found throughout the primary and secondary lymphoid organs. In the spleen and lymph nodes (LNs), the CD8\(^-\) cDCs constitute the majority of the resident DCs, whereas the CD8\(^+\) cDCs are the predominant DC subset within the thymus. Initially termed interferon-producing cells (IPCs) in humans, pDCs are known for their hallmark function of detecting virus by TLR7 or TLR9 and producing vast amounts of type-I interferons\(^4,5\). CD8\(^+\) cDCs are specialized for efficient cross-presentation of antigen to CD8\(^+\) T cells, resulting in heightened viral and antitumor responses\(^6,7\). Since cross-presentation has been associated with more efficient negative selection (reviewed in \(^8\)), it is likely that the higher proportion of CD8\(^+\) cDCs within the thymus can be attributed to this unique function\(^9,10\). Although thymic DCs (tDCs) can participate in negative selection\(^11\) a definitive requirement for tDCs in this process is still debated\(^12\). CD8\(^-\) cDCs are distinguished by their superior phagocytic abilities which lead to enhanced presentation of antigen to MHC class II-restricted CD4\(^+\) T cells\(^13,14\).

1.1.2 Non-lymphoid Tissue-Resident Dendritic Cells

In non-lymphoid organs, the roles of CD103\(^+\) CD11b\(^-\) DCs and CD103\(^-\) CD11b\(^+\) DCs mirror the specialized functions of CD8\(^+\) and CD8\(^-\) cDCs, respectively. A unique CD103\(^+\) CD11b\(^+\) subset also exists, but only in the lamina propria of the intestine\(^15\). There are also CD103\(^+\) (dermal DCs) and CD11b\(^+\) subsets, which monitor peripheral locations and migrate to draining LNs upon activation. The epithelium-resident LCs are another type
of DC that responds to activation by migrating to skin-draining LNs where they present antigen to T cells\textsuperscript{16,17}.

\subsection*{1.1.3 Human Dendritic Cells}

Human DC subsets within the peripheral blood, where pDCs were first discovered, have been extensively studied, but due to practical limitations lymphoid and nonlymphoid tissue-resident DCs are less well understood. However, the vast amounts of data on murine DC subsets has enabled the identification of equivalent human DC populations by correlative functional characterization, gene profiling and by the identification of genetic mutations resulting in human DC deficiency (reviewed in \textsuperscript{18,19-23}). A summary of the designations of murine DC subsets as defined by cell surface molecules and the transcriptional regulators involved in the development of each subset is shown in Table \textbf{1}. The equivalent human populations of cDCs and pDCs are also summarized.

\subsection*{1.1.4 Flaws with Current Dendritic Cell Classification}

Although DC classification has typically been defined by cell surface markers, it is important to note that molecules, such as B220, CD8\textgreek{a} and DEC-205, can be upregulated or downregulated following activation or stimulus. DC researchers remain in a quandary, as it is difficult to ascertain whether the identification of DC subsets by surface marker expression relates to discrete lineages or specific physiological states due to the plasticity of DC populations. For example, cells displaying a pDC phenotype can upregulate CD8\textgreek{a}, downregulate B220 and manifest a classical DC morphology upon
stimulation with CpG$^{24,25}$. Similarly, although Langerin is historically a marker for skin-resident or migratory DCs, it was recently shown that the majority of CD8$^+$ tDCs also express Langerin$^{26}$. In order to truly understand the capabilities of these DC subsets, we will need to move beyond cell surface markers and define the transcriptional regulators that govern their genetic programming. Here, I will focus on the origins and development of CD8$^+$ cDCs, CD8$^-$ cDCs and pDCs, with an emphasis on the transcription factors that control lineage choice and differentiation of these DC subsets.

### 1.2 Dendritic Cell Development and Precursors

Although considerable advances have been made in identifying upstream DC precursors in the past decade, much is still unknown. Over the years, many precursors have been shown to have DC potential in addition to their potential for other cell lineages, whereas other progenitors have been classified as committed DC lineage-specific. It is probable that all of the following precursors contribute to the DC populations throughout the body, but are prompted to develop through distinct mechanisms and at different sites of the body.

#### 1.2.1 Common Lymphoid Progenitor and Common Myeloid Progenitor

An understanding of the cellular origins of peripheral lymphoid tissue-resident DCs largely began with the advent of the identification of common lymphoid progenitors (CLP; Lin$^-$ IL-7R$^+$ Thy-1$^-$ Sca-1$^{int}$ c-Kit$^{int}$) and common myeloid progenitors (CMP; Lin$^-$ IL-7R$^α$ Sca-1$^-$ c-Kit$^+$ FcRγRII/III$^{lo}$ CD34$^+$) at the turn of the century$^{27,28}$. Following
intravenous injections into lethally irradiated recipients, CLPs, CMPs and granulocyte/macrophage precursors (GMPs; Sca-1\(^-\) c-Kit\(^+\) IL-7Rα\(^-\) FcRγRII/III\(^+\) CD34\(^+\)) all gave rise to splenic DCs\(^{29-31}\). Interestingly, CLPs produced greater absolute numbers of DCs and a higher proportion of CD8\(^+\) DCs in the spleen than CMPs\(^{31}\). Moreover, Flt3, a cytokine receptor required for peripheral lymphoid tissue DC development\(^{32}\), was expressed at higher levels on CLPs relative to CMPs\(^{33}\). Fate-mapping mice, in which cells expressing IL-7R were irreversibly labeled with YFP, revealed that only one tenth of thymic and splenic CD8\(^+\) and CD8\(^-\) cDCs had arisen from IL-7R\(^+\) precursors, suggesting most of these cells did not arise from CLPs\(^{34}\). In contrast, the majority of thymic and splenic pDCs were YFP\(^+\). However, these pDCs also expressed \(IL7r\) mRNA, thereby confounding the determination of whether they had arisen from CLPs. Nevertheless, the reconstitution of irradiated recipients with each of these precursors did not collectively regenerate the same numbers of DCs observed following injection of whole bone marrow (BM), foreshadowing the presence of unidentified DC precursor(s)\(^{31}\).

1.2.2 The Macrophage/DC Precursor and the Common DC Precursor

The identification of a more defined DC precursor was inspired by observations that Flt3 ligand (Flt3L), GM-CSF and M-CSF could support DC development \textit{in vitro}. Subsequent pursuits of DC-lineage precursors identified a bipotent macrophage/DC precursor (MDP; Lin\(^-\) c-Kit\(^{hi}\) CD115\(^+\) CX3CR1\(^+\) Flt3\(^+\))\(^{35}\) that gives rise to a common DC precursor (CDP; Lin\(^-\) c-Kit\(^{lo}\) CD115\(^+\) CX3CR1\(^+\) Flt3\(^+\))\(^{36-38}\) in which macrophage lineage potential is lost. The CDP can then diverge into pre-cDCs (Lin\(^-\) CD11c\(^+\) MHC Class II\(^-\) SIRPα\(^{int}\) Flt3\(^+\)) or a yet
unidentified precursor leading to pDCs\textsuperscript{38}. All cDC populations in lymphoid organs and tissue-resident CD103\textsuperscript{*} DCs can arise from pre-cDCs\textsuperscript{38,39}. However, this pathway is not mutually exclusive from the CLP or CMP pathways nor does it eliminate other alternative pathways of DC differentiation. Instead, it appears that there are different developmental routes that converge to give rise to the same functional subsets of DCs (Fig. 1).
**Figure 1. Precursors with dendritic cell lineage potential.** MDP and CDP populations are found only in the bone marrow, whereas pre-cDCs are present in bone marrow, lymph nodes, spleen and blood. MDP possess macrophage and DC potential, CDPs are bipotent for cDCs and pDCs, and pre-cDCs are committed to the cDC lineage. An immediate pDC precursor has not been identified. ETP and DN1 subsets, as well as MDPs, CDPs and pre-cDCs can give rise to tDCs. CLP and CMP populations can give rise to all splenic DCs. Abbreviations: LMPP (Lymphoid-Primed Multipotent Progenitor); ELP (Early Lymphoid Progenitor); LSK (Lin- Sca-1+ c-Kit+); MP (Myeloid Progenitor); MDP (Macrophage/DC Precursor); CDP (Common DC Precursor); Pre-cDC (committed precursor of conventional DCs); Pre-pDC (committed precursors of pDCs); ETP (Early T Lineage Progenitor).
1.2.3 Thymic Dendritic Cell Progenitors

There has been much controversy over the origins of the three major subsets of tDCs (CD8^+ cDCs, CD8^- cDCs and pDCs) and whether they develop within the thymus\textsuperscript{40-44,34,26,45}. There are three major developmental routes by which these tDCs could arise. First, they could develop extrathymically and migrate in as mature DCs. Secondly, they could arrive in the thymus as committed DC precursors and differentiate within the thymus. Thirdly, they could arise within the thymus from an uncommitted precursor that shares T cell and DC potential. Development into tDCs has been proposed to occur outside of the thymus for some subsets, namely CD8^- cDCs and pDCs\textsuperscript{42,43}. In fact, BM-derived MDP, CDP and pre-DC populations can give rise to tDCs following intravenous injections\textsuperscript{26}. In addition, a model of CCR9-dependent pDC migration to the thymus suggests that peripheral self-antigen can be transported from the periphery to the thymus by pDCs and cDCs, in the absence of activation\textsuperscript{46}. An earlier study demonstrated that CD8^- cDCs can migrate from peripheral blood to the thymus, contribute to negative selection and induce the generation of regulatory T cells (Tregs)\textsuperscript{47}. However, other studies have suggested that intrathymic DC development occurs, as well\textsuperscript{48,26,45}. The environment of the thymus, which is the primary site of T cell development, provides a vastly different set of microenvironmental cues for DC development than those available to other peripheral tissue-resident DC precursors\textsuperscript{49,50}. Fortunately, the ongoing search for thymic seeding progenitors has resulted in the progressive elucidation of putative tDCs precursors as well. The populations that are thought to seed the thymus include multipotent progenitors (MPPs), lymphoid-primed multipotent progenitors
(LMPPs), CLPs, and circulating T cell progenitors (CTPs)\textsuperscript{51}. Early studies showed that the majority of thymic and splenic pDCs had undergone IgH gene D-J rearrangements, and that they expressed CD3 and preTα, which provided evidence for DC development from CLPs or a similar precursor\textsuperscript{52}. A minority population of CD8\textsuperscript{+} tDCs also exhibited these characteristics, which would coincide with the low percentage of CD8\textsuperscript{+} tDCs labeled in the IL-7R fate-mapping experiments\textsuperscript{34}. Overall, it appears that cDCs do not arise from a CLP or CLP-similar precursor, whereas pDCs likely do.

### 1.2.4 Intrathymic Dendritic Cell Development

The ability of some T-cell precursors to develop into DCs when removed from the thymus has suggested that these cells could be physiological precursors of tDCs. T-cell precursors within the thymus are characterized as double negative (DN; CD8\textsuperscript{-} CD4\textsuperscript{-}) and develop from DN1 (CD44\textsuperscript{+} CD25\textsuperscript{-}) into DN2a cells (c-Kit\textsuperscript{hi} DN44\textsuperscript{+} CD25\textsuperscript{+}), which is the point of T-cell specification. DN2a cells retain the ability to differentiate \textit{in vitro} into natural killer (NK) cells and DCs\textsuperscript{53-55}. Next, DN2a cells give rise to T-lineage committed DN2b cells (c-Kit\textsuperscript{+} CD44\textsuperscript{+} CD25\textsuperscript{+}) and eventually differentiate to DN3 cells (c-Kit\textsuperscript{-} CD44\textsuperscript{-} CD25\textsuperscript{+}), which must receive survival signals through the pre-T cell receptor to progress further through T cell development. The DN1 cells can be further subdivided into ETP (early T lineage progenitors; DN1a/b; c-Kit\textsuperscript{hi} CD24\textsuperscript{-/lo}), DN1c (c-Kit\textsuperscript{int} CD24\textsuperscript{hi}), DN1d (c-Kit\textsuperscript{-} CD24\textsuperscript{+} and DN1e (c-Kit\textsuperscript{-} CD24\textsuperscript{-}) subsets based on their surface expression of c-Kit and CD24\textsuperscript{56}. ETPs are the canonical T cell precursors and contain some NK cell potential,
whereas DN1c and DN1d cells exhibit B cell potential. Little is known about the lineage potential of DN1e cells.

Many studies have provided evidence that T cell precursors have DC\textsuperscript{57} and myeloid\textsuperscript{58,59} lineage potential. During specification, T-lineage genes are upregulated and genes influencing development towards other lineages are downregulated. Interestingly, the minimal myeloid potential present in DN1 subsets is lost in DN2 cells, whereas DC potential is still present in DN2 cells which have not yet upregulated the T cell specific gene, \textit{lck}\textsuperscript{57}. Moreover, numerous \textit{in vitro} studies have shown that intrathymic precursors, prior to T cell commitment at the \textit{β}-selection checkpoint, can develop into tDCs\textsuperscript{40,60,45}.

Additional \textit{in vivo} studies have supported the ability of distinct T-cell precursors to give rise to DCs. Early studies characterized a “low-CD4 precursor” (CD4\textsuperscript{lo} CD8\textsuperscript{−} CD3\textsuperscript{−} CD24\textsuperscript{hi}), which contained what are now referred to as DN1c and DN1d cells, that could give rise to CD8\textsuperscript{+} tDCs following intravenous injections into irradiated mice\textsuperscript{40}. One progenitor within the thymus expressing CD24, c-Kit, CD11c and Langerin can arise from MDPs, CDPs and pre-DCs from the BM and spleen and has been shown to give rise to Langerin\textsuperscript{+} CD8\textsuperscript{+} tDCs\textsuperscript{26}. Studies by our laboratory have shown that ETP, DN1d and DN1e subsets can all give rise to tDCs \textit{in vivo}, which localize to the medulla in non-irradiated mice\textsuperscript{45}. Unquestionably, there are many developmental routes by which DCs can arise, depending on a variety of factors such as their localization and surrounding stimuli, which in turn influences the transcriptional regulators that orchestrate cellular fate.
1.3  Context-Dependent Transcriptional Regulators of Lymphoid Tissue-Resident Dendritic Cells

Despite the differences in the location of DC development, specific subsets share transcriptional regulatory programs, which indicates an intrinsic requirement for certain transcription factors for the DC lineage\textsuperscript{61}. Interestingly, to date there is no known single transcription factor that is universally required for the development of all DCs, analogous to the requirement of Pax-5 for the development of all B cells\textsuperscript{62}, highlighting the versatility and plasticity of DC development and homeostasis.

1.3.1  The Multitasking Transcriptional Regulators: Ets Transcription Factors

1.3.1.1  PU.1

The two Ets transcription factor family members PU.1 and Spi-B have been intensely studied in myeloid and lymphoid cells owing to their expression in many progenitors and their roles in multiple lineages. PU.1 is expressed during the earliest stages of hematopoiesis onwards in CMP, CLP, CDP, preDC, DN1 cells, cDCs and pDCs\textsuperscript{28,63,64}. Early studies of the functions of PU.1 in DCs were conflicting due to the generation of two independent lines of PU.1 knockout mice, one of which was embryonic lethal whereas the other one allowed survival until about two weeks after birth\textsuperscript{65,66}. Neither PU.1-deficient mouse strain, however, enabled analysis of the adult splenic and thymic DC compartments that are established 3-5 weeks after birth\textsuperscript{67}. PU.1 (Sfpi-1)-deficient E14.5
and E16.5 embryos exhibited a lack of CD11c⁺ CD8⁻ tDCs while CD11c⁺ CD8⁺ tDCs remained intact in one study. However, another study demonstrated a reduction in DEC-205⁺ tDCs (equivalent to CD8⁺ tDCs; see Table 1) in 10- to 12-day old mice. Subsequently, a polyI:C inducible PU.1-knockout confirmed the requirement for PU.1 in splenic and thymic cDC and pDC populations and in the generation of these subsets from CDPs. However, the involvement of PU.1 in other DC subsets and the generation of upstream precursors remain unclear. Interestingly, the context-dependent roles of PU.1 are emphasized by its ability to upregulate Flt3 in DCs, while exhibiting an equally important role in upregulating IL-7R in B cells. Moreover, the dose of PU.1 is critical for lineage determination, as highlighted by a higher level of PU.1 favouring macrophage development over B cell and granulocyte development. PU.1 also plays a role in the macrophage/DC lineage decision, in part by binding to and inhibiting Mafb, which is a bZip transcription factor that promotes macrophage and monocyte development.

The roles of PU.1 in early thymocyte development are complex. PU.1 inhibits T cell development from DN2 cells, but is required for the generation of T cell precursors. Initially, PU.1 is important for the upregulation of IL-7R and Flt3 in T cell precursors. Interestingly, there is an accumulation of CD24hi c-Kitint Sca1⁻ DN1 precursors, corresponding phenotypically to the DN1c population, in PU.1⁻/⁻ animals, suggesting that it is needed for the developmental progression of DN1c cells to CD8⁺ tDCs. PU.1 induces the expression of many DC-promoting factors, such as M-CSFR, GM-CSFR and CD11b. It was recently shown that Notch signaling does not downregulate or alter PU.1, but reinforces the expression of T-cell-specific transcription factors which
would be downregulated by high levels of PU.1\textsuperscript{79}. Thus, the decrease of PU.1 during early T cell development correlates with the loss of DC potential, likely results in the downregulation of a DC-specific gene program and an upregulation in T-specific transcription factors. The complexity of the functions of PU.1 in the intrathymic T/DC lineage choice is highlighted by a recent study, which amalgamated global transcript analysis with chromatin structure data over the early stages of T cell development. These results revealed, surprisingly, that during the stages of PU.1 expression from DN1 to DN2b cells, there were just as many targets of PU.1 in T cells as there were in B cells and macrophages. Importantly, however, these targets were unique and corresponded to genes active in early T cell development, which highlights the importance of chromatin remodeling through histone marking in transcriptional regulation\textsuperscript{80}. Therefore, PU.1 plays very important but divergent roles in DC and T cell development, by coordinating the expression of target genes required for each lineage. The ability of PU.1 to direct T-lineage gene expression is likely due to collaboration with Notch signals\textsuperscript{81}. Other factors that may collaborate with PU.1 in the T/DC choice are under investigation.

1.3.1.2 Spi-B

Spi-B is another Ets family transcription factor that is closely related to PU.1. Initially, Spi-B was identified as a lymphoid-specific factor involved in B cell receptor signaling\textsuperscript{82}. Surprisingly, however, a knock-in of Spi-B into the PU.1 locus showed that it was able to rescue myeloid but not B cell development\textsuperscript{83} and it was subsequently found to be
expressed specifically in pDCs\textsuperscript{84}. Thus, in certain lineages Spi-B and PU.1 do not play compensatory or redundant roles. Further studies using RNA interference techniques showed that Spi-B is required for pDC development from human precursors\textsuperscript{85} and it has been recently been shown to be influential in BM-derived pDC development\textsuperscript{86}. Curiously, Spi-B does not appear to play a role in the generation of splenic pDCs, suggesting that its main roles are developmentally upstream of the immature DC precursors found in the spleen. Interestingly, Spi-B activates the production of type I IFN in concert with interferon regulatory factor-7 (IRF-7), a factor important for pDC function\textsuperscript{86}. Unlike PU.1, which is normally expressed in DN1 and DN2 cells and decreases as T cells develop, Spi-B increases in expression during the DN1-3 stages, suggesting a role in T cell commitment\textsuperscript{64}. Furthermore, Spi-B\textsuperscript{−/−} animals exhibit slightly lower cellularity and delayed T cell development in the thymus\textsuperscript{87}. However, overexpression of Spi-B at the DN3 stage interrupts β-selection resulting in greater DC development within fetal thymic organ culture (FTOC)\textsuperscript{87}, and inhibits T cell, B cell and NK cell development from human precursors in vitro\textsuperscript{84}. The impact of Spi-B overexpression on lymphocyte development may be due to the levels driven by PU.1-locus regulatory elements or retroviral elements in these studies, enabling Spi-B, which binds to the same promoter site as PU.1, to act in a PU.1-like manner. The presence of DC subsets therefore in PU.1\textsuperscript{−/−} mice and Spi-B\textsuperscript{−/−} mice is further evidence of a compensatory role for these two factors. Accordingly, there is a complete lack of tDCs in PU.1\textsuperscript{−/−} Spi-B\textsuperscript{−/−} E18 fetal thymic lobes in contrast to a reduction of DC subsets in PU.1\textsuperscript{−/−} lobes\textsuperscript{87}. Adult Spi-B\textsuperscript{−/−} tDCs, however, appear phenotypically normal (Chapter III), suggesting that PU.1 is capable of
compensating for a loss of Spi-B specifically in tDCs, whereas the reverse relationship is not present.

1.3.2 Controlling the DC versus Macrophage Lineage Choice: Ikaros and Gfi1

1.3.2.1 Ikaros

Ikaros is a zinc finger transcription factor that acts as a dimer with itself and with the other family members, Aiolos and Helios. Ikaros is critical for early stages of hematopoiesis, which has complicated analysis of developmental defects in different lineages in Ikaros-deficient mice. Ikaros dominant negative mutant mice, which lack activity of all Ikaros family members, exhibit a loss of cDCs and an increase in monocytes and macrophages, suggesting a requirement for Ikaros in cDC development. Interestingly, however, Ikaros null mice only lack CD8− cDCs and pDCs, while retaining their CD8+ DC population, indicating that Ikaros is either needed in each lineage independently or that Ikaros-null CD8+ DCs arise independently of the CDP. In another mouse model in which only low levels of Ikaros were expressed in hematopoietic cells only pDCs were absent, indicating that pDCs require high levels of Ikaros whereas cDCs do not. This defect in pDCs was cell-autonomous, and was linked to inappropriate upregulation of a large array of genes and a failure to respond to Flt3L. Accordingly, Flt3 receptor expression was missing in Ikaros null LMPP cells. Therefore, part of the role of Ikaros in pDCs is to silence alternative lineage genes and to upregulate Flt3 on DC precursor populations. Interestingly, Ikaros can bind to promoter elements in the PU.1
gene locus to activate or repress PU.1 transcription in myeloid cells, depending on the regulatory site\textsuperscript{92}. Overall, these data support a role for Ikaros in pDC development as well as the divergence of the cDC and monocyte-derived DC lineages prior to the CDP stage of DC development.

1.3.2.2 Gfi1

Gfi1 is another transcriptional regulator with important roles in DC development. One of the main roles of Ikaros in the B/macrophage lineage choice is to upregulate Gfi1, promoting B cell development and repressing myeloid development\textsuperscript{93}. It is therefore possible that Gfi1 is downstream of Ikaros in DCs as well. However, Gfi1\textsuperscript{−/−} mice exhibit a more striking DC deficiency than Ikaros\textsuperscript{−/−} mice, with a reduction in all splenic, thymic and peripheral LN DC populations, correlated with an increase in LCs\textsuperscript{94}. Gfi1\textsuperscript{−/−} mice also exhibit defects in early T cell development, reduced thymic cellularity and increased Id2 mRNA levels\textsuperscript{95}. Gfi1 appears to repress Id2 in B and myeloid cells. This might also occur in developing T cells, since it is expressed throughout T cell development\textsuperscript{96,97}. In the context of multipotent progenitors, Gfi1 promotes the B cell lineage over the macrophage lineage by repressing PU.1\textsuperscript{93}. Moreover, in vitro experiments showed an increase in macrophage potential from Gfi1\textsuperscript{−/−} precursors. Collectively, these results indicate that Gfi1, like Ikaros, likely plays a role in the DC/macrophage lineage choice.
1.3.3 cDC-Specific Marker: Zbtb46

Recently, two independent studies identified a novel transcription factor, Zbtb46 (also known as Btbd4 or zDC), exclusively expressed in pre-cDC, CD8⁺ cDC and CD8⁻ cDC cells, but not in pDCs. Although Zbtb46 expression was restricted to these lineages it was not required for their development, but rather to modulate their activation status. Zbtb46 acts primarily as a transcriptional repressor in cDCs, with targets including many MHC class II genes. Once cDCs are stimulated with TLR agonists, Zbtb46 protein is downregulated, allowing MHC class II molecules to be expressed at higher levels, thereby conferring an activated status to these cDCs. Zbtb46 might also play a role in promoting the development of CD8⁺ cDCs over CD8⁻ cDCs in the spleen. However, the deletion of Zbtb46⁺ cells using diptheria toxin did not affect tumour or parasitic immunity, thus illuminating the compensatory roles of the remaining DC compartment in these functional capacities. Certainly, the ability to label Zbtb46-expressing cells with GFP has provided a valuable tool for clarifying DC classification, and enabling the identification of cells committed to the cDC lineage fate.

1.3.4 cDC-Specific Regulator: Bcl6

Bcl6, another zinc finger transcription factor, is also known to be a transcriptional repressor of many target genes, including p53. This transcriptional regulator is involved in modulating Th2 immune responses and inhibiting plasma cell differentiation, and has recently been implicated in DC development. Bcl6⁻/⁻ mice exhibit a reduction in the splenic CD4⁺ (CD8⁻ cDCs) and CD8⁺ cDC subsets. Additionally,
as shown by adoptive transfer studies, Bcl6−/− BM-derived precursors possessed a decreased capacity to develop into cDCs. This was attributed to increased p53 expression, leading to increased apoptosis. Bcl6−/− DCs also secreted greater amounts of IL-6 and IL-12, which led to a greater activation of CD4+ T cells, likely skewing to a Th2 inflammatory response. Thus, Bcl6 plays a role in the differentiation and survival of cDCs.

1.3.5 Controlling the cDC versus pDC lineage choice: Id2 and E Proteins

1.3.5.1 Id2

Id factors, which contain helix-loop-helix domains, can dimerize with and inhibit E proteins including HEB (HEBAlt, HEBCan), E2A (E12, E47) and E2-2 (E2-Can, E2-Alt). The major cDC-specific Id regulator is Id2. Id2 is not expressed in LSK, LMPPs or CLPs, or in the CDP or pre-cDC DC progenitors, but is present in all cDCs, regardless of anatomical location. However, Id2 is only required for epidermal LCs, splenic CD8+ and nonlymphoid tissue resident CD103+ DCs. Interestingly, the DN1e subset within the thymus also expresses high levels of Id2 indicating that these cells might have an increased propensity to develop into cDCs, in particular CD8+ tDCs. Thus, Id2 appears to have a role in the later stages of DC development. However, unlike Zbtb46, Id2 expression is not restricted to the DC lineage, since it is also important for the development of other lineages, such as NK, myeloid and lymphoid tissue inducer cells.
**1.3.5.2 E Proteins**

In contrast to cDCs, pDCs require the E protein E2-2 for their development and homeostasis\(^{114}\). Interestingly, E2-2 can activate pDC-specific regulators, such as Spi-B, IRF-7, IRF-8 as well as Bcl11a. Furthermore, the deletion of E2-2 from pDCs converts them to cDCs, as determined by surface marker phenotype, function, gene expression and morphology\(^{114,115}\). Since E2-2-dependent upregulation of these genes would be inhibited by Id2, the Id2/E2-2 dichotomy is likely at the top of the hierarchy that splits the pDC/cDC gene programs. Another E protein that is expressed specifically in thymic pDCs is HEBCan\(^ {45}\). HEBCan is also expressed throughout thymocyte development, while the shorter form of HEB, HEBAlt, is expressed only during early T-lineage developmental stages. HEBAlt has defined roles in promoting T-cell development\(^ {116,117}\), and decreasing DC development from BM precursors *in vitro*\(^ {45}\). However, constitutive expression of HEBAlt in T-cell precursors does not alter tDC development in the adult thymus, perhaps due to additional microenvironmental factors present in the thymus that are not available *in vitro* (unpublished data). Therefore, further study is needed to assess the roles of HEBCan and HEBAlt in the T cell/tDC lineage choice.

**1.3.6 CD8\(^+\) cDC-Specific Regulators: Batf3 and E4BP4**

**1.3.6.1 Batf3**

Global gene expression analyses of DC populations have led to the discovery of many DC subset-specific genes, including the transcription factor Batf3\(^7\). Studies of Batf3-deficient
mice showed that Batf3 is required for CD8⁺ cDC development during steady state. The lack of splenic and LN CD8⁺ cDCs in Batf3−/− mice demonstrated that these cells are required for cross-presentation of antigen to CD8⁺ T cells. Furthermore, these mice had defective anti-viral and anti-tumour immunity⁷. Interestingly, Batf3 was also required for the generation of CD103⁺ CD11b⁻ DCs within the skin and mesenteric LN, dermis, lung and intestine, which emphasizes the similarities in transcriptional regulation between CD8⁺ cDC and CD103⁺ nonlymphoid tissue DCs¹¹⁸. In vitro studies showed that the cultured equivalents to CD8⁺ DCs were not hampered by a lack of Batf3 until later timepoints, suggesting more of a homeostatic role than a developmental role of Batf3 in CD8⁺ DC development and also foreshadowing recent work highlighting the redundancy of Batf factors¹⁰⁸. Interestingly, when challenged by intracellular pathogens or administration of IL-12, CD8⁺ DCs were restored by 3 weeks in Batf3−/− mice by an alternative pathway whereby Batf and Batf2 compensate for the lack of Batf3¹¹⁹. This study also showed that Batf could interact directly with IRF-4 and IRF-8. Thus, it appears that Batf3 is important in the terminal stages of CD8⁺ cDC development and plays a role in maintaining this subset.

1.3.6.2 E4BP4

Recently, E4BP4 (NFIL3), a basic leucine zipper transcription factor, which was first recognized for its importance in NK cell development¹²⁰,¹²¹, has been implicated in CD8⁺ DC development. Despite higher E4BP4 mRNA expression levels in pDCs than CD8⁺ cDCs, E4BP4−/− mice specifically lacked splenic and thymic CD8⁺ cDCs¹²². The defect in
development appears to take place at the pre-cDC to CD8\(^+\) cDC developmental transition since precursors, such as LSK, CLP, CMP, GMP, CDP and pre-cDC populations are not affected by the absence of E4BP4\(^{-/-}\). *In vitro* studies showed that E4BP4\(^{-/-}\) BM cells could be partially rescued by retroviral transduction with a Batf3-containing vector into CD24\(^+\) Sirp\(\alpha\)^− DCs (CD8\(^+\) cDC equivalent), thus indicating that Batf3 is involved directly or indirectly with the CD8\(^+\) DC-promoting effects of E4BP4 expression.

### 1.3.7 CD8\(^-\) cDC-Specific Regulator: RelB

Despite the identification of many regulators for the CD8\(^+\) cDC and pDC lineages, the regulation of the CD8\(^-\) cDC subset by unique transcription factors remains elusive. Initially, tDCs were reported absent in RelB\(^{-/-}\) mice, but this was attributed to a lack of medullary thymic epithelial cells, which tDCs normally localize to\(^{123,124}\). RelB, a subunit of the NF-\(\kappa\)B complex, is a downstream signaling mediator of immune cell activation via pattern recognition receptors, such as Toll-like receptors\(^{125}\). RelB is specifically expressed in splenic CD8\(^-\) cDCs, and is required for their development\(^{124}\). Although functional roles pertaining to DC activation have been attributed to RelB in DCs\(^{126,127}\), the influence RelB has on lineage decisions is largely unknown.

### 1.3.8 Diverse Roles of Interferon Regulatory Factors

As their name suggests, IRFs are transcription factors known for their ability to induce the expression of interferons in response to stimulus, such as the activation of Toll-like
receptors (reviewed in \(^{128}\)). IRF-1, IRF-2, IRF-4, IRF-7 and IRF-8, have been implicated in DC development across many subsets.

### 1.3.8.1 IRF-8

In addition to Batf3, Id2 and E4BP4, CD8\(^+\) cDCs also require IRF-8 (ICSBP; interferon consensus-binding protein) for their development\(^{129,130}\). IRF-8 also plays a major role in CD103\(^+\) DCs and a minor role in pDC, LC and dermal DC development with a more pronounced defect in pDCs\(^{129,39}\). IRF8\(^{-/-}\) mice were unable to produce type I IFNs following viral challenge and exhibited delayed migration of LCs to the draining LNs in steady state and inflammatory conditions\(^{129,131,132}\). Interestingly, a single point mutation within the IRF association domain (IAD) of IRF-8, which confers the ability to interact with other IRFs, replicates the loss of CD8\(^+\) cDCs, but not pDCs, in IRF-8\(^{-/-}\) mice. Although the wildtype (WT) IRF-8 could interact with IRF-2 or PU.1 and Spi-B to bind to interferon-stimulated response element (ISRE) or Ets/IRF promoter sites, respectively, the mutated IRF8\(^{R294C}\) could not\(^{133}\). Therefore, IRF-8 is involved in the development of CD8\(^+\) cDCs, CD103\(^+\) DCs and pDCs, but likely acts through different mechanisms in each subset.

### 1.3.8.2 Other IRFs

Another factor implicated in DC development is IRF-4. IRF-4-deficient mice lacked the majority of splenic CD11b\(^+\) CD4\(^+\) CD8\(^-\) cDCs and had a slight reduction in pDCs\(^{134,135}\). In addition to developmental defects, the lack of IRF-4 impaired the migration of LCs and CD103\(^+\) dermal DCs to the cutaneous LN following skin inflammation\(^{136}\). IRF-1\(^{-/-}\) mice
also differ from WT mice in that they exhibit a slight reduction in CD8⁺ and CD8⁻ cDCs and an increase in pDCs. Further complexity is added by the severe decrease of CD8⁻ cDCs and a partial lack of CD8⁺ cDCs and pDCs in IRF-2⁻/- mice. Interestingly, IRF-4 mRNA expression levels were greater in E4BP4⁻/- pre-cDCs compared to the WT counterparts, suggesting that E4BP4 might act by restricting the IRF4-mediated development of other DC lineages. Thus, in addition to IRF-8, IRF-1 and IRF-2 play minor roles in CD8⁺ DC development whereas IRF-2, IRF-4 and, to a lesser extent, IRF-1 are important for CD8⁻ DC development. The increase in pDCs in IRF1⁻/- mice suggests that IRF-1 might repress or inhibit IRF-8. IRF-2 and IRF-4 also exhibit minor functions in pDC development. Interestingly, ChIP analysis has shown that human E2-2, which is required for pDC development, is capable of binding to promoter regions upstream Irf-7 and Irf-8 gene loci.

1.4 Cytokines Involved in Dendritic Cell Development

Cytokines, secreted by surrounding tissues and immune cells, provide many developmental cues that influence the transcriptional regulation and functions of the receiving cells. Initial in vitro studies of cytokines in DC development revealed distinct and important roles for the receptor tyrosine kinases, GM-CSF, M-CSF and Flt3L, in the generation of DCs. Flt3L and M-CSF, in particular, have been shown to influence many discrete DC subsets.
1.4.1 Flt3L

Flt3L stimulates the cytokine receptor Flt3, which is a receptor tyrosine kinase and is expressed on many progenitor cells, including DC precursors, LMPPs and ETPs\textsuperscript{144,145}. Flt3L-supplemented cultures can induce the differentiation of CD8\textsuperscript{+} cDCs, CD8\textsuperscript{-} cDCs and pDCs from a variety of precursors\textsuperscript{140,141,24,142,146}. M-CSF-supplemented cultures can also generate CD8\textsuperscript{+} cDCs, CD8\textsuperscript{-} cDCs and pDCs, albeit with lower efficiency than Flt3L cultures\textsuperscript{143}. Moreover, Flt3\textsuperscript{+} precursors include LMPPs, MDPs, CDPs, pre-cDCs and a proportion of CLPs, CMPs and ETPs. In addition, progenitors transduced to express Flt3 possess greater DC potential than their Flt3\textsuperscript{-} counterparts\textsuperscript{147,148,33,146,149}. Correspondingly, Flt3-deficient mice exhibit decreased cDCs and pDCs\textsuperscript{32}. However, the degree of reduction in cDC and pDC subsets in Flt3\textsuperscript{−/−} mice does not reflect the severe decrease of these populations in Flt3L\textsuperscript{−/−} mice\textsuperscript{150,24}, suggesting the presence of another, as of yet unidentified, receptor for Flt3L.

1.4.2 M-CSF

Interestingly, this speculation reflects recent findings in the M-CSF/M-CSF1R pathway. Mice carrying a mutated M-CSF gene (op/op mice) exhibited a reduction in splenic CD11c\textsuperscript{dim} B220\textsuperscript{+} pDCs, but LCs and microglia remained intact\textsuperscript{151,152}. Microglia, the resident macrophages within the central nervous system (reviewed in\textsuperscript{153}), and some LCs arise from progenitors in the embryonic yolk sac and, thus, exhibit similar developmental requirements\textsuperscript{154,155}. By contrast, LCs and microglia were completely absent from M-CSF1R\textsuperscript{−/−} mice\textsuperscript{156,154}. The disparity in DC developmental defects in M-
CSF−/− and M-CSF1R−/− mice was clarified by the discovery of an alternate ligand for M-CSF1R, IL-34. IL-34 is secreted by keratinocytes and neurons to foster the development of steady state LCs and microglia, respectively. Accordingly, IL-34−/− mice lack LCs and exhibit reduced microglia, thereby replicating the results in M-CSF1R−/− mice. Comparable populations of monocytes and DCs were observed between IL-34−/− and WT mice. By contrast, there are not significant LC deficiencies in Flt3−/− or Flt3L−/− mice. In addition to M-CSF1R expression on MDPs and CDPs, it is also expressed by yolk sac macrophages, adult macrophages, LCs and splenic cDC and pDC subsets. Therefore, although Flt3 and M-CSFR are both expressed on MDPs and CDPs, they clearly influence different DC lineage fates.

1.4.3 GM-CSF

Although GM-CSF is commonly added to many in vitro cultures to stimulate DC development from BM progenitors, GM-CSF−/− and GM-CSFR−/− mice do not show any significant deficiencies in DC populations in lymphoid tissues. Splenic CD8+ cDCs were slightly increased in GM-CSF−/− mice, indicating that GM-CSF inhibits the generation of this subset. There are many conflicting reports on the involvement of GM-CSF in nonlymphoid tissue DC subsets. One study shows that CD103+ CD11b− dermal DCs are reduced in GM-CSF−/− mice and GM-CSFR−/− mice, which is confirmed by another report whereby CD103+ CD11b+ lamina propria DCs and CD103+ DCs from skin and lung draining LN were also decreased in both GM-CSF−/− and GM-CSFR−/− mice. A third report observed that DC populations remained similar to WT in GM-CSFR−/− mice, but
CD103 surface expression was slightly downregulated on GM-CSFR−/− DCs. Although GM-CSF does not seem to be unequivocally required for many, if any, DC subsets, GM-CSFR transgenic mice exhibit an increase in cellularity in the thymus and spleen, which is echoed by an increase in cDCs as well. Conversely, the presence of GM-CSF inhibits the development of CD8+ cDC equivalent cells and pDCs in vitro. Moreover, GM-CSF does not enhance DC development from early T cell precursors as Flt3L does. GM-CSF does, however, seem to play a role in the function of DCs. The addition of GM-CSF to in vitro cultures resulted in the upregulation of CD103 and an increase in cross-presentation abilities of DCs, which was confirmed ex vivo and in vivo using GM-CSF-transgenic and GM-CSFR−/− mice.

Therefore, GM-CSF signaling directs different developmental outcomes than Flt3L signaling. Although many other cytokines, such as SCF, TGF-β, IL-3, IL-4 or IL-7, have been studied and can modify the outcomes of in vitro cultures, they do not appear to play an overarching, essential role for DC development.

1.4.4 STATs: Downstream Mediators of Cytokine Signaling

The signal transducer and activator of transcription (STAT) family of transcription factors has been implicated downstream of the cytokine receptors, Flt3 and GM-CSFR, thus bridging the gap between extracellular signals and transcriptional regulation. Signaling through the Flt3 receptor induces the phosphorylation of STAT3, which is required for DC development as evidenced by the lack of splenic DCs and reduced CLP and CMP precursors in STAT3−/− mice. This defect was not restored by treating mice with Flt3L,
indicating that the requirement for STAT3 is downstream of Flt3 signaling\textsuperscript{169}. Interestingly, Flt3-phosphorylated STAT3 could bind to the proximal promoter sites upstream to the *Tcf4* promoter, inducing E2-2 expression\textsuperscript{170}. STAT1, STAT3 and STAT5 are all phosphorylated in response to administration of GM-CSF to BM cultures\textsuperscript{169}. GM-CSF blocks pDC development *in vitro* and *in vivo* through STAT5 by inhibiting IRF-8 transcription and upregulating Id2 gene expression\textsuperscript{171}. The effects of phosphorylated STAT5 must be context dependent and include other regulatory factors since it has been shown to inhibit IRF-8 transcription and CD103\textsuperscript{+} DCs in one study, but was shown also to be required for CD103\textsuperscript{+} DCs by the upregulation of Id2 gene expression in another study\textsuperscript{171,170}. This can, in part, be attributed to differential histone modifications in pDCs versus CD103\textsuperscript{+} DCs, thereby allowing disparate transcriptional control by STAT5\textsuperscript{170}. Another pathway known to exist is the STAT-independent induction of CEBPβ expression from Flt3L and GM-CSF signaling, which can then upregulate Id2 gene expression\textsuperscript{170}. Clearly, the Flt3L and GM-CSF pathways are connected since Flt3 can induce the transcription of GM-CSFR, as well as M-CSFR and PU.1\textsuperscript{149}. Thus, this experimental evidence suggests that Flt3 is required during earlier stages of DC development, whereas the function of GM-CSF might be to favour the cDC lineage over pDCs. The point in DC differentiation at which M-CSF influences developmental outcomes is likely during the MDP to CDP conversion when M-CSF1R is expressed, but this has not yet been directly examined. Determining the cellular sources of Flt3L, GM-CSF and M-CSF will provide important insights into the homeostatic versus infection-induced mechanisms of DC development.
1.5 The Role of Notch Signaling on Dendritic Cell Development

Another signaling pathway that can influence cell lineage choices is the Notch pathway. In mammals, there are four Notch receptors, Notch-1, -2, -3 and -4, and five Notch ligands, Delta-like-1 (Dll1), Dll3, Dll4, Jagged-1 and Jagged-2. The Notch signaling cascade involves the translocation of an intracellular cleaved Notch fragment to the nucleus where it interacts with RBP-J, and catalyzes binding of other cofactors to activate the transcription of a variety of genes (summarized in 173).

1.5.1 Expression of Notch Ligands

The tissue-specific spatial expression of Notch ligands provides additional cues that influence the differentiation and the functionality of hematopoietic cells. The BM stroma, which interacts with many DC progenitors, such as CLPs, CMPs, MDPs, CDPs and pre-cDCs, predominantly express Jagged1, in addition to Dll1 and Dll4. Splenic stroma express Jagged1, Jagged2, Dll1 and Dll4, with the predominant ligand, Dll1, expression concentrated in the marginal zone where CD8− cDCs reside. The thymic stroma, which supports T cell development, can be categorized broadly into the cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs). Dll4 is expressed throughout the thymus primarily on cTECs, but also at lower levels on mTECs, whereas Dll1 is expressed mostly within the cortex with the highest levels expressed in the cortico-medullary junction (CMJ). However, overall the expression levels of
Dll4 are vastly greater than Dll1\textsuperscript{176}. Interestingly, hematopoietic cells express Notch ligands and are also involved in propagating Notch signaling\textsuperscript{179}. Binding studies have revealed a preference of the Notch1 receptor for Dll4\textsuperscript{180}, both of which are required for T cell development\textsuperscript{181,176}, while it has been suggested that Notch2 preferably binds to Dll1. Although little is known of the differences between signaling from different Notch ligands and receptors, the presence of and type of Notch ligand, as well as the relative expression, likely induces unique intracellular Notch signaling outcomes in the receiving cell.

### 1.5.2 The Role of Notch Signaling on Dendritic Cell Differentiation

Notch signaling appears to play different roles in DC lineage potential and DC maintenance at different stages throughout DC development, due to its varied influence on uncommitted precursors and developing DCs. The precursors primarily tested have been hematopoietic stem cells (HSCs) and the general consensus from \textit{in vitro} studies is that Notch signaling in uncommitted HSCs promotes self-renewal, not lineage commitment\textsuperscript{173,182}. Thus, the variety of Notch ligands expressed by the BM stroma likely have a role in HSC self-renewal. Interestingly, surface expression levels of Notch2 are greater than Notch1 on HSCs\textsuperscript{183}. However, at some point in development Notch signaling input changes, which might be due to the availability of Notch ligand and receptor, and/or the signaling output at the transcriptional level.

\textit{In vitro} studies have shown that hematopoietic progenitors co-cultured with Jagged-1-expressing fibroblasts results in the accumulation of immature precursors,
whereas fibroblasts withDll1 promotes DC differentiation\textsuperscript{184}. In agreement with these results, Notch1-deficient embryonic stem cells or hematopoietic progenitors with half the normal level of Notch1 expression exhibited a decreased potential to become DCs \textit{in vitro}\textsuperscript{185,184}. However, the cytokines GM-CSF and IL-4 were used in the aforementioned \textit{in vitro} studies, which likely promoted the differentiation of monocyte-derived DCs instead of cDCs and pDCs\textsuperscript{185,184,174}. Nevertheless, \textit{in vivo} studies using mice with poly(I):(C)-inducible or CD11c-specific deletion or RBP-J similarly showed that splenic CD8\textsuperscript{−} cDCs were markedly reduced, whereas pDCs were slightly increased in the absence of all Notch signaling\textsuperscript{186}. Although splenic CD8\textsuperscript{+} and CD8\textsuperscript{−} cDCs have similar surface expression levels of Notch receptors, with the exception of Notch4 which is exclusively expressed on the CD8\textsuperscript{+} cDC population, the two DC populations are regulated very differently by Notch signaling\textsuperscript{175}. The administration of inhibitory antibodies specific to Notch ligands in mice elucidated a role for all four Notch ligands expressed in the spleen for CD8\textsuperscript{−} cDC development since inhibition of only one Notch ligand did not result in a DC defect\textsuperscript{175}. These results could indicate the redundancy of Notch ligands or a unique and essential role of all four Notch ligands. The receptor Notch2, specifically, is essential for Notch signaling in splenic CD8\textsuperscript{−} cDCs in addition to a CD103\textsuperscript{+} CD11b\textsuperscript{+} DC population within the intestinal lamina propria\textsuperscript{187}. Interestingly, although splenic CD8\textsuperscript{+} cDCs were present in normal proportions when RBP-J was deleted from DCs\textsuperscript{186}, there was a slight decrease in this population following DC-specific deletion of Notch2\textsuperscript{187}, indicating a potential role for non-canonical Notch signaling on the homeostasis of splenic CD8\textsuperscript{+} cDCs. Notch signaling
was also shown to be required for the development a unique Thy1^+ CD8^+ DC subset within the thymus\textsuperscript{188}.

Although there is clearly a DC-promoting role for Notch signaling in the generation of splenic CD8^- cDCs, other studies have shown that Notch signaling has no effect or inhibits DC differentiation. In vivo studies have shown that although T cell development is abrogated in Notch1^-/- conditional knockout mice, DCs can still develop within the BM, spleen and thymus\textsuperscript{41,44}. This was shown when Notch1 was deleted from all hematopoietic cells\textsuperscript{41}, or cells expressing Cpa3\textsuperscript{189,44}, which is expressed by hematopoietic progenitor cells\textsuperscript{190}. Cpa3-expressing Notch1-deleted cells, which were marked by RFP expression, had an increased propensity to develop into cDC and pDCs in the thymus, but not in the spleen suggesting that Notch1 plays an inhibitory role on tDC development\textsuperscript{44}. An in vitro study of Notch influence on human pDCs showed that sustained Dll1-Notch signaling blocked pDC development, whereas Jagged-Notch did not. In fact, precursor cells capable of developing into pDCs had downregulated Notch1 mRNA, possibly rendering them ignorant to Dll1\textsuperscript{191}.

1.53 Notch Target Genes and Their Role in Dendritic Cell Lineage Commitment

The target genes of Notch signaling in DC precursors and subsets are largely unknown. Although, many Notch target genes are known in other cell subsets, evidence shows that the target genes vary depending on the cell type (reviewed in\textsuperscript{173}).
The most intensely studied hematopoietic subsets in Notch signaling experiments have been thymic precursors, namely ETP, DN2a, DN2b and DN3 cells. Notch signaling through the receptor Notch1 is required for T cell development and induces the upregulation of T lineage-specific genes, such as HEBAlt and Bcl11b, or enables proper functioning of important T cell transcription factors, such as GATA-3. Notch signaling is also thought to simultaneously downregulate genes that promote development to other lineages. However, it has recently been shown that Notch signaling does not downregulate PU.1, but rather acts to enforce T lineage development while PU.1 is expressed by preventing the downregulation of T cell genes. Interestingly, Dll-Notch signaling and PU.1 expression are competing factors, whereby the one with the greater signaling strength or expression level is capable of directing lineage potential. Specifically in this study, PU.1 inhibited E proteins by the upregulation of Id2, an E protein-antagonist. However, the presence of Notch signaling restored T cell lineage fate, even when cells expressed both PU.1 and Id2, likely by restoring E protein expression and activity. Notch-specific transcriptional control of PU.1 is clearly context-dependent as evidenced by the upregulation of PU.1 by Notch in MPP cells. Another putative target gene of Notch signaling is NF-κB2 (p100/p52), which can heterodimerize with RelB to propagate NF-κB downstream signals. Intriguingly, both Notch signaling and RelB are required for splenic CD8− cDC development. It is possible that the NF-κB and Notch signaling pathways have a synergistic effect on marginal zone splenic CD8− cDC development, as has been evidenced in marginal zone B cells.
1.6 Thesis Goals

Developmental cues, such as growth factors or cell-to-cell communication, that are received by precursors in a specific microenvironment influence the upregulation and downregulation of transcription factors, which ultimately control their developmental outcome and function. The goal of my thesis is to understand how DCs arise in the thymus during their development. Specifically, I set out to 1) determine the transcription factors involved in the generation of the three tDC subsets (CD8⁺ cDCs, CD8⁻ cDCs and pDCs), 2) address the roles of thymus microenvironment-specific factors that direct their development, and 3) identify precursors that tDCs originate from.

Over the years, multiple transcription factors have been elucidated as important or required for DC development (Table 2). However, in many cases only sDC subsets were examined. Thus, I first examined the relative mRNA expression of known important DC-promoting transcription factors in tDC populations. Since developmental plasticity has clearly been shown for ETPs, DN1 and DN2a subsets, I applied the tDC subset gene profiles to infer DC lineage potential in the earliest T cell progenitors: ETP, DN1c, DN1d and DN1e cells.

The thymus microenvironment provides a vastly different set of growth signals and developmental cues than other organs, such as the expression of Dll1 and Dll4. I have examined the influence of Dll4 on precursors to develop into DCs in vitro, in addition to the input Dll1-Notch and Dll4-Notch signaling has on the expression of DC-promoting transcription factors.
Few progenitor cells have been shown to give rise to tDCs\textsuperscript{40,48,26,45}. Thus, I tested the ability ETP, DN1d and DN1e cells to develop into DCs \textit{in vivo} in unperturbed recipient mice to determine if they possessed DC lineage potential.

Collectively, these experiments contribute to our overall understanding of how DC developmental and transcriptional programs operate in different microenvironments, they shed light on the influence of Dll4-Notch signaling on DC development and have led to the identity of additional tDC precursors\textsuperscript{45}.
Table 2. List of important transcriptional regulators for conventional and plasmacytoid DC subsets.

<table>
<thead>
<tr>
<th>Regulators</th>
<th>CD8⁺ cDC</th>
<th>CD8⁻ cDC</th>
<th>pDC</th>
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<td>Gfi1</td>
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<td>✔</td>
<td>✔</td>
<td>All splenic, thymic &amp; LN-resident DCs</td>
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<td>✔</td>
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<td>110</td>
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<tr>
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<td>✔</td>
<td></td>
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<td>Nonlymphoid tissue CD103⁺, LCs</td>
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<tr>
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Chapter II

Methods
# Methods

## 2.1 Mice

C57Bl/6 WT mice, Lck-HEBAI1g mice (Tg)\(^{196}\), Rag1\(^{-/-}\) and Ly5.1 mice (originally obtained from Jackson Laboratories: B6.SJL-Ptpc\(^{a}\)Pep3\(^{b}\)/BoyJ) were maintained at the Sunnybrook Research Institute (SRI), Toronto, Ontario, Canada. In Tg mice, HEBAI1g is under the control of the lck-proximal promoter. Spi-B\(^{-/-}\) mice were obtained from Dr. Rodney DeKoter, University of Western. CCR7\(^{-/-}\) mice (B6.129P2(C)-Ccr7\(^{tm1Rfor}\)/J) and IL7R\(\alpha\)\(^{-/-}\) mice (B6.129S7-Il7tm1Imx/J) were obtained from Jackson Laboratories. All animal protocols were approved by the animal care committee at SRI. Mice aged 4-7 weeks old were used for splenic and thymic DC and DN1 subset analyses and mice older than 7 weeks were used for LSK isolation from BM precursors. Lck/CD2-CAR mice express the coxsackievirus and adenovirus receptor (CAR) under the control of the lck-proximal promoter/human CD2 enhancer Tg construct\(^{197,198}\).

## 2.2 Dendritic Cell Isolation and Flow Cytometric Analysis

For *ex vivo* sorting experiments, thymocytes and splenocytes were depleted of RBCs using ACK solution (155 mM NH\(_4\)Cl, 0.1 mM disodium EDTA, 10 mM KHCO) and stained in HBSS, 2.5% BSA buffer at 4\(^\circ\)C. DCs were positively selected from thymocyte samples using CD11c-conjugated-microbeads (Miltenyi Biotec) and an AutoMACS\(^a\) Pro Separator using the “posseld” program (Miltenyi Biotec). All samples were incubated with anti-Fc\(\gamma\)R antibodies (clone 2.4G2) prior to antibody incubation to eliminate non-specific
staining. Thymic CD11c-selected cells and RBC-depleted splenocytes were stained with the following antibodies: MHC II-FITC, CD11c-PE, CD45-PerCP eCy5, CD8α-APC, CD11b-Alexa700, B220-APCCy7, CD19-Bio, F4/80-Bio, Gr1-Bio, NK1.1-Bio, Thy1-Bio, and streptavidin-eFluor450 (antibodies obtained from eBioscience, BD Biosciences, and SRI antibody core facility). DAPI staining was used to differentiate live and dead cells. DC subsets were sorted on a FACS Aria using the following parameters: CD8+ cDCs (CD45+, Lin−, CD11c+, B220−, MHC II+, CD8+, CD11b−), CD8− cDCs (CD45+, Lin−, CD11c+, B220−, MHC II+, CD8−, CD11b+), pDCs (CD45+, Lin−, CD11cint, B220+, MHC II+int), where Lin = Gr1, F4/80, Thy1, NK1.1 and CD19 (Figures 2, 3, 10, 12, 15, 16, 17, 18, 19, 20, 21, 23A, 24, 25, 26) or Lin = Gr1, F4/80, CD3ε, NK1.1, CD19, DX5 (Figures 4, 6, 22, 23B, 27, 29, 30). In in vitro-derived DC analysis, cells were first gated on CD45+ Lin− cells. Non-DC myeloid cell analysis was performed after gating on total CD45+ CD11c− cells, without the Lin− gate.

2.3 DN1 Thymocyte Subset Isolation

Thymocytes were harvested and depleted of CD4+ and CD8+ cells using biotin-conjugated antibodies (SRI Antibody Core Facility, Toronto, Ontario), streptavidin-microbeads and an autoMACS® Pro Separator using the “depletes” program (Miltenyi Biotec). Depleted cells were incubated with anti-FCyR antibodies, washed, then incubated with the following antibodies: CD44-FITC, CD24-PE, NK1.1-Bio, CD8α-Bio, CD4-Bio, TCRβ-Bio, CD11b-Bio, CD11c-Bio* (see note below), Ter119-Bio, CD25-APC, CD117-Alexa750, and streptavidin-PerCP (antibodies obtained from eBioscience, BD Biosciences and SRI antibody core facility). DN1 subsets were sorted with a FACS Aria
using the following parameters: ETP (Lin− CD44+ CD25− c-Kit+ CD24−/int), DN1c (Lin− CD44+ CD25− c-Kitint CD24+), DN1d (Lin− CD44+ CD25− c-Kit− CD24−), DN1e (Lin− CD44+ CD25− c-Kit− CD24−), where Lin− = NK1.1−, CD8α− CD4−, TCRβ−, CD11b−, Ter119−. *Note: CD11c+ cells were not specifically gated out in all experiments, but FACS analysis showed that the percentage of CD11c-expressing cells in each population were as follows: ETP: 6.40% ± 0.74%, DN1c: 66.93% ± 6.79%, DN1d: 8.98% ± 1.12%, DN1e: 8.16% ± 0.80%, and we found that CD11c− DN1 subsets did not differ significantly in gene expression from the unfractionated DN1 subsets.

2.4 Gene Expression Analysis

RNA was extracted from cells using TRIzol (Life Technologies) and first-strand cDNA was generated from total RNA using Superscript Reverse Transcriptase III (Life Technologies) and 250 ng random primers (Life Technologies). The addition of DNase I (Life Technologies) reduces genomic DNA contamination. Quantitative real-time RT-PCR (qRT-PCR) was performed using gene-specific primers (Table 3; 0.1 μM of each primer per 25 μL reaction), iTaq™ SYBR® Green Supermix with ROX (BioRad) and an ABI Prism® 7000 Sequence Detection System (Applied Biosystems). Gene-specific primers, which span exon-exon boundaries of mRNA to reduce genomic DNA detection, were standardized with a melting temperature of 60°C and a PCR product size of 80-155 bp. A BLAST search was performed for each primer set to eliminate nonspecific product amplification. Relative gene expression was determined using β-actin to normalize for cDNA input and the ΔCt-method was performed. Using the ΔCt-method the relative
gene expression was determined by the equation \( \Delta Ct = 2^{-(Ct_{\text{sample}} - Ct_{\beta\text{-actin}})} \). cDNA dilutions yielding \( \beta\text{-actin} \) Ct values of \(~16-17\) were used as templates to normalize cDNA input.

2.5 Id2-Expressing Adenovirus Amplification and Infection

Id2 was cloned into the pAd/PL-DEST\textsuperscript{TM} vector using a pENTR-Ubc shuttle vector (designed by Moemeni B. and Julius M.) and using ViraPower\textsuperscript{TM} Adenoviral Expression System (Life Technologies). Following the ViraPower\textsuperscript{TM} Adenoviral Expression System protocol, 293A cells were transfected with control (pAd-control) or Id2-expressing (pAd-Id2) adenovirus and crude virus was collected following 10 days. A fresh batch of 293A cells was infected with crude virus and cultured for 3-5 days. Virus was collected and viral titre was determined. Thymocytes were infected with optimal multiplicity of infection (MOI) of 10 (based on preliminary experiments to determine the greatest increase in Id2 transcript in target cells, see Figure 5B). Thymocytes were harvested from Lck-CAR\textsuperscript{+} mice and CD44\textsuperscript{+} cells were positively selected using magnetic sorting (streptavidin-microbeads and LS columns; Miltenyi Biotec). CD44\textsuperscript{+}-enriched cells were infected pAd-control and pAd-Id2 adenovirus (MOI 10 for both) overnight while cultured in 10 ng/mL each of SCF, IL-7 and Flt3L. Cells were washed and injected intravenously into the tail vein of Ly5.1 mice irradiated with a dose of 450 Rads using a cesium-137 irradiator 1 hour prior to injection.
2.6 Dendritic Cell In Vitro Culture System

BM cells were depleted of Lin\(^+\) cells using biotin-conjugated antibodies against CD19, F4/80, Gr1, Ter119, streptavidin microbeads and LS MACS columns (Miltenyi Biotec). Lin\(^-\) cells were cultured overnight in OP9 media (RPMI, 10% FBS, 100 U penicillin, 100 \(\mu\)g/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM non-essential amino acids, 0.055 mM 2-mercaptoethanol) supplemented with 10 ng/mL each of SCF, IL-7 and Flt3L. LSK precursors were sorted using a FACSaria. 2000 LSK cells/well were seeded onto OP9-derived monolayers or placed in stroma-free (S-F culture) in 6-well plates supplemented with 5 ng/mL SCF, IL-7 and Flt3L, unless otherwise indicated. Cells were seeded onto fresh monolayers approximately every 4 days. Flow cytometry for DC phenotype was performed as described above on an LSR II (BD Biosciences). In some experiments, \(\gamma\)-secretase inhibitor X (Millipore), which was diluted in DMSO, was added at final concentrations of 0.03 \(\mu\)M, 0.1 \(\mu\)M, 0.3 \(\mu\)M, 1 \(\mu\)M or 1.3 \(\mu\)M. Only DMSO was added to control samples.

2.7 Fetal Thymic Organ Culture

Fetal thymic lobes from embryos of gestation 14.5 days were isolated and cultured on Surgifoam (Ethicon), which was covered with Nucleopore Track-Etch membrane (Whatman). FTOCs were cultured with AMEM supplemented with 20% FBS, penicillin, streptomycin and glutamate. For Figure 12, DN1 subsets were added to fetal thymic lobes by the hanging drop method. Droplets of 20 \(\mu\)L each containing 5000 cells of DN1a/b, DN1c, DN1d or DN1e cells in media were placed in Terasaki microwell plates.
(Nunc) and fetal thymic lobes were added to each well. Each plate was inverted and cultured overnight. Fetal lobes were then placed in standard FTOC conditions, as explained above, for six days. For Figure 24, fetal thymic lobes were cultured with media containing DMSO, or 0.03 μM, 0.1 μM, 0.3 μM, or 1.3 μM γ-secretase inhibitor for 7 days.

2.8 Intravenous and Intrathymic Injections and Immunofluorescence Microscopy

ETP, DN1d and DN1e subsets were purified from C57Bl/6 mice, as described above, with these additional lineage markers: CD11c, F4/80, CD19, B220, Gr1, CD3ε. 30,000 cells (ETP), 180,000 cells (DN1d) and 160,000 cells (DN1e) were injected per mouse by tail vein intravenous injection. For intrathymic injections, 100,000 cells (ETP), 75,000 cells (DN1d) and 100,000 cells (DN1e) were injected per thymic lobe of each recipient mouse. Five week old CD45.1 congenic mice were used as recipients. Thymii were harvested seven days post-intravenous injection and five days post-intrathymic injection and frozen in OCT. Thin sections (10 μm) were fixed in 2% paraformaldehyde, blocked with PBS/5% FBS/0.05% triton and stained with CD11c-FITC, CD45.2/anti-mouse Cy3, anti-Keratin-5/anti-rabbit-Cy5 (antibodies obtained from eBioscience and Jackson Immunoresearch Laboratories) and DAPI. Images were acquired with a Zeiss Axiovert 200 Fluorescence Microscope with a final magnification of 200x. Hematoxylin and eosin (H&E) staining was performed by Petia Stefanova in the SRI histology core facility.
2.9 Calculations and Statistics

Percentages of DC populations were calculated by dividing the number of each population by the number of CD45\(^+\) cells within the sample. Standard deviation was performed on all data represented with error bars. Unpaired t test statistical analysis of replicates was used to determine statistical significance.
Table 3. List of sense (S) and antisense (A) qRT-PCR primers.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| Batf3            | S: AGAAGGCTGACAAGCTCCAC  
|                  | A: GCACATCTTCTCGTGCTCCCT |
| CCR4             | S: AGGCAAGGACCCTGACCTAT  
|                  | A: GCAGTACGTTGTTGTGCT |
| CCR7             | S: GTGGTGCTCCTCCTTGTCAT  
|                  | A: AGTCCACCGTGTTATTTCTCG |
| CD8α             | S: AGGATGCTCTTGCTCTCCCA  
|                  | A: CAGGTTGACGAAATTCAGCTTC |
| CXCR4            | S: ACGGCTGTAAGACGAGTGTT  
|                  | A: AGGGTTCCCTGTTGGAGTCA |
| E2-2Alt          | S: GAAAAGGCGGTCTACGCTCCT  
|                  | A: TGCCCATCTTGGAAGAAAG |
| E2-2Can          | S: AACAGCGAATGGCTGCTTTA  
|                  | A: TGTCACCTCGCAAAAGATTT |
| E4BP4            | S: GAACTCTGCCTTAGCTGAGGT  
|                  | A: ATTTGCTTTTCTCCGACACG |
| Flt3             | S: CTTTGTGCTTGGTGATCAAATG  
|                  | A: GTGGTGACCAACAACATTTCT |
| HEBAlt           | S: GTGCTTATCTCTGTTGCCCTGAATG |
| HEBCan           | S: TCAATTCTTGCCCAAGTTCCA |
| HEB*             | A: TGGGTTGGGAGATGGGTAAC |
| Hes1             | S: TCCGTACGCCAATTTTCG  
|                  | A: GGAAGTTACGTTGCTGTTGAAG |
| Id2              | S: TCCGTTGAGGTCCGTTAGGAA  
|                  | A: GCTGGAGATGCACTGCTGTACTG |
| Ikaros           | S: TGGGGAGGCAAGTCTGGTTA  
|                  | A: ACAGGTACCAGGGACAGGCA |

*HEBCan and HEBAlt have the same antisense primer.
<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| IRF-8           | S: GAGCGAAGTTTCTGAGATGG  
A: TGGGCTCCTCTTTGTCATAC |
| Notch1          | S: AGTGCAACCCTCGATGAC  
A: CTAGGCCATCCCACCTCAC |
| Notch2          | S: CGTGAGAGATCGCTGTGT  
A: TTGTGGCAGACACCATTGTT |
| Notch3          | S: GCCGTACGGGTAGTCACTGT  
A: CATAGCCAGCTGGACACTCA |
| Notch4          | S: GCCGATGAGGAAAGAGACAT  
A: GGGGCTACTGAGACATTG |
| PU.1            | S: ACACCATGTCCACAACAGA  
A: GTGCATCTGTTCAGCTCCAT |
| RelB            | S: TGCTTCCATATCCCTGCTG  
A: GGCAGCAGGTCACACATAGT |
| SpiB            | S: CTGCAAGCCCTCCAGTTACC  
A: AAAGGCAGCAGTAGCAGGAT |
| XCR1            | S: TCTAGCAGTGGAGGAGATCAA  
A: GGGATGCAGGATACGTGAGA |
| Zbtb46          | S: AGAGAGCAATGAAGCGACA  
A: CTGGGCTGCAGACATGAACAC |
Chapter III

Regulatory Factors that Condition Thymocyte Precursors for Dendritic Cell Development

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All assays were performed by AJ Moore. J Sarmiento helped to perform intrathymic injections of DN1 subsets. B Meomeni designed the adenoviral shuttle vector and aided in experiments with adenovirus. SK Li helped to assay DC populations in SpiB\(^{-/}\) mice. Spi-B\(^{-/}\) mice were obtained from Rodney P. DeKoter’s laboratory.

Figures 2, 3, 8, 10, 13 and 14 have been published in Development. 2012. 139(2): 373-84. Part of Figure 3 and 8 have been published in Advances in Hematology. 2013. Feb 18. Volume 2013. Article ID 949513. 1-16. PMID: 23476654.
3 Regulatory Factors that Condition Thymocyte Precursors for Dendritic Cell Development

3.1 Abstract

Specialized DCs within the thymus are important for the deletion of autoreactive T cells. The question of whether these cells arise from intrathymic precursors with T-cell potential has been hotly debated, and the regulatory pathways and signals that collectively direct their development remain unclear. Here, I compared the gene expression profiles of tDC subsets with those of four early thymic precursor subsets: ETP, DN1c, DN1d and DN1e cells. I found that the DN1d subset expressed Spi-B and HEBCan, similar to thymic pDCs, whereas the DN1e subset expressed high levels of Id2, similar to thymic cDCs. When Id2 was overexpressed in vivo in thymocyte precursors the proportion of NK cells and DCs increased, illustrating its role in NK and DC lineage choice. However, T cell development was unperturbed by transient Id2 overexpression. Hes1, a transcriptional indicator of Notch signaling, was expressed by ETPs and mature thymic pDCs, demonstrating they were likely undergoing active Notch signaling. To directly evaluate the lineage potential of the ETP, DN1d and DN1e subsets, I injected them into nonirradiated congenic hosts intrathymically or intravenously, and found that they were all able to form medullary DCs in vivo. Interestingly, in vitro cultures and FTOC experiments did not foster DC development from ETP, DN1c, DN1d or DN1e cells, indicating that an adult, structured thymus is required.
3.2 Introduction

Multiple upstream precursors have been identified for splenic DCs\textsuperscript{199,36,38}, whereas thymic DC precursors remain elusive. The earliest T-cell precursors in the thymus are DN1 cells (CD4\textsuperscript{−} CD8\textsuperscript{−} CD44\textsuperscript{+} CD25\textsuperscript{−}), which are further subdivided into ETP (c-Kit\textsuperscript{+} CD24\textsuperscript{low/+}), DN1c (c-Kit\textsuperscript{int} CD24\textsuperscript{high}), DN1d (c-Kit\textsuperscript{−} CD24\textsuperscript{+}), and DN1e (c-Kit\textsuperscript{−} CD24\textsuperscript{−}) cells\textsuperscript{200,56}. In the presence of strong Dll-Notch signals, ETPs give rise to T-lineage specified cells which continue to require Dll-Notch signals to become committed T cell precursors\textsuperscript{201}. However, DC lineage potential is clearly present in ETPs and DN2a cells\textsuperscript{45,48,42,57,43,53}. CD11c\textsuperscript{+} DN1c cells, which are thought to derive from blood-borne myeloid CDPs, can give rise to CD8\textsuperscript{+} cDCs in the thymus\textsuperscript{26}. Although DN1d and DN1e cells have very little T-cell potential\textsuperscript{56}, the developmental relationships between DN1d cells, DN1e cells and tDCs are unclear.

It is hypothesized that the role of Dll-Notch signaling in T-cell development is to upregulate T lineage-specific genes and constrain the expression or activity of transcription factors that direct development to other lineages. However, recently it was shown that Dll-Notch signaling does not downregulate the myeloid-promoting transcription factor, PU.1, but rather that Dll-Notch signals restrain the ability of PU.1 to inhibit T lineage genes, such as GATA-3, TCF-7 and Myb\textsuperscript{79}. Interestingly, many of the major DC regulators, such as PU.1, Spi-B, Gfi1, Id2 and IRF-4 are expressed by developing T cell precursors\textsuperscript{64}. In fact, PU.1 is required for the early stages of normal T cell development\textsuperscript{73}. However, with the exception of PU.1\textsuperscript{80}, the gene targets and roles of each factor have not been explored in T cell progenitors versus DC progenitors. Here, I
examined the gene expression of DC-promoting transcription factors in mature splenic (sDC) and tDC subsets, as well as T cell precursors. Collectively, defining gene expression and the responsiveness of each subset to Notch receptors will aid in the determination of whether T cell precursors are equipped to develop into tDCs. I hypothesize that the presence of Notch receptors, E proteins, Id2, and the Ets family members PU.1 and Spi-B in distinct precursors are likely to be critical for determining the downstream consequences of microenvironmental signals during DC development within the thymus.

3.3 Results

3.3.1 Regulatory factors expressed by thymic and splenic DC subsets

Analysis of the transcriptional signatures of splenic and thymic DC subsets using microarray techniques indicate that DC function is heavily shaped by the microenvironments in which the cells reside. However, little is known about the regulatory factors that control DC development within the thymus. I therefore set out to characterize the expression of a set of key regulatory factors in DC populations in order to identify subset-specific patterns. Due to the small percentage of DCs within the thymus, I first positively selected cells expressing CD11c, a global marker of DCs, from the thymii of WT mice using magnetic sorting. Next, CD8⁺ cDC, CD8⁻ cDC and pDC subsets were sorted from the DC-enriched fraction using fluorescence-activated cell sorting (FACS) according to the following parameters: CD8⁺ cDCs (CD45⁺ Lin⁻ CD8α⁺ CD11b⁻ B220⁻ CD11c⁺ MHC II⁺), CD8⁻ cDCs (CD45⁺ Lin⁻ CD8α⁻ CD11b⁺ B220⁻ CD11c⁺ MHC
Il^+) and pDCs (CD45^+ Lin^- B220^+ CD11c^int MHC II^int) (Fig. 2A). Other lineages (Lin = CD19, NK1.1, Thy1, F4/80, Gr1, Ter119) were electronically excluded during sorting. The equivalent DC populations were also sorted from splenocytes according to the same parameters for a comparative analysis. Since DCs are known to pull off surface antigens from surrounding cells, to confirm that the CD8^+ tDC sorted cells were bona-fide CD8^+ tDCs I assessed the CD8α mRNA expression (Fig. 2B). Indeed, CD8^+ tDCs, but not CD8^- tDCs, expressed CD8α transcripts and at comparable levels to CD8^+ single positive (SP) T cells.

To assess gene expression in each sDC and tDC subset, qRT-PCR was performed. PU.1, Spi-B and Id2 were robustly expressed within one or more of the DC subsets (Fig. 3). Since these three factors have been shown to be required for DC development in some or all subsets^65,68,85,109, they were chosen as a diagnostic tool to indicate DC potential in precursor subsets. PU.1 was expressed in all subsets except the thymic pDCs. Interestingly, PU.1 levels were higher in CD8^- splenic and CD8^- thymic cDCs than in the other subsets, consistent with a myeloid gene program for these cells. Spi-B, a hallmark for pDCs, was expressed in both thymic and splenic pDC subsets. Id2 levels, by contrast, were higher in the CD8^+ thymic and splenic DC subsets as compared with the other DC populations. I also examined the expression of HEBCan and HEBAlt in these six subsets. Unexpectedly, HEBCan was expressed at very high levels within thymic pDCs, but not in splenic pDCs, in contrast to Id2 and HEBAlt, which were undetectable in thymic pDCs. Interestingly, HEBAlt was present at low levels within the splenic CD8^- cDCs and splenic pDCs, and essentially absent in thymic CD8^- cDCs. However, the HEBAlt
Figure 2. Sorting parameters for thymic and splenic DCs. (A) CD11c⁺ cells were magnetically selected from pooled thymii. Specific DC subsets were sorted from RBC-depleted splenocytes and CD11c-selected thymocytes. DC subsets were gated first by FSC/SSC profiles and DAPI⁻. Lin⁻ (Lin = CD19, F4/80, Gr1, NK1.1, Thy1) CD45⁺ MHC II⁺ cells were separated into pDC and cDC populations. cDCs were further differentiated into CD8⁺ cDCs and CD8⁻ cDCs. Numbers refer to percentages. (B) CD8α transcript levels were tested on sorted CD8⁺ and CD8⁻ cDC subsets, as well as CD8⁺ T cells, CD4⁺ T cells and double positive (DP; CD8⁺ CD4⁺) T cells. mRNA expression levels are relative to β-Actin. Values shown are mean ± s.d. (n=3).
Figure 3. Regulatory factor mRNA expression in mature thymic and splenic DC subsets.
Transcription factor expression in thymic and splenic DC subsets. Gene expression levels, as
determined by qRT-PCR, were normalized to β-actin. Values shown are mean ± s.d. (n=3).
expression by DC subsets was much lower than that observed in DN subsets (data not shown). The canonical and alternative forms of E2-2 (E2-2Can and E2-2Alt) as well as IRF-8 were expressed mostly by thymic pDCs, as expected, due to their roles in pDC development. IRF-8 was also expressed at higher levels by CD8^+ tDCs and sDCs than their CD8^- population counterparts. Batf3, which is important for CD8^+ cDCs, was expressed by cDCs in the spleen and thymus, albeit at higher levels within the splenic subsets. Although RelB has been proven to be imperative for splenic CD8^- cDCs, it was only expressed at very low levels in mature DCs (data not shown). Low expression of Ikaros and undetectable levels of Gfi1, which play more of a role in DC precursors than in mature DCs, were observed (data not shown).

To determine the potential for Notch signaling in mature thymic and splenic DC subsets, I evaluated the presence of all four Notch receptors. Notch receptors 1, 2, 3 and 4 were all expressed predominantly in thymic pDCs, but not in splenic pDCs (Fig. 3). Our results therefore demonstrate that although both thymic and splenic pDCs express high levels of Spi-B, mature thymic pDCs are unique by virtue of their repertoire of Notch receptors and HEBCan.

3.3.2 Spi-B is not required for thymic or splenic DC development or homeostasis

To determine if Spi-B is imperative to thymic or splenic DC development or homeostasis I compared the proportions of DCs in WT and Spi-B^-/- mice (Fig. 4A, B). There were differences in the proportion of gated subsets in flow cytometry plots in both thymic
Figure 4. Splenic and thymic DCs do not require Spi-B. 

(A) Splenic and thymic DCs were examined in WT and Spi-B−/− mice. DC subsets were gated as previously described: pDCs (CD11c^{int} B220^+), CD8^+ cDCs (CD11c^+ B220^− CD8α^+ CD11b^−) and CD8− cDCs (CD11c^+ B220^− CD8α^− CD11b^+). Numbers refer to percentages. 

(B) Percentages of the indicated subset, normalized to the number of CD45^+ cells, were calculated. Means were calculated from triplicate sample ± s.d. *p<0.05
and splenic populations (Fig. 4A). However, there was considerable variability among different mice and there were no statistically significant differences, with the exception of CD8⁺ cDCs, between subsets from WT and Spi-B⁻/⁻ mice (Fig. 4B). These results agreed with a recently published study, whereby only BM-resident pDCs were affected in Spi-B⁻/⁻ mice. Interestingly, however, there was a significant increase in the percentage of splenic CD8⁺ cDCs in Spi-B⁻/⁻ mice when compared to WT mice, which suggests that Spi-B might play a role in promoting pDC development over cDC development.

3.3.3 Optimization for adenoviral-mediated overexpression of Id2

The influence of Id2 expression at the T/DC/NK lineage checkpoint within the thymus was examined by adenoviral-mediated transient overexpression of Id2 within thymocyte precursors. The E-proteins, HEBAlt, HEBCan and E2A, which are important for early T cell development, are inhibited at the protein level by Id2²⁰³,²⁰⁴,¹¹⁶. Lck/CD2-CAR mice, which express the coxsackievirus and adenovirus receptor (CAR) under the control of the lck-proximal promoter and the human CD2 enhancer transgene, significantly enhances the infection of cells by adenovirus by increasing viral entry into CAR⁺ cells¹⁹⁸. Therefore, I chose to use this vector to force expression of Id2 in thymic precursors. I first assayed the thymocyte populations, including the DN1 subsets, for CAR protein expression by flow cytometry. The majority of the DN1 subsets, and DN2 cells expressed CAR, demonstrating that infection efficiency would be high in our cells of interest (Fig. 5A).

Using a multiplicity of infection (MOI) of 10 for both pAd-control and pAd-Id2, I
Figure 5

(A) Percentage of CAR$^+$ Cells

(B) Id2 mRNA Levels Relative to β-Actin

(C) Fold Difference Above Control

Figure 5. Optimization of adenoviral-mediated overexpression of Id2. (A) The percentage of thymocyte subsets that express CAR on the surface, determined by flow cytometry. (B) CAR$^+$ thymocytes were harvested, infected with pAd-control or pAd-Id2 and cultured for 3 days in 5 ng/mL each of SCF, IL-7 and Flt3L. Samples were taken every 24 hours and assayed for Id2 transcript expression. Id2 transcript levels and fold differences above pAd-control values are shown. (C) WT mice were irradiated with varying doses (225 Rad, 450 Rad, 675 Rad, 900 Rad) and thymic structure was analyzed by H&E staining 24 hours later. Cortex (C) and medulla (M) regions are labeled. (DP = double positive, SP = single positive).
infected CAR+ whole thymocytes overnight and determined the Id2 transcript expression 24 hours, 48 hours and 72 hours post-infection. Id2 was specifically upregulated above the transcript level expressed in uninfected and control-infected cells at 48 hours and 72 hours post-infection (Fig. 5B) and at levels comparable to what is expressed in mature DC subsets (Fig. 3). The fold increase of Id2 transcript levels in Id2-treated cells compared to control-treated cells was greatest 48 hours following infection but persisted at 72 hours post-infection (Fig. 5B).

Since the experimental system involved irradiating host Ly5.1 mice, to allow for engraftment of CD45.2+ adenovirus-treated cells, I next determined the dose of irradiation that would retain the medulla and cortex distinctions. The majority of tDCs reside in or in close proximity to the medulla, suggesting that the thymic medulla is important for their development or survival. Thymic sections were taken from WT mice 24 hours after they were treated with 250 Rad, 450 Rad, 675 Rad or 900 Rad of irradiation. H&E stain treatment revealed that although mice treated with 250 Rad, 450 Rad and 675 Rad still had distinctive cortex and medulla regions, they were less numerous and less scattered than WT mice (Fig. 5C). The standard for lethal irradiations to remove all hematopoietic cells, 900 Rad, did not retain cortex and medulla regions (Fig. 5C). Thus, the optimal irradiation dose chosen was 450 Rad to ensure room for engraftment, while also providing donor-derived cells a thymic architecture similar to WT mice.
3.3.4 Transient Id2 overexpression increases the proportion of thymic DCs and NK cells, but T cell subsets are intact

Thymocytes were harvested from C57Bl/6 Lck-CAR$^+$ mice, CD44$^+$ cells were purified using magnetic sorting, and these were infected with pAd-control or pAd-Id2 overnight. The adenovirus-treated cells were injected intravenously into the tail vein of irradiated Ly5.1 recipient mice. Thymii were analyzed 8 days following injection for NK and DC development (Fig. 6) or T cell development (Fig. 7) using flow cytometry. The NK, DC, and T cell populations of host-derived CD45.1$^+$ cells appeared normal, indicating that a dose of 450 Rad did not alter development and/or homeostasis of host cells (data not shown). For this experiment, SiglecH was used to identify pDC populations. There was an increase in the percentage and absolute number of NK cells (CD11c$^{-/\text{int}}$ SiglecH$^-$ DX5$^+$) in Id2-treated cells compared to controls. Similarly, all DC populations (pDC and cDC, including CD8$^+$ cDC and CD8$^-$ cDC) were increased in Id2-treated cells with the most striking increase observed in the CD8$^-$ cDC population. The absolute numbers of NK cells or DC subsets did not differ between control- or Id2-infected cells, suggesting that Id2 overexpression allows for lineage diversion in thymocyte precursors but does not offer a proliferative advantage.

T cell development was also examined from host- (CD45.1$^+$) and donor-derived (CD45.2$^+$) cells. Interestingly, although the total (CD45.1$^+$ and CD45.2$^+$) thymocyte cell numbers did not differ between control and Id2-infected mice, the number of donor-derived (CD45.2$^+$) cells from Id2-infected thymocytes was approximately half that of control (Fig. 6C). This suggests a decrease in cell survival, T cell expansion or thymic
homing from progenitors with transient Id2 overexpression. Host-derived T cell development appeared normal, while donor-derived T cells included a larger proportion of DN (CD4<sup>+</sup> CD8α<sup>-</sup>) thymocytes, likely due to the enrichment of CD44<sup>+</sup> cells prior to intravenous cell transfer (Fig. 7). There were no observed differences between control-or Id2-infected cells in CD44 and CD25 expression (Fig. 7), indicating that transient Id2 expression did not perturb T cell development. Similar DN populations, based on CD44 and CD25 expression, were observed in CD45.1<sup>+</sup> host-derived cells as well (data not shown).
Figure 6

(A) Thymocytes harvested from Lck-CAR^+ mice → Selection for CD44^+ cells → Infect CD44-enriched cells with pAd-control and pAd-Id2 overnight → Cells injected in irradiated Ly5.1 recipient mice

(B) Thymic NK and DC subsets were analyzed by flow cytometry 8 days following injection. Subsets were gated as follows: NK cells (Lin^- DX5^+), pDCs (Lin^- DX5^- CD11c^+ SiglecH^-), cDCs (Lin^- DX5^- CD11c^+ SiglecH^-), CD8^+ cDCs (Lin^- DX5^- CD11c^+ SiglecH^- CD8a^+ CD11b^-) and CD8^- cDCs (Lin^- DX5^- CD11c^+ SiglecH^- CD8a^- CD11b^+). Numbers refer to percentages.

Figure 6. Transient overexpression of Id2 in thymocyte precursors directs development to the NK and DC lineages. (A) Thymocytes from Lck-CAR^+ mice were harvested and CD44^+ cells were positively selected using magnetic sorting. CD44^+ enriched cells were infected with pAd-control and pAd-Id2 adenovirus overnight while cultured in 10 ng/mL each of SCF, IL-7 and Flt3L. Cells were injected into the tail vein of Ly5.1 mice irradiated with 450 Rads. CD45.2^+ cells are the donor-derived cells and CD45.1^+ cells represent the host-derived cells. (B) Thymic NK and DC subsets were analyzed by flow cytometry 8 days following injection.
Figure 6 continued. (C) Total thymocyte cell number (CD45.1+ and CD45.2+ cells), absolute number of host (CD45.1+) and donor (CD45.2+) cells for each replicate. (D) The percentage of donor cells that gave rise to specified populations were determined based on gating parameters outlined in (B) divided by the total number of CD45.2+ donor cells. (E) Absolute cell numbers were calculated by multiplying the percentage of each subset to the total cell number and to the fraction of CD45.2+ cells. Means were calculated from duplicate sample ± s.d.

(Absolute cell number = % of subset × total cell number × number of CD45.2+ cells
CD45.2+ cells + CD45.1+ cells)
Figure 7. Transient overexpression of Id2 in thymocyte precursors does not perturb T cell development. Using the same experimental setup as Figure 6, thymocytes were harvested from injected mice 8 days following intravenous injection of adenovirus-treated cells. Donor cells (CD45.2+) and host cells (CD45.1+) were gated and DN populations (CD4−CD8α−) are shown for both. DN1 (CD44+CD25−), DN2 (CD44+CD25+), DN3 (CD44−CD25+) and DN4 (CD44−CD25−) populations were shown for CD45.2+ donor-derived cells. Numbers refer to percentages.
3.3.5 Thymic DCs and specific DN1 subsets share transcriptional regulators

I next set out to identify candidate precursors of tDCs. Previous studies have suggested that thymic precursors can give rise to DCs as well as T cells$^{40,60,42,43}$. I reasoned that uncommitted T-cell precursors that exhibit similar transcriptional profiles to mature tDCs would be expected to have greater DC-lineage potential and decreased T-cell potential. I therefore examined gene expression in the ETP, DN1c, DN1d, and DN1e subsets, and compared these patterns to those in mature tDC subsets. To obtain DN1 subsets, I first enriched for DN cells by depleting thymocytes expressing CD8α and/or CD4 by magnetic sorting. ETP, DN1c, DN1d and DN1e subsets were then isolated by FACS based on the expression of c-Kit and CD24, as previously described (Fig. 8A)$^{56}$.

PU.1 was present in all DN1 subsets (Fig. 9B), with the highest levels in the DN1c subset. By contrast, Spi-B was expressed at much higher levels within the DN1d cells than in the other DN1 subsets. Similarly, DN1d cells contain the highest levels of IRF-8 when compared to the remaining T cell precursors. Id2 exhibited a different expression profile, with the highest levels in the DN1e subset. HEBCan was present in all populations, but was expressed at lower levels in the DN1c subset. Like Id2, HEBAlt was expressed almost exclusively in DN1e cells. Batf3 was not expressed at high levels, if at all, in these T cell precursors (data not shown). However, Ikaros was expressed by DN1 subsets at higher levels (Fig. 8B) than the DC subsets (data not shown) and increased as precursors became committed to the T cell lineage.
To infer potential responsiveness to Notch signaling, Notch receptors were also examined. Comparatively among the DN1 subsets, Notch1 was expressed primarily in the ETPs, with low level expression in the DN1d and DN1e subsets as well. Notch2 was more widely expressed, also peaking in the ETP subset. Like Spi-B, Notch3 was the highest in the DN1d subset, whereas Notch4 was highest in the DN1c and DN1e subsets (Fig. 8B). Thus, with the exception of ETPs and Notch3 expression by DN1d cells, the DN1 subsets do not appear to express high levels of Notch receptors.

The DN1 subsets examined above were sorted as CD11c− subsets to exclude the possibility of including DC subsets or DC precursors that have already upregulated CD11c. However, a previous study indicated that DN1c cells expressing CD11c are the CD8+ tDC precursors. Thus, I also examined the gene expression of important DC regulatory factors in CD11c+ DN1 subset populations for consistency (Fig. 9B). In agreement with previously published results, DN1c cells exhibited the greatest percentage of CD11c surface receptor expression, followed by DN1d, DN1e and ETP cells (Fig. 9A). I found that the majority of gene expression levels were similar between CD11c− DN1 subsets and their CD11c+ counterparts, with the exception of Id2 expression in DN1e cells and the collective expression of PU.1 in CD11c+ and CD11c− DN1c cells compared to the ETP subset. Although Id2 expression in CD11c+ DN1e cells was lower than CD11c− DN1e cells, it was still expressed at comparatively higher levels than the remaining DN1 subsets, in concordance with previous observations (Fig. 9B). Thus, the transcriptional profiles of CD11c− and CD11c+ DN1 subsets were similar. Discrepancies between the relative expression levels observed in Figure 8 and Figure 9 could be due to
the fact that CD11c^+ cells were excluded from DN1 sorted subsets in a grouped lineage gate for Figure 8, whereas CD11c was used as an identification marker in a singular flow cytometry channel for Figure 9. For the remainder of experiments I sorted and examined CD11c^- DN1 cells since they are the developmentally earlier precursors.
Figure 8. Regulatory factor mRNA expression in DN1 early thymocyte subsets. (A) Sorting parameters for ETP, DN1c, DN1d and DN1e subsets. DN2, DN3 and DN4 subsets were also assayed for Ikaros and IRF-8 gene expression. (B) Gene expression levels, as determined by qRT-PCR, were normalized to β-actin. Values shown are mean ± s.d. (n=3). nd, not detectable.
Figure 9

(A) ETP, DN1c, DN1d, and DN1e subsets were sorted and assayed for CD11c expression. Numbers refer to percentages.

(B) Gene expression levels of CD11c⁻ and CD11c⁺ DN1 subsets, as determined by qRT-PCR, were normalized to β-actin. Only CD11c⁻ ETP cells were assayed due to the low proportion of CD11c⁺ ETP cells. Values shown are mean ± s.d. (n=3).

Figure 9. CD11c⁻ and CD11c⁺ DN1 subsets share transcriptional profiles. (A) ETP, DN1c, DN1d, and DN1e subsets were sorted and assayed for CD11c expression. Numbers refer to percentages. (B) Gene expression levels of CD11c⁻ and CD11c⁺ DN1 subsets, as determined by qRT-PCR, were normalized to β-actin. Only CD11c⁻ ETP cells were assayed due to the low proportion of CD11c⁺ ETP cells. Values shown are mean ± s.d. (n=3).
3.3.6 Predicting Notch signaling in thymic DC and DN1 subsets

To infer whether Notch signaling was functional in thymic pDCs, I analyzed the expression of the Notch target gene, Hes-1, and found that it was expressed at high levels in thymic pDCs and at low levels in thymic cDCs (Fig. 10A). These results indicate that pDCs are receiving Notch signals within the thymus, and suggest that Notch signaling plays a role in thymic pDC function and/or homeostasis.

Notch1 and Notch2 were primarily expressed by ETPs, whereas Notch3 was expressed by ETP and DN1d cells. DN1c and DN1e cells expressed Notch4 (Fig. 8B). However, Hes1 expression, which suggests active Notch signaling, was observed only in the ETPs (Fig. 10B). Therefore, DN1c, DN1d, and DN1e cells appear to be transcriptionally primed towards the DC lineage(s), and awaiting the proper signals to fully differentiate.
Figure 10. Hes1 mRNA expression in DC and DN1 subsets. qRT-PCR of Hes1 transcripts of (A) thymic DC subsets and (B) DN1 subsets. Gene expression levels, as determined by qRT-PCR, were normalized to β-actin. Values shown are mean ± s.d. (n=3)
3.3.7 **Only ETPs can give rise to DCs in vitro**

Although B cell and NK cell potential have been tested for the DN1 subsets *in vitro*, DC potential was not examined. Thus, I sorted ETP, DN1c, DN1d and DN1e cells, as previously described (Fig. 8A), and placed them in culture for 6 days. Each precursor was cultured with SCF, IL-7, Flt3L and medium Dll4-expressing stroma (Fig. 11A) or with IL-3 in stroma-free (S-F) conditions (Fig. 11B). I also examined the development of each DN1 subset in the presence of low and high Dll4 expression levels, OP9 control stroma and in S-F conditions with SCF/IL-7/Flt3L, or SCF/Flt3L (data not shown), but none of these conditions produced CD11c+ cells. Interestingly, ETP cells gave rise to CD11c+ cells in the presence of medium levels of Dll4 (Fig. 11A) and IL-3 (Fig. 11B). Although DN1c cells did not survive in the presence of Dll4 (Fig. 11A), a small percentage of Thy1+ CD11c+ was generated when cultures were supplemented with IL-3 (Fig. 11B). DN1d and DN1e cells did not give rise to DCs and the majority of cells were CD8α+ in all culture conditions (data not shown). Thus, with the exception of ETPs, DN1 cells have limited capability of developing into DCs *in vitro*, which is likely due to missing factors in the *in vitro* system that are normally found in the thymus.
Figure 11. Only ETP cells can derive DCs in vitro. ETP, DN1c, DN1d and DN1e subsets were cultured in vitro for 6 days in the presence of (A) medium-expressing Dll4 OP9 stroma supplemented with 5 ng/mL each of SCF, IL-7 and Flt3L or in (B) stroma-free (S-F) conditions with 5 ng/mL IL-3. T cell (Thy1+ CD11c−) development and DC (Thy1− CD11c+) development was assayed by flow cytometry.
3.3.8 DN1 subsets do not give rise to DCs in fetal thymic organ culture

Next, I examined DC developmental potential of DN1 subsets in FTOC to determine whether thymic-specific cytokines or factors were required for DC development. Although there are numerous differences between fetal and adult thymic microenvironments, a fetal thymic microenvironment might be sufficient to promote DC development. Similarly, it should be noted that fewer DCs are present in the fetal thymus and the majority are CD11b[+] CD8α[−]67. DC populations are not normally found in high proportions in FTOCs (data not shown). The DN1 subsets were sorted from CD45.1[+] mice and added to CD45.2[+] embryonic timepoint E14.5 fetal thymic lobes by a hanging drop method. DC development was assayed from DN1 subsets following 7 days of culture by flow cytometry (Fig. 12). Total cells (CD45.1[+] and CD45.2[+]) are represented (Fig. 12), but no significant differences in the DC populations were observed when gating on CD45.1[+] donor and CD45.2[+] host cells separately (data not shown). Compared to control thymic lobes, in which no precursor cells were added, I did not observe an increase in DC development from any DN1 subset. We did, however, observe the presence of CD8α[+] CD11c[−] cells from each DN1 subset, indicating that the cells did engraft and develop into T cells. Thus, DN1 subsets were incapable of developing into DCs in a fetal thymic environment, which strengthens the case that a mature intact thymic structure is necessary to create the conditions required for intrathymic DC development.
Figure 12. DCs do not develop from DN1 subsets in a fetal thymic environment. Fetal thymic organ culture (FTOC) were generated using undepleted E14.5 fetal thymii. CD45.1+ DN1 subsets were administered to CD45.2+ FTOCs by the hanging drop method and were cultured for 7 days. CD8− cDC (CD11b+ CD11c+) and CD8+ cDC (CD8α+ CD11c+) populations from total cells (CD45.1+ and CD45.2+) were assayed by flow cytometry.
3.3.9 Thymic DCs can develop from ETPs, DN1d and DN1e subsets in vivo

Our data suggests that DN1d and DN1e subsets are transcriptionally primed towards the DC lineage. Although many precursors have been shown to contain DC potential,\textsuperscript{40,42,43} in vivo transfer studies demonstrating direct developmental pathways to tDCs are lacking. I first sorted ETPs, DN1d, and DN1e precursors from WT CD45.2\textsuperscript{+} mice and injected them intrathymically into nonirradiated congenic CD45.1\textsuperscript{+} recipients. Thymus sections were taken 5 days post-injection. Nonirradiated recipient mice were used to provide unperturbed thymic microenvironments.\textsuperscript{67} I performed immunofluorescence staining using antibodies that detected CD45.2 (donor origin), CD11c (DC phenotype), and cytokeratin-5 (mTECs). All three subsets gave rise to CD11c\textsuperscript{+} DCs within the thymus, which indicates that DC potential is present in each subset and that this potential is realized within the setting of the mature structured thymus (Fig. 13). Interestingly, the majority of DCs were present within the medulla or in close proximity to mTECs, regardless of the donor subset. The presence of mature T cells in the medulla was not examined since the short duration of in vivo development would not have allowed for full T-cell differentiation at that time point. These results show that placement of these precursors directly into the thymus allows them to differentiate into DCs, and suggests that those injected near the medulla might have been preferentially induced to become DCs.

Precursors that enter the thymus through the CMJ would be recruited into either the medullary or cortical environments depending on their repertoire of chemokine
receptors. Since DN1d and DN1e cells express CCR7 whereas ETPs do not\textsuperscript{45}, I forced these cells to enter the thymus through the circulation, thereby more closely mimicking the route by which thymic precursors are initially sorted, as well as testing their ability to home to the thymus. Sorted CD45.2\textsuperscript{+} ETPs, DN1d and DN1e cells were injected intravenously into nonirradiated congenic CD45.1\textsuperscript{+} recipients, and their presence and phenotype were monitored seven days later by immunofluorescence staining. Although all three subsets again had the capacity to generate CD11c\textsuperscript{+} DCs within the thymus, donor-derived DCs were slightly more frequently observed in the thymic medullary regions of animals injected with DN1d or DN1e subsets (Fig. 14). Therefore, our work shows that ETPs, DN1d, and DN1e cells can home to the thymus, develop into DCs, and that these precursors or DCs can localize to the medulla.
Figure 13

Figure 13. Intrathymic DC development from DN1 subsets following intrathymic injections. PBS, ETP, DN1d or DN1e CD45.2 donor cells were injected intrathymically into nonirradiated CD45.1 recipient mice. Thymus sections were analyzed for the presence of donor-derived DCs (CD11c+ CD45.2+) in proximity to medullary thymic epithelial cells (keratin 5+) 5 days following injection. Cells were stained with DAPI (blue) and for CD11c (green), CD45.2 (red), keratin 5 (purple). Images are representative of triplicate experiments. Arrowheads indicate donor-derived DCs.
Figure 14. Intrathymic DC development from ETP, DN1d and DN1e subsets following intravenous tail vein injections. PBS, ETP, DN1d or DN1e CD45.2 donor cells were injected intravenously into nonirradiated CD45.1 recipient mice. Thymus sections were analyzed for the presence of donor-derived DCs (CD11c+ CD45.2+) in proximity to medullary thymic epithelial cells (keratin 5+) 7 days following injection. Cells were stained with DAPI (blue) and for CD11c (green), CD45.2 (red), keratin-5 (purple). Images are representative of triplicate experiments. Arrowheads indicate donor-derived DCs.
3.4 Discussion

I have shown that the DN1 subsets, ETP, DN1d and DN1e cells, are capable of giving rise to tDCs in vivo, thereby providing another precursor source for intrathymic DCs. Our gene expression studies support the ability of DN1d cells and DN1e cells to act as precursors of tDCs. The similarity between DN1d cells and thymic pDCs is particularly striking: both have little to no PU.1 or Id2, and high levels of Spi-B, IRF-8 and Notch3. DN1c cells, by contrast, have high levels of PU.1 and undetectable levels of Spi-B. Interestingly, DN1c cells do not express Notch1, which could render them ignorant to strong Dll. The transcriptional differences between DN1 subsets suggest that there may be distinct routes by which intrathymic precursors adopt different DC lineage fates. Although ETPs clearly have DC potential they also express high levels of Notch1\textsuperscript{205}. PU.1 is required early in hematopoiesis to activate Flt3, which is critical for DC development\textsuperscript{63}. However, our studies suggest that in thymic pDCs (and perhaps splenic pDCs), this function may be mediated by Spi-B, thus allowing Flt3 expression without activation of PU.1-driven myeloid genes. Additionally, the influence of graded Dll-Notch signaling on Spi-B expression and pDC development is unclear\textsuperscript{191,206}. However, our results indicate that although Hes1 expression is low in DN1d cells suggesting weak Notch signaling, mature thymic pDCs are likely receiving strong Dll-Notch signals. Therefore, collaboration between Spi-B and Notch factors might be involved in pDC maturation within the thymus. Spi-B might also play a similar role to PU.1 in the thymus\textsuperscript{79}, whereby it inhibits T-cell-specific transcription factors more strongly in the absence of Notch signaling. Although mice lacking Spi-B do not have drastic DC
deficiencies\textsuperscript{96}, it does not exclude the possibility that Spi-B plays redundant roles to PU.1, a related Ets transcription factor family member. In this case, Spi-B might still be important in directing the development of precursors to the DC lineage when it is expressed, but PU.1 might be able to compensate when it is absent.

I also investigated the expression of the E proteins HEBCan and HEBAlt in tDCs. Although the impact of HEB-deficiency on tDC development has not been formally tested, our results suggest that studies on DC development using currently available HEB-knockout mouse models, in which both HEBAlt and HEBCan are deleted, could be misleading. I found that thymic pDCs express very high levels of HEBCan, but lack HEBAlt altogether, whereas both thymic cDC subsets are high in Id2 expression. Moreover, transgenic expression of HEBAlt interferes with DC development even under conditions of low Dll-Notch signaling\textsuperscript{45}. These results suggest that whereas HEBCan might play a role in segregating the pDC and cDC phenotypes, HEBAlt acts downstream of high Dll-Notch signaling to constrain DC potential in developing T cells.

The effect that transient overexpression of Id2, an E-protein antagonist, has on DC, NK and T cell development was revealed \textit{in vivo}. The competition between DC and T cell potential in the thymus is likely regulated by PU.1 and Notch signaling levels, and Id2 is an intermediary between these two pathways. PU.1 upregulates Id2 expression, which can then antagonize E proteins to inhibit T-cell development\textsuperscript{79}. Here, I show that with transient overexpression of Id2 during the early stages of T cell development the percentage of NK cells and DCs increases, whereas T cell development appears normal. It is probable that those cells receiving high levels of Dll-Notch signaling within the
thymus are able to maintain E-protein levels, whereas cells not receiving sufficient Dll-Notch signals are more heavily influenced by the presence of Id2. Surprisingly, the proportion of thymic pDCs was also increased in Id2-treated cells. However, since Id2 is only expressed transiently, the initial inhibition of T cell development likely increased the ability for precursors to develop into any DC subset. Overall, initial expression of Id2 is a DC-promoting factor within the thymus, regardless of the DC subset.

The presence of Ikaros in DN1 subsets could upregulate Gfi1, which is known to be expressed in T cell precursors\textsuperscript{96}, to inhibit Id2 and promote pDC development. Interestingly, mature splenic and thymic DC subsets do not express high levels of Ikaros or Gfi1, agreeing with the theory that these two factors play roles early in DC development but not in mature DCs. Overall, the expression of multiple DC-essential transcription factors within T cell precursors suggests that these cells are partially equipped to develop into DCs.

Our work has defined a new set of DC precursors within the thymus. Previous studies showed a low-CD4 precursor population in the thymus, which contains both DN1c and DN1d cells, can give rise to CD11c\textsuperscript{+} CD8\textsuperscript{+} cells \textit{in vivo} following intravenous transfer\textsuperscript{40,60}. Moreover, a recent study showed that CD11c\textsuperscript{+} DN1c cells give rise to CD8\textsuperscript{+} CD207\textsuperscript{+} DCs in the thymus\textsuperscript{26}. However, cells that already express CD11c are likely already committed to the DC lineage, which would not represent the most upstream precursor. It would be interesting to know if DN1c cells are the immediate precursors of the identified CD11c\textsuperscript{+} DN1c cells. Other studies have shown that DN1c and DN1d cells also have some B cell potential, suggesting that they are not all committed to the DC
lineage. Here, I have shown definitively that DN1d and DN1e cells, in addition to ETPs, can independently home to the thymus and differentiate into thymic medullary DCs. Introducing the cells intravenously confirmed that the cells could enter the thymus through the CMJ and traffic to the medulla, whereas the intrathymic injections confirmed that the developmental events by which DN1 subsets became DCs could occur within the thymus. Interestingly, although ETP, DN1d and DN1e cells were capable of developing into tDCs in vivo, they did not arise throughout FTOC or in vitro conditions, indicating a requirement for unknown developmental factors provided by an adult thymus. The major difference between E14.5 fetal thymic lobes and an adult 5 week-old thymus is the fetal environment lacks DP thymocytes, and, thus, lacks medullas. Thymic crosstalk studies revealed that later stages of T cell development, beginning at the DN3 to DN4 cell transition are required for the development of mTECs and cTECs.
Chapter IV

Interplay Between Dll-Notch and Flt3 Signaling Specifies Dendritic Cell Fate

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4 Interplay Between Dll-Notch and Flt3 Signaling Specifies Dendritic Cell Fate

4.1 Abstract

DCs throughout the body encounter a variety of extracellular signaling molecules including Notch ligands, which influence their development and homeostasis. More specifically, tDCs are exposed to varying levels of Dll1 and Dll4, depending on their microenvironmental localization within the thymus. I examine the influence of Notch signaling on DC development using an in vitro culture system designed to express varying levels of Dll1 or Dll4 on OP9 stromal cells. Here, I show that in the absence of Dll1, Jagged1 enhances DC precursor proliferation and restricts CD8\(^-\) DC differentiation. I also show that whereas medium levels of Dll1 and Dll4 are permissive for DC development, they are inhibitory to myeloid cells, indicating a higher threshold of Dll-Notch signaling for the DC lineage. Although CD8\(^-\) cDCs were the predominant population generated in vitro, as determined by transcriptional profiling and cell surface phenotypes, pDCs were derived in altered culture conditions supplemented with high Flt3L concentrations in the presence of Notch ligands. These results illustrate the competitive roles of Dll-Notch signaling and Flt3/Flt3L signaling in DC generation.
4.2 Introduction

The thymus is a specialized organ that uniquely supports T-cell development, in part by providing Dll for Notch signaling to uncommitted precursors. The thymus is also thought to support the development of several types of DCs, but the regulatory pathways and precursors of these cells have been controversial. Three DC subsets, the CD8+ cDCs, CD8− cDCs and pDCs, reside within or in close proximity to the medulla. In the event of intrathymic DC development either from DC precursors that enter the thymus or from intrathymic precursors with T/DC potential, it is inevitable that the developing precursor cells will encounter Dll. The predominant ligand, Dll4, is expressed throughout the thymus primarily on cTECs, but also at lower levels on mTECs, whereas Dll1 is expressed mostly within the cortex with the highest levels expressed in the CMJ. Thus, as developing cells migrate they experience different expression levels of Dll1 and Dll4, which likely shapes their development.

Notch signaling within the thymus is known to direct the developmental potential of precursors towards the T cell lineage and away from the B cell lineage. However, the influence of Notch signaling on tDCs, specifically, is still largely unknown. In vivo studies have determined that Notch1 is not involved in tDC development, but the involvement of the remaining Notch2-4 receptors is undetermined. In vitro studies have only elucidated the roles of Notch signaling directed by Jagged1 or Dll1 in monocyte-derived DC commitment and maturation. Similarly, the use of stromal monolayers with single, discrete levels of Dll1 does not reflect the varying expression levels of Dll1 within the thymus. I hypothesize that DC precursors migrate from the CMJ...
to the medulla, thereby exposing them to lower levels of Dll1 and Dll4 than T cell precursors, which migrate to the high levels of Dll4 within the cortex. Thus, the downstream Notch signaling events that ensue are likely different from low and high ligand levels and, thus, influence DC development uniquely in each thymic region.

It is widely accepted that Flt3L is required for cDC and pDC development, based on the absence of these populations from Flt3L−/− mice150,24. Flt3, the Flt3L receptor, is expressed by a fraction of ETPs within the thymus210. Although Flt3L plays a role to induce thymocyte precursor proliferation early in T cell development, Flt3 is downregulated in cells following persistent Notch signaling at the DN2 stage210. Thus, although they likely antagonize each other, Flt3/Flt3L and Dll-Notch signaling are important at different stages in T cell development and play distinct roles in lineage choice of early T cell precursors.

4.3 Results

4.3.1 Jagged1 inhibits DC development

Cells within the thymus are exposed to differing levels of Notch ligand, depending on their localization. Thus, I tested the influence of the Dll1-expressing OP9 stromal cell monolayers on DC differentiation. OP9 cells, which was derived from the BM of the op/op M-CSF-deficient mouse211, expresses low levels of Jagged-1 and secretes an array of cytokines and other regulatory factors212 (unpublished data, Mohtashami, M and Zúñiga-Pflücker, JC). OP9-DL1 cell monolayers were engineered to express high levels of Dll1 to effectively produce T cells from hematopoietic precursors in vitro213. LSK
hematopoietic precursors were sorted from adult BM and co-cultured with OP9 or OP9-DL1 stromal cells in the presence of SCF, IL-7 and Flt3L (Fig. 15A). As expected, the presence of Dll1 on OP9 stroma was inhibitory to DC development (Fig. 15A). Next, to determine whether graded levels of Dll1 ligand had different effects on DC development, I administered γ-secretase inhibitor (GSI) to high Dll1-expressing OP9 stroma cultures (Fig. 15A, B). GSI inhibits γ-secretase, which is a multi-subunit protease complex that is required to cleave the transmembrane domain of all Notch receptors to release intracellular Notch and is present when Notch is actively signaling. Thus, by adding increasing concentrations of GSI to OP9-DL1 co-cultures all Notch signaling induced by Dll or Jagged ligands are inhibited. Previous work has shown that 1 μM GSI inhibits T cell development at the DN1 to DN2 transition in vitro. I, therefore, co-cultured LSK cells on OP9-DL1 stroma in the presence of increasing amounts of GSI. There was an increase in the percentage of CD11c^+ cells (Fig. 15A, B) as increasing concentrations of GSI were administered to OP9-DL1 co-cultures. The trend of increasing DC development as Notch signaling was inhibited was repeated in two separate and independent experiments (Fig. 15B, C). When 0.3 μM GSI was administered to OP9-DL1 co-cultures, the percentage of CD11c^+ cells was similar to the proportion generated in OP9 co-cultures. Thus, low levels of Notch signaling through Dll1 are not inhibitory for DC development. However, the percentage of CD11c^+ cells in 1 μM GSI-supplemented OP9-DL1 cultures was greater than the proportion of DCs present in OP9 cultures. These results indicate that even Jagged1, which is present on OP9 cells, might have an inhibitory effect on DC development or maturation.
Figure 15.

Incremental increase in DC potential by inhibition of Notch signaling. LSK cells were cultured on OP9 or Dll1-expressing OP9 stroma for (A) 8 days (B) 7 days or (C) 9 days in the presence of 5 ng/mL each of SCF, IL-7 and Flt3L. (A) Cells gated on FSC/SSC, DAPI−, Lin−, CD45+ are shown. Numbers refer to percentages. (B, C) Cultures were treated with DMSO (control), 0 μM, 0.03 μM, 0.1 μM, 0.3 μM or 1 μM γ-secretase inhibitor (GSI). Percentages of CD11c+ cells were calculated and normalized to CD45+ cells. Absolute CD11c+ numbers are shown. (B) and (C) represent independent experiments.
4.3.2 An in vitro system for assessing the impact of graded Dll1- and Dll4-mediated Notch signaling on DC development

Although using GSI-supplemented cultures shed light onto the influence of varying levels of Notch signaling, it involved the inhibition of Notch signaling rather than mimicking the thymic environment through different Dll expression levels. Thus, to further determine how graded levels of Dll1 and Dll4 signaling impact DC development, I used an in vitro culture system whereby OP9 stromal cells express low, medium or high levels of Dll1 or Dll4 \(^{215}\). Control OP9 monolayers provided a baseline of DC development on stromal cells in the absence of Dll, whereas stroma-free (S-F) cultures allowed us to monitor DC development in the absence of stromal cell-derived factors. In all conditions, the cells were provided with SCF, IL-7 and Flt3L. SCF and Flt3L foster progenitor cell survival and proliferation, usually in a synergistic manner\(^ {216}\). IL-7 is provided to early thymocyte precursors to allow efficient expansion\(^ {217}\). This system allows us to evaluate the effect of Notch signaling on DC development in a controlled environment, which minimizes confounding effects of other signals present in the various thymus niches. The in vitro system used was initially developed by our collaborators in the Zúñiga-Pflücker laboratory using fetal liver (FL) precursors\(^ {215}\). In this study, I extended its use to examine the effects of Dll-Notch signaling on DC development from BM-derived hematopoietic precursors. Preliminary experiments were performed with FL-derived precursors, which yielded similar results to BM-derived precursors, but were less efficient at differentiating DCs (data not shown). Correspondingly, all three tDC populations are not generated until the adult stages of development in the mouse\(^ {67}\).
4.3.3 Medium levels of Dll4 preferentially support immature DCs

LSK hematopoietic precursors were sorted from adult BM and co-cultured with OP9 stromal cells expressing low, medium or high levels of Dll1 or Dll4. After defined timepoints, DC development was assessed using flow cytometry. Immature (CD11c+ MHC II−) and mature (CD11c+ MHC II+) DCs can be differentiated by their levels of MHC Class II\textsuperscript{218}. Following 8 days of culture, mature DCs were observed (Fig. 16A, C). The percentages of mature DCs fell as the levels of either Dll1 or Dll4 rose. However, cultures expressing medium levels of Dll4 at day 8 also contained an immature DC population (CD11c+ MHC II−) (Fig. 16B). Therefore, exposure of precursors to intermediate levels of Dll4 preferentially supported the generation or expansion of immature DCs. Thus, it is possible that DCs within the thymus need to migrate to a niche with low Dll4 levels to terminally differentiate.
Figure 16. DCs can develop in the presence of low and medium levels of Dll1 and Dll4. LSK cells were cultured with low, medium, and high Dll1- and Dll4-expressing OP9 stroma, control OP9 stroma, or in stroma-free (S-F) conditions for 8 days. Cultures were supplemented with SCF, IL-7, and Flt3L (A) DCs were gated on by FSC/SSC, DAPI−, CD45+ and Lin− (Lin = CD19, F4/80, Gr1, NK1.1, Thy1). Numbers represent percentages. Percentage and absolute number of (B) immature DCs (CD11c+ MHC Class II−) and (C) mature DCs (CD11c+ MHC Class II+) were calculated. Means were calculated from triplicate sample ± s.d. Data is representative of three independent experiments. Statistically significant differences from the OP9 control were determined. *P<0.05, **P<0.01.
Figure 16
(continued)

(B) Immature Dendritic Cells (CD11c⁺ MHC I⁻)

Percentage of CD45⁺ Cells

Absolute Number

(C) Mature Dendritic Cells (CD11c⁺ MHC I⁺)

Percentage of CD45⁺ Cells

Absolute Number
4.3.4 High levels of Dll1 inhibit the generation of DC-primed precursors

Multiple developmental stages occur during the differentiation of the LSK precursor cells into DCs or T cells during normal development. However, on high levels of Dll1 or Dll4, LSK precursors undergo accelerated T-cell development\textsuperscript{215}, and are thought to bypass earlier developmental stages due to their immediate exposure to Dll-Notch signaling. Therefore, I examined DC development in switch cultures to determine whether high levels of Dll1 inhibited the generation of DC precursors or the maturation of DCs. I co-cultured LSK cells with OP9 stroma for 2 days (OP9(2d)Dll1) or 4 days (OP9(4d)Dll1) before transferring the cells to high Dll1-expressing stroma in the presence of SCF, IL-7 and Flt3L (Fig. 17A). Following 8 days of culture, the percentage of DCs generated on OP9(2d)Dll1 was less than that on OP9, but greater than the amount of DCs that had developed on high Dll1-expressing stroma (Fig. 17B,C). High levels of Dll1 were inhibitory for the \textit{in vitro} formation of DC precursors, but once these precursors had formed, they were refractory to high levels of Dll1. These results indicate that the migration route of precursors within the thymus, which will provide exposure to different Dll levels, is likely imperative in determining cell fate outcomes.
Figure 17

(A) LSK cells were cultured with OP9 stroma for 2 or 4 days followed by culture with high-expressing Dll1 OP9 stroma for a total of 8 culture days. (B) DC populations were assayed by MHC II and CD11c expression. Numbers represent percentages. (C) Percentages and absolute numbers of DCs (CD11c⁺) were calculated and compared with OP9 and Dll1-high stroma co-culture controls. Means were calculated from triplicate samples ± s.d. Statistically significant differences from the OP9 control were determined. *P<0.05, **P<0.01.
4.3.5 Medium levels of Dll4 support DC development but not myeloid development

I first assessed the appearance and phenotype of DCs in these cultures by monitoring the expression of CD11c, CD11b, CD8α and B220 using flow cytometry. Preliminary experiments indicated that the proportion of CD11c⁺ cells peaked following 8 days of *in vitro* culture. S-F cultures generated the highest percentage of CD45⁺ Lin⁻ CD11c⁺ DCs (Fig. 18A, B), whereas OP9 cultures had the highest absolute numbers of DCs (Fig. 18E). Exposure of LSK cells to high levels of either Dll4 or Dll1 strongly inhibited the appearance of DCs (Fig. 18A, B, E), which correlated with enhanced T-cell development (data not shown), similar to results from FL-derived precursors²¹⁵.

In all Dll1 cultures, the percentages of DCs at day 8 decreased as the Dll1 expression levels increased (Fig. 18A, B), whereas the absolute numbers of cells in each Dll1-expressing culture did not differ significantly (Fig. 18D). Thus, higher levels of Dll1 expression inhibited DC development, while also promoting the development of other lineages, such as T cells. However, Dll4-medium cultures supported the development of DCs to a greater degree than any of the other Dll-bearing stroma, including Dll-low stroma, in terms of absolute numbers (Fig. 18A, E). I also analyzed the presence of non-DC myeloid cells (CD11c⁻ CD11b⁺) in these cultures. Interestingly, the percentages of CD11c⁻ CD11b⁺ cells, which represent macrophages, monocytes and granulocytes, were more negatively impacted by medium levels of Dll1 and Dll4 than DCs were (Fig. 18A, C). Therefore, thymic niches presenting intermediate levels of Dll4 would be expected to support DC development while excluding myeloid development. Although macrophages,
neutrophils and eosinophils are also found within the thymus, DCs represent the largest myeloid population and Notch signaling is likely important in regulating these lineages\textsuperscript{219,220}. 
Figure 18. Non-DC myeloid cells are inhibited by low levels of Dll. All data was taken from day 8 of culture. (A) LSK cells were cultured with low, medium and high Dll1- and Dll4-expressing OP9 stroma, control OP9 stroma, or in stroma-free (S-F) conditions. All cultures were supplemented with SCF, IL-7 and Flt3L. Non-DC myeloid cells were assessed by gating on FSC/SSC, DAPI−, CD45+ cells. (B,C) Percentage of (B) DCs and (C) myeloid cells were calculated. Statistically significant differences from the OP9 control, where indicated, were determined. **P<0.01. (D) Total cell numbers in culture and (E) absolute number of DCs are shown. The percentage of CD11c+ cells, as determined by flow cytometry, in each condition were multiplied by the total numbers of cells in each culture. Means were calculated from triplicate samples ± s.d. Data is representative of two independent experiments.
4.3.6 Transcriptional signatures of \textit{in vitro}-derived DCs

Classification of DCs by flow cytometry can be troublesome since cell surface markers are subject to upregulation and downregulation depending on their environment. Therefore, I sorted CD11c\textsuperscript{+} cells generated in low-, medium- and high-Dll4 expressing monolayers in addition to S-F and OP9 conditions to determine the transcriptional influence of varying levels of Notch ligand (Fig. 19A). The majority of the sorted CD11c\textsuperscript{+} cells generated in all culture conditions expressed CD11b, whereas only DCs generated in S-F conditions exhibited a small percentage of B220\textsuperscript{+} cells, indicating the presence of a small population of pDCs. The transcripts of genes known to be expressed by certain DC lineages were measured in each sample by qRT-PCR. Spi-B, CCR7, Flt3 and IL-7R\alpha were expressed at highest levels in S-F conditions (Fig. 19B), consistent with the pDC lineage. The lower expression levels of Flt3 and IL-7R\alpha on DCs generated by monolayers suggests that stroma-derived factors might be inhibitory to the expression of these cytokine receptors and thus, the ability of these cells to respond to Flt3L and IL-7. PU.1, which is universally expressed by all DC subsets, was expressed by all \textit{in vitro}-derived DCs; the highest levels of PU.1 were apparent in medium-Dll4 expressing co-cultures (Fig. 21B). Curiously, Id2, a marker for cDCs and inhibitor of the pDC factor E2-2, was expressed at highest levels in S-F conditions, suggesting that cDCs are also present. Interestingly, Notch1 and Notch2 were present at similar levels in all DCs, whereas low Hes1 expression suggests that only a small fraction of cells cultured with Dll4-expressing monolayers were undergoing Notch signaling. HEBCan, which is present in thymic pDC populations (Fig. 3), was expressed at higher levels by DCs in S-F and OP9 cultures.
Interestingly, with the exception of PU.1, there were no major differences in transcript levels between DCs generated in low-, medium- and high Dll4-expressing monolayers, which suggests that the level of Notch signaling does not affect gene expression in a committed DC. However, Notch signaling obviously plays an imperative role in precursor cells by determining cell lineage outcome. This is demonstrated by a decrease in non-T lineage potential in the presence of high Dll1 or Dll4 levels, which takes precedence over the influence of Flt3L (Fig. 16, 18). Overall, DCs generated in vitro expressed PU.1, Id2, Spi-B, Zbtb46 and, in some cases, E2-2 and E4BP4 (Fig. 19, data not shown), which suggest the presence of cDC and pDC subsets in cultures rather than monocyte-derived populations. Collectively, based on surface marker phenotype (Fig. 16, 18) and transcriptional profiling (Fig. 19), the majority of the DCs derived correspond to CD8\(^{-}\) cDCs, with the exception of some pDCs arising in S-F conditions.
Figure 19. Transcriptional regulation of in vitro-derived DCs. LSK cells were cultured with low (L), medium (M), and high (H) DL4-expressing OP9 stroma, control OP9 stroma, or in stroma-free (S-F) conditions for 8 days in cultures containing 5ng/mL SCF, IL-7 and Flt3L. DCs were sorted by FSC/SSC, DAPI−, Lin− (Lin = CD19, F4/80, Gr1, NK1.1, Thy1), CD45+ and CD11c+. (A) Pattern of CD11c+ expression in each sample. (B) B220 and CD11b expression by CD11c+ sorted cells. (C, D) Gene transcript levels, as determined by qRT-PCR, were normalized to β-actin. Values shown are mean ± s.d. (n=3). (C) qRT-PCR of CD11c+ cells from S-F, OP9, L-, M- and H- DL4. (D) qRT-PCR of CD11c+ cells from S-F, OP9, L- and M-DL4.
4.3.7 HEBAlt inhibits DC development

During normal T-cell development, HEBAlt levels rise until the β-checkpoint after which it is downregulated\(^\text{116}\). We have previously shown that HEBAlt-expressing FL-derived LSK cells are inhibited from becoming B cells and myeloid cells in the presence of high levels of Dll1\(^\text{221}\). HEBAlt is upregulated by Notch signaling, suggesting that it could influence DC development. I therefore examined the development of DCs in cultures of precursors from HEBAlt-transgenic (Tg) mice. In these mice, HEBAlt expression is under the control of the Lck promoter, which drives HEBAlt expression in some DN1 cells and most DN2 and DN3 cells\(^\text{196,222}\). Following 8 days of culture there were no significant differences between the WT and Tg cultures. On day 18 WT cultures had both immature and mature DCs on the low and medium levels of Dll1 and Dll4 (Fig. 20A). However, the percentage of DCs on low Dll1 and medium Dll4 in Tg cultures were lower than in WT cultures (Fig. 20B), suggesting that HEBAlt inhibited DC development even in the presence of low Dll-Notch signaling.

I also examined the surface expression of B220 and CD8\(\alpha\) to determine whether pDCs and CD8\(^+\) DCs, respectively, were generated in these cultures. A lack of B220 expression indicated that canonical pDCs were not present on day 18. However, CD8\(\alpha\) expression on CD11c\(^+\) cells was observed on WT cells in the presence of medium Dll4 levels (Fig. 20C). Interestingly, HEBAlt inhibited the generation of both CD8\(^+\) and CD8\(^-\) DCs when compared to WT cells. Since lowest levels of Dll4 on OP9 stroma are thought to be most similar to Dll4 expression levels within the medulla\(^\text{215}\), these results provide evidence that CD8\(^+\) and CD8\(^-\) DCs can develop within the thymic microenvironment, and
suggest that the upregulation of HEBAlt at the DN2 stage may be in part responsible for driving the T/DC fate choice towards the T-cell lineage.
Figure 20. HEBAlt inhibits DC development. (A) LSK cells from WT and HEBAlt-transgenic (Tg) BM were co-cultured with the indicated stroma for 18 days. Cultures were supplemented with SCF, IL-7 and Flt3L. DCs were gated on FSC/SSC, DAPI−, CD45+ and Lin− (Lin = CD19, F4/80, Gr1, NK1.1, Thy1) cells. Numbers represent percentages. (B) Percentage of mature DCs was calculated. Means were calculated from triplicate samples ± s.d. (C) CD8+ DCs (CD8α+ CD11c+) were only generated on medium levels of Dll4 from WT precursors.
4.3.8 The role of IL-3 in DC development in the absence of Flt3L

In addition to SCF, IL-7 and Flt3L, I investigated the influence of IL-3 on DC development in vitro. IL-3R is in the same cytokine family as GM-CSFR and has been shown to be expressed by human pDCs, as well as human DC precursors, and can enhance human pDC development when added to in vitro cultures. Other groups have identified a role for IL-3 in the development of monocytes, similar to the cells induced by GM-CSF and IL-4 (summarized in 189). Therefore, I investigated a possible role for IL-3 in DC development using our culture system. In addition to SCF, which is present in all cultures, three different combinations of cytokines were added to S-F, OP9 co-cultures or OP9 co-cultures expressing different levels of Dll4: (1) SCF, IL-7 and Flt3L (IL-7/Flt3L), or (2) SCF and Flt3L (Flt3L), or (3) SCF and IL-3 (IL-3; Fig. 21). Whereas B220⁺ CD11c⁺ pDC cells arose in vitro in various conditions (Fig. 21A), the proportion of B220⁺ CD11c⁺ cells was not enhanced by the removal of IL-7 and addition of IL-3. Thus, IL-3 does not promote murine pDC development in vitro. Interestingly, however, cells cultured with IL-7 and Flt3L or with IL-3 generated more heterogenous populations of DCs than those cultured in Flt3L alone, including CD11c⁺ CD11b⁺ and CD11c⁺ CD11b⁻ populations (Fig. 21B). As expected, the addition of IL-3 increased the proportion of non-DC myeloid cells (CD11c⁻ CD11b⁺), especially on medium levels of Dll4. Interestingly, cells cultured with Flt3L alone exhibited the greatest proportion of CD11c⁺ cells (Fig. 21B, C) likely due to the failure of other lineages, such as T cells, B cells and NK cells, to develop in the absence of IL-7. However, the absolute numbers of CD11c⁺ cells were much greater in cultures supplemented with IL-7 and Flt3L or IL-3 rather than Flt3L alone (Fig. 21C),
suggesting that the addition of IL-7 or IL-3 offered proliferative advantages. The addition of IL-3 enhanced the number of CD11c+ cells more than IL-7 and Flt3L in S-F conditions, and in the presence of medium or high Dll4-expressing monolayers (Fig. 23C). I also observed similar results to SCF/IL-3 when IL-3 was added to SCF/IL-7/Flt3L supplemented cultures (data not shown). Thus, this data indicates that IL-3 does not play the same role in murine pDC development as it does with human pDC generation, but it does enhance the proliferation of developing cells.
Figure 21. DC development in the absence of Flt3L. LSK cells were cultured with low, medium, and high Dll4-expressing OP9 stroma, control OP9 stroma, or in stroma-free (S-F) conditions for 8 days in cultures containing 5ng/mL SCF, IL-7 and Flt3L (SCF/IL-7/Flt3L), or 5 ng/mL SCF and Flt3L (SCF/Flt3L), or 5 ng/mL SCF and IL-3 (SCF/IL-3). DCs were gated by FSC/SSC, DAPI−, Lin− (Lin = CD19, F4/80, Gr1, NK1.1, Thy1) and CD45+. (A) pDCs (CD11cint/− B220+) and (B) CD8− cDCs (CD11c+ CD11b+) are shown. Numbers refer to percentages. (C) Percentage of DCs (CD11c+ cells) in each condition and (D) absolute numbers of DCs are shown. The percentage of CD11c+ cells, as determined by flow cytometry, in each condition were multiplied by the total numbers of cells in each culture. Means were calculated from triplicate samples ± s.d. Data is representative of two independent experiments.
Figure 21 (continued)

(B)

S-F

OP9

DL4 Low

DL4 Med

DL4 High

SCF/Flt3L/IL-7  SCF/Flt3L  SCF/IL-3

CD11b  CD11c
Figure 21 (continued)

(C) Percentage of CD11c⁺ Cells

(D) Absolute Number of CD11c⁺ Cells
4.3.9 Flt3L enables DC development in the presence of high Dll1 and Dll4 ligand expression levels

Although Flt3L has been shown to be imperative for cDC and pDC development \textit{in vivo}\textsuperscript{32}, the role of Flt3L in DC development in the presence of Notch ligands has not been thoroughly examined. Here, I tested the ability of DCs to develop from LSK progenitors supplemented with 100 ng/mL of Flt3L only (Fig. 22). CD11b\textsuperscript{+} CD11c\textsuperscript{+} DCs were capable of developing in every culture condition (Fig. 22A), however, to a lesser degree than cultures supplemented with 5 ng/mL SCF, IL-7 and Flt3L (Fig. 18). It is probable that this difference can be attributed to the proliferative effects of SCF and/or IL-7 on precursor proliferation. I also examined the generation of CD8\textalpha\textsuperscript{+} DCs in the presence of high concentration of Flt3L. CD8\textalpha\textsuperscript{+} DCs represented only a minor population in a few culture conditions (Fig. 22B). I did observe the emergence of a CD8\textsuperscript{+} DC population in the presence of medium Dll4-expressing stroma with 5 ng/mL IL-7, SCF and Flt3L, but only following 18 days of culture (Fig. 20C). Thus, CD8\textsuperscript{+} DCs only arose in specific conditions, whereas CD8\textsuperscript{−} CD11b\textsuperscript{+} DCs were capable of developing in nearly every condition I imposed on them, with varied efficiency (Fig. 18, 21B).
Figure 22. Generation of CD8$^+$ DCs in vitro with Dll1-expressing stroma. LSK cells were cultured with low, medium, and high Dll1- and Dll4-expressing OP9 stroma, control OP9 stroma, or in stroma-free (S-F) conditions for 7 days in cultures containing 100 ng/mL Flt3L. DCs were gated on by FSC/SSC, DAPI$^-$, CD45$^+$ and Lin$^-$ (Lin = CD19, F4/80, Gr1, NK1.1, CD3ε, DX5) cells. (A) CD11b$^+$ DCs (CD11c$^+$ CD11b$^+$) or (B) CD8$^+$ DCs (CD11c$^+$ CD8α$^+$) are shown. Numbers represent percentages. Percentages and absolute numbers of (C) CD11c$^+$ CD11c$^+$ DCs and (D) CD8α$^+$ CD11c$^+$ DCs.
Figure 22
(continued)

(C) LSK Cells Cultured with 100 ng/mL Flt3L

Percentage of CD11c⁺ CD11b⁺ Cells

Absolute Number of CD11c⁺ CD11b⁺ Cells

(D) LSK Cells Cultured with 100 ng/mL Flt3L

Percentage of CD11c⁺ CD8α⁺ Cells

Absolute Number of CD11c⁺ CD8α⁺ Cells
4.3.10 Plasmacytoid DCs are enhanced by Dll in the presence of Flt3L

Next, I examined the development of pDCs (B220+ CD11c\textsuperscript{int/+}) in the absence or presence of Notch ligands when co-cultured with 5 ng/mL each of SCF, IL-7 and Flt3L. A distinct pDC population was only generated in S-F conditions (Fig 23A), indicating that low Notch and Jagged1 signals and/or other stroma-derived factors can be inhibitory to pDC development. Curiously, pDCs (CD11c\textsuperscript{int}, Siglec H\textsuperscript{+}), which were also PDCA-1\textsuperscript{+} (data not shown), were observed in greater proportions in cultures supplemented with 100 ng/mL of Flt3L (Fig 23B) than those receiving 5 ng/mL of Flt3L in addition to SCF and IL-7 (Fig 23A). Similar to co-cultures provided with SCF, IL-7 and Flt3L, pDCs developed in the presence of S-F conditions, albeit at a much lower efficiency. However, with greater amounts of Flt3L, pDCs were also capable of developing in the presence of medium and high expression levels of Dll1, as well as medium levels of Dll4 (Fig 23B). Surprisingly, however, pDCs did not develop in cultures expressing low levels of Dll1 or Dll4. Thus, Notch ligands expressed at intermediate levels may synergize with high levels of Flt3L to support pDC development.
Figure 23. Plasmacytoid DCs can develop in the presence of Dll1- and Dll4-expressing stroma with Flt3L. LSK cells were cultured with low, medium, and high Dll1- and Dll4-expressing OP9 stroma, control OP9 stroma, or in stroma-free (S-F) conditions for (A) 8 days or (B) 7 days in cultures containing (A) 5 ng/mL each of SCF, IL-7 and Flt3L or (B) 100 ng/mL Flt3L. DCs were gated by FSC/SSC, DAPI−, Lin− (Lin = CD19, F4/80, Gr1, NK1.1, Thy1) and CD45+. DX5+ cells were additionally excluded from cells in (B). Numbers refer to percentages. Percentages and absolute numbers of (C) pDCs (CD11cint B220+) generated with 5 ng/mL SCF, IL-7, and Flt3L or (D) pDCs (CD11cint SiglecH+) with 100 ng/mL Flt3L.

115
Figure 23
(continued)

(C) LSK Cells Cultured with 5 ng/mL SCF, Flt3L, IL-7

Percentage of pDCs

Absolute Number of pDCs

(D) LSK Cells Cultured with 100 ng/mL Flt3L

Percentage of pDCs

Absolute Number of pDCs
4.4 Discussion

Here, I have shown that medium levels of Dll1 and Dll4 are permissive for DC development, whereas they are inhibitory to myeloid development. Our results also suggest that Jagged1, which is expressed at basal levels by OP9 stroma, can negatively impact DC generation. Notch receptors and Hes1 transcript levels suggest that at least some in vitro-derived DCs undergo and respond to Notch signaling. Our finding that a lower threshold of Dll-Notch signaling is required to inhibit myeloid development than DC development is consistent with other studies showing that myeloid potential within intrathymic precursors is lost before DC potential\textsuperscript{224,57,53}. However, while latent DC potential can be revealed in multiple precursors when they are removed from the thymic environment or from the inhibitory impact of high Dll-Notch signaling\textsuperscript{58,44,210}, the question of whether any of these constitute the normal precursor pools for thymic medullary DCs remains open.

Previous in vitro studies have indicated that Jagged1-expressing fibroblasts promote the accumulation of immature precursors, but not fully differentiated DCs\textsuperscript{174}. Our observation of an increased percentage of DCs generated from cultures with 1 μM GSI, which inhibits all Notch signaling transmitted from Jagged or Dll ligands, when compared to OP9 co-cultures suggests that Jagged1 plays a limiting role in the generation of CD11c\textsuperscript{+} cells. Similarly, the greatest percentage of DCs was generated when LSK precursors were first co-cultured with OP9 for 4 days to allow precursor development then seeded onto high Dll1-expressing monolayers. This succession likely enhanced the development of precursors in the presence of Jagged1 and DC maturation.
with Dll1. Additionally, high levels of Dll1 were inhibitory for the *in vitro* formation of DC precursors, but once these precursors had formed, they were refractory to high levels of Dll1.

Cytokine conditions proved to be a critical element in promoting the development of pDCs and CD11b⁺ DCs in the presence of high Dll ligands. When 5 ng/mL Flt3L was administered to cultures in addition to SCF and IL-7, the majority of DCs derived corresponded to CD8⁻ cDCs (CD11c⁺ CD8α⁻ CD11b⁺ MHC II⁺), which was confirmed by transcriptional profiling. Interestingly when 100 ng/mL Flt3L was administered to cultures, pDCs (CD11c⁺⁺ Siglec H⁺ PDCA-1⁺) and, to a very minor extent, CD8⁺ cDCs (CD11c⁺⁺ CD8α⁺ MHC II⁺), were capable of developing, but at the cost of decreased proliferative capacity. This experimental condition reflects a standard in the DC research field³⁶,³⁷,²²⁵, but the physiological reason for using 100-200 ng/mL Flt3L is unclear. Many reports suggest that pDCs are inhibited by Notch signaling¹⁹¹, which might be an artifact of improper culture conditions that do not foster pDC development. It is also known that the three cytokines, SCF, IL-7 and Flt3L, have synergistic proliferative effects²¹⁶. Interestingly, one report has shown that CD8⁺ cDCs do require some extent of Notch signaling, but likely from noncanonical Notch signaling through the Notch2 receptor¹⁸⁷. Here, I show that CD8⁺ cDCs are generated at low frequencies by Dll1-expressing stroma only, which interestingly is the hypothesized preferred ligand of Notch2.

There is emerging evidence highlighting the competitive interplay between Flt3/Flt3L and Notch signaling. ETPs express Flt3, which contribute to increased
proliferation that is not detrimental to T cell development\textsuperscript{210}. Once Notch signaling is initiated, subsequent downregulation of Flt3 occurs\textsuperscript{210}. Either Notch signaling directly inhibits Flt3 expression or Flt3 inhibition is a result of Dll-Notch signaling competition with other factors, such as PU.1\textsuperscript{79}, which is known to upregulate Flt3\textsuperscript{68}. The dual influence of Dll-Notch signaling and Flt3/Flt3L signaling might also play a role in determining whether precursors develop into cDCs or pDCs, since Notch signaling is thought to be inhibitory to pDC generation\textsuperscript{191,186}. However, the inhibitory effect of Notch signaling on pDCs has been shown only \textit{in vitro} with human-derived pDCs or \textit{in vivo} with splenic pDC populations. Our observation of greater Notch receptor expression by mature thymic pDCs indicates that they might be regulated differently than pDCs in other anatomical locations. Interestingly, when Dll4-Notch signaling is inhibited by α-Dll4 antibodies, which does not obliterate thymic architecture, the percentage and absolute number of tDCs increases\textsuperscript{226}. These results agree with previous findings that Notch1 does not play a role in tDC development or maintenance\textsuperscript{41,44}. Surprisingly, the increased DC population does not depend upon Flt3L and appear to be similar to tDCs in unperturbed mice\textsuperscript{226}. This differs from Notch-dependent splenic DC populations, which still require Flt3L for their development\textsuperscript{187}. Clearly, Flt3/Flt3L signaling is important for early stages of T cell precursor development, but the role in tDC generation needs to be examined more thoroughly.

In addition to Flt3L, the roles of IL-7 and IL-3 were examined and were found to offer proliferative advantages to precursors \textit{in vitro}. I found that IL-3, which has been shown to foster myeloid cell or human pDC development, promoted DC expansion.
Interestingly, IL-7 also increased the absolute number of DCs. *In vivo* studies have shown that although cDC and pDC subsets do not express IL-7R on their surface, IL-7 is involved in their development from BM precursors through a CLP intermediate\(^{227}\). Thus, supplementing *in vitro* cultures with IL-7 most likely increases the number of CLPs generated from LSK cells, in agreement with the presence of substantial B and T cell populations. Similarly, the growth factor cytokine, IL-3, likely induces the expansion of precursors with multi-lineage potential.

In conclusion, I have shown a novel role for Dll4 in CD8\(^-\) DC development, as well as a novel role for Dll1 and Dll4 in pDC development. The flexibility of this *in vitro* system will enable us to determine the transcriptional influence of Notch signaling within DC subsets, which will be important to decipher how it differs from Notch-directed transcriptional input in other cell lineages. Further investigation into the microenvironmental cues of each DC subset *in vivo* is also imperative in determining the spatial and context-dependent signaling inputs in DC precursors and mature subsets. This information will provide invaluable insight into how DCs are formed, how they can be enhanced and will allow further investigations into their roles in negative selection.
Chapter V

Thymic Microenvironmental Requirements for Dendritic Cell Development and Homeostasis

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5 Thymic Microenvironmental Requirements for Dendritic Cell Development and Homeostasis

5.1 Abstract

Thymic precursors, which include ETP, CD11c+ DN1c, DN1d and DN1e cells, have recently been identified for the intrathymic generation of tDC populations. The mechanism of tDC development, including the factors that direct their development, still remain elusive. Here, I address the roles of thymic medulla, the chemokine factors produced by mTECs and the presence of developing thymocytes on optimal tDC development. I found that low tDC frequency in fetal thymic microenvironments is not enforced by inhibitory Notch signaling, as illustrated by fetal thymic organ cultures supplemented with γ-secretase inhibitor. CCR7−/− mice exhibit slight reductions in all three mature tDC subsets (pDCs, CD8+ cDCs and CD8− cDCs) and an increase in immature CD11cint cells, which indicates reduced terminal tDC differentiation. RAG−/− thymii, which lack mature developing thymocytes and medulla, and IL-7Rα−/− thymii, which exhibit very low thymic cellularity, display significant reductions in thymic pDC, CD8+ cDC and CD8− cDC populations. Collectively, these results indicate that both medulla and an optimal developing thymocyte pool are imperative for maximal tDC development and/or homeostasis.
5.2 Introduction

Previous work has shown that latent DC potential can be revealed in multiple precursors when they are removed from the thymic environment or from the inhibitory impact of high Dll-Notch signaling\textsuperscript{210,58,44}. However, the question of whether any of these constitute the normal precursor pools for thymic medullary DCs remains open. Moreover, there has been a long-standing debate about whether the DCs that exist within the thymus arise from a common precursor with both T-cell and DC potential, or whether they enter the thymus as committed DC progenitors. We\textsuperscript{45} and others\textsuperscript{26} have shown that DCs can arise from ETP, DN1d, DN1e\textsuperscript{45} and a subset of DN1c cells\textsuperscript{26}, respectively, \textit{in vivo}. The stepwise mechanism of intrathymic DC development, however, has not been thoroughly examined. The thymus is organized into multiple regions, each of which can differ by epithelial cell content, growth factor availability and Dll expression levels (summarized in \textsuperscript{49}). Although the thymic architecture excels in fostering T cell development, I hypothesize that niches are also available to promote the development of other cell lineages found within the thymus, such as DCs and NK cells.

Thymic seeding progenitors enter the thymus at the CMJ\textsuperscript{49,228}. Although the majority of these precursors transit to the cortex for T-cell development\textsuperscript{229}, some subsets may preferentially migrate to the medulla and, thus, escape the T-cell fate as a requisite step for non-T lineage differentiation. Dll4 is expressed throughout the thymus primarily on cTECs, but also at lower levels on mTECs\textsuperscript{176}, whereas Dll1 is expressed mostly within the cortex with the highest levels expressed in the CMJ\textsuperscript{177,178}. The mTECs secrete the chemokines CCL17, CCL19 and CCL21, which ensure proper trafficking of SP
T cells to the medulla\textsuperscript{230,231}. XCR1 and CXCR4 have also been implicated in the localization of tDCs to the medulla\textsuperscript{232}. Although the majority of mature tDCs are present within the medulla\textsuperscript{231}, the influences of Dll1 and Dll4 on DC development were largely unknown until I examined the effect of varying Dll1 and Dll4 ligand levels on DC generation \textit{in vitro}\textsuperscript{215,45}. Here, I examine the role of CCR7, the receptor for CCL21, which is the main chemokine secreted by mTECs\textsuperscript{50}, in intrathymic DC development. Similarly, I also characterized the tDC subsets in RAG\textsuperscript{−/−} mice, which lack medulla, and IL-7Rα\textsuperscript{−/−} mice, which contain developing T cells, albeit at markedly reduced levels and thus, hypothetically, contain cortical and medullary distinctions. Using these mouse models, I was able to infer the influence of the medullary environment, and developing thymocytes, respectively, on intrathymic DC generation.

5.3 Results

5.3.1 Inhibition of Notch signaling in fetal thymic organ cultures does not enhance thymic DC development

High levels of Dll1 and Dll4 have been shown to be imperative for efficient T cell development\textsuperscript{215}, but inhibitory to DC development\textsuperscript{45}. In the fetal thymic environment, DCs are first detected at embryonic day 17\textsuperscript{67}, which coincides with the increase in DP thymocytes, which then, in turn, contributes to the formation of medulla by the differentiation of mTECs\textsuperscript{208}. Maximal tDC numbers are reached between 4 and 5 weeks after birth\textsuperscript{67}, concurrent with the maximum thymocyte cellularity in adult mice. The
three major factors that might influence the ontogeny of tDCs are Notch signaling, the presence of full thymic architecture and T-DC crosstalk. Here, I address whether Notch signaling plays a role in shaping the ontogeny of tDC subsets in the fetal thymic environment by adding increasing concentrations of GSI to E14.5 fetal lobes cultured in FTOC conditions for 7 days. The majority of the DCs present were CD11b⁺ CD8α⁻ (Fig. 24A), corresponding to the DC subset present during this embryonic gestation timepoint, which I approximated to E21.5 (E14.5 fetal lobes cultured for 7 days). Although the percentage of CD11c⁺ cells decreased when 0.03 μM and 1.3 μM doses of GSI were administered, there were no significant differences compared to the control (Fig. 24B). Thus, Notch signaling is not the limiting factor for tDC development in fetal thymic lobes, which suggests that thymic architecture or T-DC crosstalk might play roles in tDC generation.
Figure 24

(A) Inhibiting Notch signaling does not increase DC development in fetal thymic environment. γ-secretase inhibitor (GSI) was added to E14.5 FTOCs in incremental concentrations (0.03 μM, 0.1 μM, 0.3 μM, 1.3 μM). DMSO was added to FTOCs as a control. (A) DC populations (CD11b+ CD11c+) were assayed by flow cytometry following 7 days of culture. (B) The percentage of CD11c+ cells (DAPI− Lin− CD45+ CD11c+) normalized to the number of CD45+ cells, was calculated for each condition. Means were calculated ± s.d. (n=5).
5.3.2 **CCR7 is expressed by thymic DCs, DN1d and DN1e subsets**

Migration within the thymus results in differential exposure to Dll4 and Dll1, depending on the route taken. ETPs migrate from the CMJ to the outer cortex, which contains high levels of Dll4. However, if DN1 subsets migrated directly to the medulla from the CMJ, they would encounter a different microenvironment with lower levels of Dll1 and Dll4. To identify a potential mechanism of migration and localization of DCs in the thymic medulla I analyzed the mature tDC subsets for the expression CCR4, CCR7, XCR1 and CXCR4 chemokine receptors, which respond to the ligands CCL17, CCL19/21, XCL1, and CXCL12, respectively. Interestingly, I found that CCR7 was expressed by all tDCs (Fig. 25A), whereas CCR4 transcripts were not detected in any tDC subset (Fig. 25A). Interestingly, CD8\(^-\) tDCs expressed the highest transcript levels of XCR1 and CXCR4 as well, although it was still detected at low levels in CD8\(^+\) tDCs and pDCs (Fig. 25A). I also examined the presence of CCR4 and CCR7 mRNA in DN1 subsets. Intriguingly, DN1d and DN1e cells expressed higher levels of both CCR4 and CCR7 than ETPs and DN1c subsets (Fig. 25A), suggesting they may be able to home directly to the medulla. These results are consistent with the previously reported absence of CCR7 from DN1c cells 26. Varying levels of XCR1 mRNA was also expressed by DN1 subsets, whereas DN1c cells were the only precursor to express CXCR4 transcripts (Fig. 25A). Collectively, these results indicate that cells within the heterogeneous DN1 subsets have the capacity to home to the medulla.

Since I cannot decipher the expression level of CCR7 at the single cell level using qRT-PCR, I examined CCR7 surface protein expression by flow cytometry (Fig. 25B, C).
Interestingly, all tDC subsets expressed CCR7 on the surface (Fig. 25B), regardless of their relative CCR7 mRNA expression levels. I also discovered very small fractions of ETP, DN1d and DN1e cells with varying levels of CCR7 expression (Fig. 25C). Given the very small numbers of tDCs residing within the thymus\textsuperscript{67}, it is possible that these CCR7\textsuperscript{+} precursor cells contribute to these tDC populations.
Figure 25. Chemokine receptor expression in mature thymic DCs and DN1 subsets. (A) CCR7, CCR4, XCR1 AND CXCR4 transcript levels were determined by qRT-PCR, which were normalized to β-actin. Values shown are mean ± s.d. (n=3). nd, not detectable. CCR7 surface expression was examined on (B) thymic DC subsets and (C) DN1 subsets by flow cytometry.
5.3.3 CCR7 deficiency causes a decrease in mature thymic conventional DC subsets

Next, I wanted to examine the requirement for CCR7 in tDC development and homeostasis. Thus, I compared the thymic pDC and cDC populations in WT and CCR7−/− mice (Fig. 26). Once Lin+ cells were excluded from the CD45+ pool, three populations were gated on: pDCs (CD11cint B220+), immature DCs (CD11cint), and cDCs (CD11c+ B220−). Since NK cells (NK1.1+), T cells (CD3ε+), macrophages (F4/80+) and granulocytes (Gr1+) were excluded in the lineage gate, the CD11cint cells are likely immature DC subsets prior to terminal differentiation. These cells do not express high levels of MHC class II (data not shown), which would be consistent with this status. cDCs were further separated into CD8+cDC (CD8α+ CD11b−) and CD8− cDC (CD8α− CD11b+) populations. Once more, although the percentages of DC populations varied in flow cytometric plots (Fig. 26A), the proportions of DCs as normalized to CD45+ cells were not significantly different between WT and CCR7−/− mice (Fig. 26B). Nonetheless, I observed a trend in which all mature DC subsets (pDCs, CD8+cDCs and CD8− cDCs) were decreased in CCR7−/− mice, whereas CD11cint cells were increased in CCR7−/− mice when compared to WT controls. This suggests that CCR7 might play a role in the differentiation of tDCs, likely through directed migration of precursors or mature DCs to the medulla. Interestingly, there was a significant reduction in the absolute cell numbers of CD8− cDCs in CCR7−/− mice compared to WT, indicating an important role for CD8− cDC development or homeostasis. However, it is also very probable that other chemokines
expressed in the medulla, CCL17, XCL1 and CXCL12, play compensatory roles in the absence of CCR7 for all tDC subsets.
Figure 26. CCR7 is required for tDC development or homeostasis of CD8^- cDCs. (A) Thymic DCs were examined in WT and CCR7^-/- mice. Flow cytometry profiles are shown for three mice of each genotype. DC subsets were gated first by FSC/SSC profiles, DAPI^-, Lin^- and CD45^+. pDCs (CD11c^int B220^-), CD11c^int (CD11c^int B220^-), cDCs (CD11c^+ B220^-), CD8^+ cDCs (CD11c^+ B220^- CD8^+ CD11b^-) and CD8^- cDCs (CD11c^+ B220^- CD8^- CD11b^+) were gated. Numbers refer to percentages. (B) Percentages and absolute numbers of the indicated subset were calculated. Means were calculated from triplicate samples ± s.d. *P<0.05, **P<0.01.
Figure 26
(continued)

(B)

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WT CCR7−/−
5.3.4 Disrupted thymic DC development in RAG−/− mice

I hypothesize that the medulla, which tDCs localize to, provides a conducive environment for intrathymic DC development. Due to the complete block at the DN3 stage of T cell development in RAG−/− mice, the absence of DP thymocytes leads to improper thymic architecture lacking medullary and cortical regions\textsuperscript{233}. Thus, by examining tDC subsets in RAG−/− mice, I was able to address the question of whether tDCs require a medulla for their generation or maintenance. Populations of NK cells (DX5\textsuperscript{+}), pDCs (DX5\textsuperscript{−} CD11c\textsuperscript{int} SiglecH\textsuperscript{+} PDCA-1\textsuperscript{−}), CD8\textsuperscript{+} cDCs (DX5\textsuperscript{−} CD11c\textsuperscript{+} SiglecH\textsuperscript{−} CD8α\textsuperscript{+} CD11b\textsuperscript{−}) and CD8\textsuperscript{−} cDCs (DX5\textsuperscript{−} CD11c\textsuperscript{+} SiglecH\textsuperscript{−} CD8α\textsuperscript{−} CD11b\textsuperscript{+}), as determined by flow cytometry, were present in both RAG−/− and WT mice (Fig. 27A). Primarily due to the complete lack of maturing T cells, there were statistically significant increases in the proportions all three DC subsets and NK cells within the thymus and spleen (Fig. 27B, C). Curiously, however, there were also significant decreases in the absolute numbers of pDCs, CD8\textsuperscript{+} cDCs and CD8\textsuperscript{−} cDCs within the thymus exclusively (Fig. 27B). By contrast, the numbers of thymic NK cells were not altered in RAG−/− thymi (Fig. 27B). These results could indicate that a medulla is required for tDC development, homing or homeostasis, or that RAG-deficiency alters the intrathymic precursors that normally give rise to tDCs. It is also possible that crosstalk between developing T cells and DCs is required for optimal tDC development.
Figure 27

(A) Thymic DCs were examined in WT and RAG−/− mice. DC subsets were gated first by FSC/SSC profiles, DAPI−, Lin− and CD45+. pDCs (CD11cint PDCA-1+), CD8+ cDCs (CD11c+ PDCA-1− CD8α+ CD11b−) and CD8+ cDCs (CD11c+ PDCA-1− CD8α− CD11b+) were gated. Numbers refer to percentages.

(B) Percentages and absolute number of the indicated subset were calculated. Means were calculated from triplicate sample ± s.d. (n=5) *** p<0.001 (extremely statistically significant), ** p<0.01 (very statistically significant), * p<0.05 (statistically significant), ns (not significant).

Figure 27. Reduced thymic DC subset absolute numbers in RAG−/− mice.
Figure 27
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(B) Thymic Populations

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(C) Splenic Populations

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5.3.5 Reduction of cKit-expressing thymocyte precursors in RAG$^{-/-}$ thymus

I next examined the DN1 subsets in RAG$^{-/-}$ mice to determine whether thymocyte precursor deficiencies could explain the decreased tDC subsets. DN1 subsets were analyzed, as previously described (Fig. 8A), in WT and RAG$^{-/-}$ thymii. Although the populations present in RAG$^{-/-}$ thymii varied from mouse to mouse, it was clear that cKit$^+$ DN1 cells, namely ETPs, were disproportionately smaller in RAG$^{-/-}$ thymii compared to WT thymii (Fig. 28), in agreement with previously published results$^{234}$. Similarly, cKit$^{int}$ DN1c cells were also absent from RAG$^{-/-}$ mice, whereas the presence of DN1d and DN1e cells varied drastically from mouse to mouse (Fig. 28). Thus, it is likely that tDC development is adversely affected by the perturbation of the precursors from which they arise.
Figure 28. ETPs and DN1c cells are reduced in RAG−/− thymi. DN1 subsets were analyzed in WT and RAG−/− thymi. Cells were gated first by FSC/SSC profiles, DAPI−, Lin−, CD45+, CD4−, CD8α−, CD44+, CD25−. ETP (cKit+ CD24−/int), DN1c (cKitint CD24+), DN1d (cKit− CD24+) and DN1e (cKit− CD24−) cells were gated from DN1 cells (CD44+ CD25−). Numbers refer to percentages.
5.3.6 Similar splenic and thymic DCs and NK cells in RAG\textsuperscript{−/−} and wildtype mice

The tDC subset most affected in RAG\textsuperscript{−/−} mice were pDCs, as indicated by the significant decrease in their absolute numbers (Fig. 27B). I observed that, based on flow cytometry profiles, pDCs and NK cells exhibited similar staining patterns, with the exception of WT thymus. In WT and RAG\textsuperscript{−/−} mice, at least some DX5\textsuperscript{+} cells also exhibit intermediate expression levels of CD11c (Fig. 29A) and B220 (data not shown). Similarly, DX5\textsuperscript{+} cells also possess intermediate expression of PDCA-1 and SiglecH, albeit at lower levels than pDCs (data not shown). Interferon-producing killer DCs (IKDCs) have been identified as a novel DC lineage characterized by the phenotype CD11c\textsuperscript{int} B220\textsuperscript{+} DX5\textsuperscript{+}. However, some argue that this phenotype represents a unique NK cell population\textsuperscript{238}. Thus, to determine which subsets were present in WT and RAG\textsuperscript{−/−} mice, I sorted NK and pDC subsets from each strain. NK cells were sorted according to the phenotype CD45\textsuperscript{+} DX5\textsuperscript{−} CD11c\textsuperscript{−}, with the exception of WT thymic NK cells, whereby the predominant NK cell population expressed intermediate levels of CD11c (Fig. 29A). Thymic and splenic pDCs (Lin\textsuperscript{−} DX5\textsuperscript{−} CD45\textsuperscript{+} CD11c\textsuperscript{int} B220\textsuperscript{+}) were sorted as in previous analyses (Fig. 29A). I tested the transcript expression levels of Spi-B, Id2, E2-2 and E4BP4, which can differentiate between pDCs and NK cells. Spi-B and E2-2 are expressed at high levels in pDCs\textsuperscript{84,114}, whereas Id2 and E4BP4 are highly expressed by NK cells\textsuperscript{120,121,110}. Thymic and splenic NK cell subsets from WT and RAG\textsuperscript{−/−} mice expressed similar patterns of these four transcription factors, as did thymic and splenic pDC populations (Fig. 29B). Thus, IKDCs are not an emergent population in RAG\textsuperscript{−/−} mice that differ appreciably from the other
tNK cells. These results also demonstrate that mature tDCs generated in RAG<sup>−/−</sup> thymii exhibit the same transcriptional profile as those in WT control mice.
Figure 29

Similar splenic and thymic pDCs and NK cells are generated in WT and RAG−/− mice. (A) NK cell populations were sorted from thymus and spleen (DX5+ CD11c−), except for WT thymus whereby the predominant NK cell population (DX5+ CD11cint) was sorted. pDCs (DX5− CD11cint B220+) were also sorted from WT and RAG−/− thymii and spleens. Numbers refer to percentages. (B) Expression levels of Spi-B, Id2, E2-2 and E4BP4 mRNA, determined by qRT-PCR, were normalized to β-actin. Values shown are mean ± s.d. (n=3).
5.3.7 Thymic DC populations are severely affected in IL-7Rα−/− mice

I next examined the tDC populations in IL-7Rα−/− mice, which exhibit significantly decreased thymic cellularity, but contain DP and SP thymocytes and, thus, hypothetically possess proper corticomedullary thymic structure. Interestingly, cKit+ precursors were absent from IL-7Rα−/− thymi, similar to our observations of RAG−/− mice (unpublished). If the number of tDCs are regulated according to thymocyte cell numbers there would be reduced T-DC crosstalk in IL-7Rα−/− thymi due to low thymic cellularity. Also, IL-7 would be present at elevated levels due to the lack of uptake from developing IL7Rα−/− thymocytes. Although the phenotype of pDCs (CD11cint PDCA-1+), CD8+ tDCs (CD11c+ PDCA-1− CD8α+ CD11b−) and CD8− tDCs (CD11c+ PDCA-1− CD8α− CD11b+) were normal and there were an increased percentage of each subset (Fig. 30A), there were significant reductions in the absolute number of tDCs in IL-7Rα−/− mice compared to WT mice (Fig. 30B). Interestingly, this was not observed in splenic DC populations. Many implications can be derived from these observations. IL-7 might play drastically different roles in splenic and thymic DC subsets, whereby it is required only for tDC development and/or homeostasis. Another possibility is that T-DC crosstalk is important for DC development and/or maintenance, as it is severely reduced in IL7Rα−/− mice.
Figure 30

(A) Thymic DCs were examined in WT and IL-7Rα−/− mice. DC subsets were gated first by FSC/SSC profiles, DAPI−, Lin− and CD45+. pDCs (CD11cint B220+), CD8+ cDCs (CD11c+ B220− CD8α+ CD11b−) and CD8+ cDCs (CD11c+ B220− CD8α− CD11b+) were gated. Numbers refer to percentages. (B) Percentages and absolute number of the indicated subset were calculated. Means were calculated from triplicate sample ± s.d. (n=5) *** p<0.001 (extremely statistically significant), ** p<0.01 (very statistically significant), * p<0.05 (statistically significant), ns (not significant).
Figure 30
(continued)

(B) Thymic Populations

(C) Splenic Populations
5.4 Discussion

In order to fully understand the generation and, ultimately, role of DCs within the thymus, deciphering the microenvironmental requirements and niches in which they develop is equally as important as identifying their precursors. Interestingly, although ETP, DN1d and DN1e cells were capable of developing into tDCs in vivo, they did not arise throughout FTOC or in vitro conditions, indicating a requirement for unknown developmental factors provided by an adult thymus. Here, I show that the limited numbers of tDCs in the fetal environment is not controlled by Notch signaling. Observations have correlated the increase in tDC populations with the escalating thymocyte cellularity as mice age. Thymic crosstalk studies, which refers to crosstalk between T cells and epithelial cells, revealed that the formation of the cortex by cTEC generation is due to the appearance of DP thymocytes, whereas the emergence of SP thymocytes instigates the formation of the medulla. Interestingly, although RAG−/− thymocyte precursors are inhibited from becoming T cells, they are not diverted to the DC lineage, as shown by the decrease in absolute cell numbers of tDCs in RAG−/− mice compared to WT mice. Due to T cell developmental block at the DN3 stage, RAG−/− thymii do not possess normal thymic structure. It is also possible that RAG-deficiency in precursors causes cell-intrinsic differences in gene expression or that the altered microenvironment causes a cell-extrinsic effect on thymocyte precursors, also altering gene expression. However, pDC and NK cell subsets sorted from RAG−/− thymii were similar to the corresponding populations from WT mice according to their expression of Spi-B, Id2, E2-2 and E4BP4. Thus, the same pDC subsets, as determined by surface
receptor phenotype and gene profiling, were generated in WT and RAG−/− mice. Similarly, there was not a significant decrease in the absolute number of splenic DC subsets, proving that the DC developmental defect is restricted to the thymus. These results strongly indicate that the thymic environment in RAG−/− mice is not optimal for tDC development.

However, it is also possible that crosstalk occurs between DCs and T cells, and that the reduction in tDCs is a direct correlation to diminished T cells. An even more drastic reduction in tDC populations was observed in IL-7Rα−/− mice, which should hypothetically contain medulla regions due to the presence of DP and SP thymocytes. However, the DP and SP cellularity is significantly decreased in IL-7Rα−/− mice compared to WT mice239. Since all cells are deficient in IL-7Rα, the reduction in tDCs is not due to an inhibitory role of IL-7 on DC development. Multiple reports have shown conflicting data on the involvement of IL-7 in DC development240,241,227,34. Two studies show that, with the exception of the very early developmental stages before precursors become committed to the DC lineage, IL-7Rα is not required for tDC development240,241. Since IL-7R-dependent CLPs242 give rise to a fraction of the splenic DC subsets31, any DC defects in IL-7R−/− mice are likely an effect from the reduced survival of CLPs. Another study using a mixed BM chimera demonstrated that there is an intrinsic DC defect in spleens and LNs from IL-7R−/− BM227. However, since mature splenic and LN-resident DCs do not express IL-7R, these results also reflect developmental defects in precursors. Interestingly, in IL-7 transgenic mice, whereby IL-7 transcripts are overexpressed 10-30 fold more than WT mice, cDC and pDC populations are increased in secondary lymphoid
organs similar to the increase in thymocytes. Again, since mature DCs do not express IL-7R, this could be an effect of the increase in DC-generating CLPs or it could result from a cell extrinsic effect by the increase of thymocytes if DC-T crosstalk does exist.

Conversely, the cDC and pDC subsets in the thymus are more uniquely regulated by IL-7/IL-7R. Fate-mapping mice, in which cells expressing IL-7R were irreversibly labeled with YFP, revealed that only one tenth of thymic and splenic CD8+ and CD8− cDCs had arisen from IL-7R+ precursors, suggesting that most of these cells did not arise from CLPs or IL-7R+ thymocyte precursors and were not dependent on IL-7. However, nearly all thymic and splenic pDCs exhibited a history of IL-7R expression, indicative of a developmental transition through the CLP stage. In another study, only CD8+ and CD8− cDCs were examined in the thymus of irradiated mice reconstituted IL-7Rα−/− BM, which revealed no developmental deficiencies. This illustrates that the reduction of tDCs, at least within the cDC compartment, in IL-7Rα−/− thymii is not a cell intrinsic effect. Thus, it is likely that either altered thymic architecture or some degree of DC-T crosstalk exists to regulate the expansion of tDC populations.

I predict that migration of thymocyte precursors with DC potential to the medulla enhances intrathymic DC development by providing a DC-promoting microenvironmental niche. The expression of CCR7 and CCR4 in DN1d and DN1e cells suggested that they might be able to migrate towards the medulla (low in Dll proteins) and away from the cortex (high in Dll proteins) where early T-cell development occurs. Thus, I conducted an analysis of tDC subsets in CCR7−/− mice, but found that although all mature tDCs express CCR7, CCR7−/− mice did not exhibit significant differences from WT
mice. However, slight reductions in all three tDC subsets and an increase in CD11c\textsuperscript{int} cells was observed. This may be due, in part, to the compensatory ability of CCR4, XCR1 and CXCR4 to respond to chemokines secreted by mTECs. Moreover, others have shown that although CCR7\textsuperscript{−/−} mice exhibit a more disorganized thymus, medullas are still present\textsuperscript{243}. Therefore, a CCR7\textsuperscript{−/−} CCR4\textsuperscript{−/−} XCR1\textsuperscript{−/−} CXCR4\textsuperscript{−/−} mouse model would be required to fully test the hypothesis of chemokine-mediated migration of intrathymic DC precursors to the medulla. A fuller understanding of the migration route of tDC precursors or homing of mature tDCs will allow us to determine the very specific niches that contribute to intrathymic DC generation.
Chapter VI

Thesis Goals and Concluding Remarks

Figure 32 has been published in Advances in Hematology. 2013. Feb 18. Volume 2013.

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6 Thesis Goals and Concluding Remarks

6.1 Regulatory factors that condition thymocyte precursors for dendritic cell development

In chapter III, I used gene profiles of mature splenic and thymic DC subsets to infer DC potential within early thymic progenitors. These gene profiles were also very informative by providing the expression levels of the regulatory factors involved in the maintenance of splenic and thymic DC populations. I found that CD8$^+$ cDCs and CD8$^-$ cDCs expressed similar transcription factors within each subset at similar levels regardless of whether they resided in the spleen or the thymus. Id2 expression was greatest in CD8$^+$ cDCs and PU.1 was expressed highly in CD8$^-$ cDC subsets. Adenoviral-mediated transient overexpression of Id2 in thymocyte precursors increased the proportion of NK cells and all DC populations in the thymus, without perturbing T cell development. Surprisingly, thymic pDC development was also increased in Id2$^+$ precursors. This indicates that transient Id2 expression might contribute to diversion of a precursor to the pDC lineage. The subsequent downregulation of Id2 would then allow for the proper function of E2-2 and HEBCan, which are thought to be involved in thymic pDC development.$^{114,45}$

Since Spi-B was expressed at highest levels in both splenic and thymic pDCs, I examined the DC subsets in Spi-B$^{-/-}$ mice. Interestingly, I found no significant differences in DC development, which is consistent with a recent report.$^{86}$ Unlike Spi-B, several other transcription factors were found to differ between the pDC populations in the
spleen versus the thymus, including HEBCan and Notch receptors, which were exclusively expressed by thymic pDCs. Although HEBCan was found to not play a role in the development or maintenance of splenic pDCs\textsuperscript{114}, it had not been previously examined in thymic subsets. Thus, our results suggest that thymic pDCs may be under differential control than splenic pDCs. Furthermore, the expression of Hes1 and all four Notch receptors in thymic pDCs indicates that they are actively signaling through Notch receptors. The question of whether thymic pDCs are influenced by Notch signaling during development or once they have matured has not been answered.

Since DC potential decreases as thymocytes develop from DN1 to DN2 cells\textsuperscript{57}, the DN1 subsets were examined for regulatory factors that are important for DC development and many similarities were observed. ETPs, which have been most extensively studied, express PU.1, HEBCan and Notch receptors. DN1c cells express the highest levels of PU.1 and low levels of Notch receptors and Hes1, revealing that Notch signaling is likely not active in this subset. I also observed many notable similarities between DN1d cells and pDCs, including the collective expression of Spi-B, IRF-8, and HEBCan, as well as low levels of Id2. On the other hand, high levels of Id2 were expressed by DN1e cells, suggesting cDC and NK cell potential. However, given the ability of transient Id2 expression to increase the proportion of all DC subsets, it is possible that pDC potential is also present in DN1e cells. Intriguingly, although ETP, DN1d and DN1e cells do not develop into DCs \textit{in vitro} or in FTOC they are capable of deriving tDCs \textit{in vivo}, which home to the medulla. These results indicate that the thymus, and more specifically the medulla, provides not yet identified factors that are
imperative for intrathymic DC development. These factors could include additional cytokines provided by thymic stroma, crosstalk between T cells and DCs, or Wnt factors, which have been shown to play a role in DC development in vitro\textsuperscript{244}.

6.2 Interplay Between Dll-Notch and Flt3 Signaling Specifies Dendritic Cell Fate

In Chapter IV, I narrowed my focus to the involvement of Notch signaling in DC generation using an in vitro culture system, whereby varying levels of Dll1 and Dll4 are expressed by OP9 stroma. These experiments allowed us to examine the role of Notch signaling without the multitude of confounding factors that are present in the thymus. Initial experiments using GSI revealed that as Notch signaling is increasingly inhibited, regardless of the whether the ligand Dll or Jagged is transmitting the signal, the proportion of DCs also increases. Although DCs are inhibited by gradual increases in Notch signaling, only high levels of Dll1 and Dll4 are completely inhibitory. Interestingly, whereas medium levels of Dll1 and Dll4 are still permissive for DC development, they are completely inhibitory to other myeloid cell development. This observation reflects and supports the paradigm of lineage potential from thymocyte precursors, whereby DC potential is greater than non-DC myeloid potential in DN subsets\textsuperscript{245}.

Although the majority of DCs generated in our in vitro studies corresponded to the CD8\texttextsuperscript{−} cDC population, according to surface phenotype profiling (CD11c\texttextsuperscript{+} MHC II\texttextsuperscript{+} CD11b\texttextsuperscript{+} CD8α\texttextsuperscript{−} B220\texttextsuperscript{−} PDCA-1\texttextsuperscript{−} SiglecH\texttextsuperscript{−}) and transcriptional signatures (PU.1 and Id2),
pDCs and CD8α⁺ DCs were generated in some conditions. Using PDCA-1 and SiglecH in our flow cytometry staining panels allowed us to identify pDCs which could develop in the presence of Dll1 and Dll4 with 5ng/mL each of SCF, IL-7 and Flt3L. Higher concentrations of Flt3L, exclusively, also fostered pDC development, and in rare cases, minor populations of CD8α⁺ DCs. Interestingly, CD8α⁺ DCs also developed following 18 days of cultures, when a second wave of in vitro-derived DCs were generated. Our results suggest that, in the case of pDCs, Notch signaling either contributes to or inhibits pDC development, depending on the concentrations of Flt3, indicating that Notch signals might not be exclusively inhibitory, as previously suggested. Instead, the correct combination of signals allows the generation of pDCs in vitro, even in the presence of Notch signaling.

I also examined the role of Notch signaling in DC precursor development as well, since previous in vitro studies focused entirely on monocyte-derived DC populations. I discovered that high levels of Dll1 were only inhibitory towards DC development if LSK precursors were exposed to high Dll1 levels immediately. If LSK progenitors were co-cultured with OP9 stroma first for 2 or 4 days before transfer to high Dll1 expression levels, DC potential increased. These results show that precursors generated from OP9 stroma were not as strongly inhibited by Notch signaling, which could indicate that Notch receptors were not expressed on these progenitors or the precursors expressed factors, such as PU.1 or Flt3, which might compete with Notch signaling. Interestingly, increasing levels of Dll might inhibit terminal differentiation of DCs since a larger fraction of immature DCs (CD11c⁺ MHC II⁻) were generated in the
presence of medium levels of Dll4. Clearly, there is an interplay between DC-promoting transcription factors and receptors, such as PU.1 and Flt3, and T cell-promoting Notch signaling, which compete for cell lineage potential. I hypothesize that the factors present in greater amounts or the factors expressed first in the progenitor cells will succeed in the lineage competition battle. For example, HEBAnt, which is a putative Delta-Notch signaling target, inhibits DC development in vitro. Consistently, as observed in Chapter III, the forced expression of Id2 in thymocyte precursors enhances DC development in vivo.

The interplay between cytokines and Notch signaling and their combined role in DC development were also examined. Although Flt3L is imperative for cDC and pDC development, cultures supplemented exclusively with high concentrations of Flt3L did not exhibit the greatest absolute numbers of DCs. SCF, IL-7 and, interestingly, IL-3 significantly enhanced the proliferative capacity of in vitro-derived DCs. Since we know that mature DC subsets do not express IL-7R or IL-3R (data not shown), I can conclusively state that this proliferative advantage is due to precursor expansion. Interestingly, high concentrations of Flt3L enabled a greater proportion of DCs to develop in the presence of high Dll1 or Dll4, which is likely due to the competition of DC- and T cell-promoting regulatory factors.
6.3 Thymic Microenvironmental Requirements for Dendritic Cell Development and Homeostasis

Results from Chapter III have shown that although the DN1 populations, ETPs, DN1d and DN1e, cannot develop in FTOC or in vitro conditions, they can give rise to tDCs in vivo, which indicates that there are unidentified factors provided by the adult thymus that are imperative for tDC development from thymocyte precursors. Since the majority of tDCs home to the medulla, I hypothesized that precursors likely localize to this region prior to or during DC commitment. Since lower Dll1 and Dll4 expression levels are expressed by mTECs, the medulla would provide a more conducive environment for DC development. Cells expressing CCR7, CCR4, XCR1 or CXCR4 would be attracted to the medulla. In Chapter V, I found that all mature tDC subsets express surface CCR7. Interestingly, CD8− tDCs expressed the highest transcript levels of XCR1 and CXCR4. CCR7 and CCR4 transcripts were expressed at highest levels in the DN1d and DN1e subsets. Only a small fraction of DN1d and DN1e cells express CCR7 on the surface, but due to the low numbers of DCs in the thymus, these CCR7+ progenitors could certainly be a source of tDCs. Although significant differences were not observed in DC subsets between CCR7−/− and WT mice, I observed a small decrease in all mature tDCs and a small increase in CD11cint cells. Terminal differentiation of DCs in CCR7−/− thymi was therefore mildly affected, likely due to impaired migration of precursors.

I observed a significant decrease in the absolute number of all three tDC subsets, but not NK cells, in RAG−/− mice compared to WT mice suggesting that the absence of a
medulla or depletion of specific T cell progenitors is detrimental to intrathymic DC development. Importantly, these differences were specific to the thymus and were not mirrored by splenic DC subsets. Curiously, ETP and DN1c populations were absent from RAG−/− thymii and the presence of DN1d and DN1e cells was variable in each age-matched sample. I also confirmed that pDCs from WT and RAG−/− thymii exhibited similar expression of Spi-B, E4BP4, E2-2 and Id2 mRNA. Thus, the pDC populations generated in RAG−/− mice were bona fide pDCs, albeit present at a much lower frequency.

Our analysis of tDC populations in IL-7R−/− mice allowed us to determine tDC frequency in a thymic environment with similar DP and SP thymocyte populations that would be found in a WT thymus, but at a significantly lower thymic cellularity239. I discovered strikingly lower frequencies of all tDCs, as well as NK cells, in IL-7R−/− mice compared to WT mice, indicating that T-DC crosstalk plays a role in tDC generation, and that IL-7 is likely needed for the generation of DC precursors. Again, these deficiencies were not mirrored in the DC populations of the spleen.

Collectively, the results from my thesis suggest an intricate model for the intrathymic development of DCs, whereby thymic precursors are directed to different microenvironments within the cortex or the medulla depending on their expression of CCR7, CCR4, XCR1 or CXCR4 (Fig. 31). The way in which they respond to those environments are regulated by their repertoire of receptors and the transcription factors that they express, such that ETPs respond to high Dll by activating a T-lineage program, whereas DN1d and DN1e cells respond to moderate levels of Dll4 by
generating DCs. Competing factors within precursor cells determine the lineage outcome, as I have shown that tDCs can still be derived from ETP progenitors in vivo. More work will be needed to confirm the precursor-product relationships of DN1d and DN1e cells with specific subsets of tDCs, and to evaluate their latent T cell potential. Our results clearly show that these cells are functionally equipped with the genetics programs necessary for differentiation into the DC lineage within the thymus.
Figure 31. Proposed model of intrathymic DC development. The thymus is separated into the cortex (light purple) and the medulla (dark purple). When thymic seeding progenitors enter the thymus at the CMJ (dashed line), which borders the cortex and the medulla, thymocyte precursors migrate towards the cortex due to chemokines and growth signals secreted by the cTECs. As they develop into DP T cells they start migrating towards the medulla, where the majority of tDCs reside. This migration is guided by the secretion of CCL17 (CCR4), CCL19 (CCR7) and CCL21 (CCR7) by mTECs. (The receptor that responds to each chemokine, expressed by DP and SP T cells, are in brackets). We propose that it is more likely that tDC precursors migrate directly to the medulla, where low levels of Dll4 are expressed, instead of transiting to the cortex which express high levels of Dll4. Chemokine receptors that could be involved in direct migration of tDC precursors to the medulla include CCR7, CCR4, XCR1 and CXCR4. The tDC precursors are likely primed to become DCs due to the expression of DC-promoting transcription factors, such as PU.1, Spi-B or Id2. Collectively, DC lineage potential can be promoted in thymocyte precursors by the expression of chemokine receptors, which enables the migration to an environment with low Dll4 expression levels, and the expression of DC-promoting transcription factors.
6.4 cDC and pDC Gene Regulatory Networks

In my thesis and my model of intrathymic DC development (Fig. 31), I show and emphasize that the genes expressed by precursors are imperative for the determination of lineage outcome. In the introduction, I have outlined the transcription factors that are important for DC development. This combined knowledge has led to us a context-dependent understanding of DC lineage commitment. Once organized into lineage-specific gene regulatory maps, the similarities and differences between cDCs and pDCs become more apparent (Fig. 32). The networks are separated based on the stage of development in which each factor is proposed to function. PU.1 is a master regulator of both cDCs and pDCs and, based on experimental evidence, it likely functions early in DC development at or immediately prior to the CDP stage. The main function of PU.1 is to turn on regulatory genes that are responsible for proper DC development, such as Id2, Flt3 and GM-CSFR. Since signaling through GM-CSFR can activate STAT5, which inhibits IRF-8 transcription, GM-CSF might be an environmental cue to favour CD8^- cDC development. Indeed, GM-CSF promotes the development of CD8^- CD11b^+ DCs in vitro. The partial restoration of a WT phenotype by transducing E4BP4^-/- cells with Batf3 suggest that either E4BP4 and Batf3 have similar transcriptional targets or Batf3 is upregulated by E4BP4. Conversely, the elevated levels of IRF-4 mRNA in E4BP4^-/- cells indicates that E4BP4 inhibits IRF-4, directly or indirectly (Fig. 32A).

Clearly, Id2 functions to inhibit pDC development by binding to and inhibiting E2-2, which is required for pDCs. However, as I have shown, initial overexpression of Id2 in a precursor cell might contribute to increased pDC lineage potential. Although the
earlier Ikaros mutant studies were contradictory, a model in which Ikaros is expressed only at low levels elucidates its role in the pDC lineage\textsuperscript{90}. In this model, Ikaros upregulates Gfi1, and Gfi1 inhibits Id2 transcription (Fig. 32B). The repression of Id2 would result in functioning E2-2 protein, which can reprogram precursors for the pDC lineage fate by upregulating Spi-B, IRF-7 and IRF-8\textsuperscript{114}. There must be mechanisms in place to restrict GM-CSF signals from inhibiting IRF-8 through STAT5 to allow for CD8\textsuperscript{+} cDC development, as well as pDCs\textsuperscript{171}. Future studies examining the environmental cues and resulting transcriptional regulation will allow us to further understand the mechanisms that govern homeostatic DC development and infection- or inflammatory-induced DC differentiation.
Figure 32. Gene regulatory networks for cDC and pDC development. Shared gene regulation patterns in (A) and (B). PU.1 upregulates many factors important for DC development, including Id2, GM-CSFR, and Flt3. The Flt3 pathway phosphorylates STAT3, which can upregulate/downregulate target genes. (A) Gene regulation in cDCs. Id2 expression inhibits E2-2 via protein interaction. GM-CSFR phosphorylates STAT5, which can inhibit IRF-8 expression. Batf3 upregulates E4BP4. Batf expression in CD8+ cDCs compensates for a lack of Batf3. E4BP4 negatively modulates IRF-4 expression. (B) Gene regulation in pDCs. Ikaros upregulates Gfi1, which can inhibit Id2 expression, allowing for E2-2 function. E2-2 binds to the promoter of Spi-B, IRF-7, and IRF-8 to upregulate gene expression. A yet unidentified mechanism prevents the downstream events of GM-CSFR in pDCs, since STAT5 has been shown to downregulate IRF-8, which is required for pDC development. Proven interactions are indicated in solid bars. Hypothesized interactions are shown in dashed lines.
6.5 Future Directions

The utilization of specific gene-knockout mouse models have revealed regulatory factors, such as PU.1, E2-2 or IRF-8, that play important roles in the development or maintenance of many DC subsets. However, in the case of Spi-B\(^{−/−}\) mice, although there are no major DC deficiencies, I cannot discount that Spi-B might still play a DC-directing role when expressed in precursors undergoing lineage commitment. A flaw associated with knockout mouse models is that in the absence of a gene, there could be compensatory genes present to maintain a particular threshold of lineage conversion. However, by overexpressing a gene in progenitor cells I am able to address the direct result of that gene’s activity in the context of an otherwise normal cell. The adenoviral system I used in Chapter III can be very useful in temporarily overexpressing specific genes to test whether a regulatory factor truly is important for DC lineage conversion from uncommitted thymocytes. I aim to test the roles of Spi-B, HEBCan and HEBAlt in promoting DCs or T cells within the thymus. I hypothesize that Spi-B and HEBCan can aid in directing thymic pDC development based on our prior observations and published reports\(^{86}\), whereas HEBAlt directs T cell development\(^{116,221}\).

To further test the role of HEBCan, I am developing many tools for \textit{in vivo} analysis of tDC subsets. I am currently generating conditional HEB knockout mice strains, whereby HEB is deleted from all hematopoietic cells, including T, DC and NK cells, using the Vav-cre transgene. One caveat to using this mouse strain is the disruption of developing thymocytes and, thus, perturbation of the thymic architecture, which has been observed in HEB knockout mouse controlled by Lck-cre\(^{246}\). To produce HEBCan-
specific knockout mice, I will introduce the expression of HEBAlt by crossing HEBAlt-tg mice\textsuperscript{196} to the Vav-cre-directed HEB conditional knockout mice. This will allow us to test the specific effects of HEBCan on DC potential, in addition to T and NK cells. Unfortunately, there are currently no cre strains to target T/DC precursors or DC precursors, specifically. CD11c-cre has been used in multiple studies, but the deletion of HEB in cells that begin to express CD11c would be too late developmentally. The generation of a Zbtb46-cre transgenic animal would allow the deletion of HEB during the pre-cDC precursor stage before cells develop into cDCs\textsuperscript{98,99}. However, pDC lineage potential is lost at the pre-cDC developmental stage and, thus, Zbtb46-cre would not allow us to determine the role of HEB in pDC development. E2-2-cre could be a candidate for the deletion of HEB in the still elusive pDC precursors; however, it is also expressed in other lineages. In addition to the required presence of E2-2 for pDC development\textsuperscript{114}, it has been shown that the loss of E2-2 in pDCs causes a spontaneous shift of these cells to the cDC lineage\textsuperscript{115}.

I am currently conducting experiments whereby DN1 subsets are transferred into congenic mouse strains to determine the specific populations of DCs that arise from each progenitor. Although I have shown that ETPs, DN1d and DN1e cells give rise to medullary-homing DCs in vivo, immunofluorescent microscopy analyses did not allow us to phenotype the subset. I am optimizing the intravenous transfer of DN1 subsets into sublethally irradiated recipients, whereby there are still intact corticomedullary regions, as well as using IL-7Rα\textsuperscript{-/-} mice as recipients. WT thymocyte precursors transferred into IL-7Rα\textsuperscript{-/-} recipient mice should develop into T cells, as well as DCs. Thus, if T-DC
crosstalk is a factor for DC development it should not be an issue in this experimental model.

The influence of IL-7 on DC development within the thymus, specifically, is still not well understood. Thus, I will perform competitive reconstitution of lethally irradiated mice with WT Ly5.1+ thymocytes and Ly5.2+ IL-7Rα−/− thymocytes to determine the role of IL-7 on the generation of all three tDC subsets. Based on previous fate-mapping models using IL-7R expression, I hypothesize that a greater percentage of pDCs will arise from WT precursors than from IL-7Rα−/− precursors. It will also be interesting to observe the developmental outcome of thymic cDC populations. I will follow this experiment by reconstituting with WT Ly5.1+ BM and IL-7Rα−/− Ly5.2+ BM since I do not exclude the possibility of tDCs developing extrathymically followed by migration to the thymus, in addition to intrathymic DC development. This experiment will also allow us to extend previously reported results on splenic DC subsets.

In addition to the generation of tDCs, I am also interested in NK cell potential from DN1 subsets. Due to high Id2 expression by DN1e cells, I hypothesize that they are predisposed to give rise to a population of thymic NK cells. However, the possibility of intrathymic NK cell development in the adult thymus is just as controversial as intrathymic DC development. Many studies have shown that NK cells can arise from thymocyte precursors in vitro55,247, but only one report has shown intrathymic NK cell development in vivo from RAG−/− thymocyte precursors248.

Collectively, a fuller comprehension of the lineage potential from thymocyte precursors and the regulatory factors that govern these developmental decisions will
grant us additional tools and a greater understanding to examine the functional role of each DC or NK cell subset within the thymus.
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